

DESCRIPTION

Title of the Invention: ANTI-SIGLEC-15 ANTIBODY

Technical Field

[0001]

The present invention relates to a substance useful as a therapeutic and/or preventive agent for abnormal bone metabolism, and a method of treating and/or preventing abnormal bone metabolism.

Background Art

[0002]

Bone is known to be a dynamic organ which is continuously remodelled by repeated formation and resorption so as to change its own morphology and maintain blood calcium levels. Healthy bone maintains an equilibrium between bone formation by osteoblasts and bone resorption by osteoclasts, and bone mass is maintained constant. In contrast, when the equilibrium between bone formation and bone resorption is lost, abnormal bone metabolism such as osteoporosis occurs (WO 07/093042 and Endocrinological Review, (1992) 13, pp. 66-80).

[0003]

As factors which regulate bone metabolism, many systemic hormones and local cytokines have been reported, and these

factors collaborate with one another to form and maintain bone (WO 07/093042 and Endocrinological Review, (1996) 17, pp. 308-332). As a change in bone tissue due to aging, the occurrence of osteoporosis is widely known, but the mechanism of its occurrence encompasses various factors such as a decrease in secretion of sex hormones and an abnormality in the receptors for the hormones; variation in cytokine expression locally in bone; expression of aging genes; and osteoclast or osteoblast differentiation failure or dysfunction, and thus it is difficult to consider it as a simple age-related physiological phenomenon. Primary osteoporosis is largely divided into postmenopausal osteoporosis due to a decrease in secretion of estrogen and senile osteoporosis due to aging, but advancement of basic research on the mechanisms of regulation of bone formation and bone resorption is essential to elucidate the mechanism of its occurrence and to develop a therapeutic agent therefor.

[0004]

Osteoclasts are multinucleated cells derived from hematopoietic stem cells, and by releasing chloride ions and hydrogen ions on a bone surface to which osteoclasts adhere, osteoclasts acidify a gap between the bone surface and the osteoclasts and also secrete cathepsin K which is an acid protease or the like (American Journal of Physiology, (1991) 260, C1315-C1324). This causes degradation of calcium

phosphate, activation of acid proteases and degradation of bone matrix proteins, resulting in bone resorption.

[0005]

Osteoclast precursor cells have been found to be differentiated into osteoclasts by stimulation with RANKL (receptor activator of NF- κ B ligand) expressed on the cell membrane of osteoblasts/stromal cells present on the surface of bone (Proceedings of the National Academy of Science of the United States of America, (1998) 95, pp. 3597-3602, and Cell, (1998) 93, pp. 165-176). It has been revealed that RANKL is a membrane protein produced by osteoblasts/stromal cells, its expression is regulated by a bone resorption factor, RANKL induces differentiation of osteoclast precursor cells into mature multinucleated osteoclasts, and the like (Proceedings of the National Academy of Science of the United States of America, (1998) 95, pp. 3597-3602, and Journal of Bone and Mineral Research, (1998) 23, S222). Further, knockout mice devoid of RANKL have been found to develop an osteopetrosis-like disease, and therefore, RANKL has been proved to be a physiological osteoclast differentiation-inducing factor (Nature, (1999) 397, pp. 315-323).

[0006]

As pharmaceutical preparations for treating bone metabolism diseases or shortening the duration of treatment,

bisphosphonates, active vitamin D₃, calcitonin and derivatives thereof, hormones such as estradiol, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), PTH, calcium preparations, and the like are used. However, these medicinal agents are not always satisfactory in terms of therapeutic outcome and the development of a medicinal agent with a more potent therapeutic effect has been demanded.

[0007]

The cell membranes of immune cells are covered with a dense coating of various glycans such as sialylated glycans which are recognized by various glycan-binding proteins. Sialic-acid-binding immunoglobulin-like lectins (hereinafter referred to as "siglecs") are a family of type I membrane proteins which recognize sialylated glycans and bind thereto. Many siglecs are expressed on the cell membranes of immune cells and recognize sialic acid similarly present on the cell membranes of immune cells and regulate cell interaction or cell function and are considered to be involved in immune responses (Nature Reviews Immunology, (2007) 7, pp. 255-266). However, there are also a lot of siglec molecules whose physiological functions have not been elucidated yet. Siglec-15 (Sialic-acid binding immunoglobulin-like lectin 15) is a molecule which has been newly reported to belong to the Siglecs (see, for example, Non-patent document 10) and is identical to a molecule called CD33L3 (CD33 molecule-like 3). This

molecule is highly evolutionarily conserved from fish to humans and has been found to be strongly expressed in dendritic cells and/or macrophages of human spleen and lymph nodes. Further, as a result of a binding test using a sialic acid probe, it has also been found that human Siglec-15 binds to Neu5Ac α 2-6GalNAc and that mouse Siglec-15 binds further to Neu5Ac α 2-3Gal β 1-4Glc, and the like (see, for example, Glycobiology, (2007) 17, pp. 838-846). Until recently, the physiological role of Siglec-15 had not been revealed, however, it has been reported that the expression of Siglec-15 increases with the differentiation and maturation of osteoclasts, and the differentiation of osteoclasts is inhibited by decreasing the expression of Siglec-15 by RNA interference (see, for example, WO 07/093042). Further, the effect of an anti-Siglec-15 antibody on osteoclast differentiation has been revealed for the first time in WO 09/48072 (published on April 16, 2009), however, an antibody sequence which can be administered to humans has not been elucidated yet.

Summary of the Invention

Problems that the Invention is to Solve

[0008]

An object of the invention is to provide a gene which is specifically expressed in various forms of abnormal bone metabolism which are seen in osteoporosis, rheumatoid

arthritis, cancer metastasis to bone or the like, a substance which inhibits the differentiation and maturation of osteoclasts and the activity thereof, and a therapeutic and/or preventive agent for abnormal bone metabolism.

Means for Solving the Problems

[0009]

The present inventors studied to elucidate the mechanism of osteoclast differentiation, maturation and activation in order to find a substance having a therapeutic and/or preventive effect on abnormal bone metabolism. As a result, the present inventors found that the expression of Siglec-15 gene increases with the differentiation and maturation of osteoclasts, and also found that the differentiation of osteoclasts is inhibited by an antibody which specifically binds to Siglec-15. Further, the present inventors humanized an obtained rat anti-mouse Siglec-15 antibody, and thus completed the invention.

That is, the invention includes the following inventions.

(1) An antibody which binds to a polypeptide comprising amino acid residues 39 to 165 of an amino acid sequence represented by SEQ ID NO: 2 and inhibits osteoclast formation and/or osteoclastic bone resorption, or a functional fragment of the antibody.

(2) The antibody or a functional fragment of the antibody according to (1), characterized in that:

a heavy chain sequence contains a variable region having CDRH1, CDRH2, and CDRH3, and the CDRH1 comprises an amino acid sequence represented by SEQ ID NO: 44, the CDRH2 comprises any one of amino acid sequences represented by SEQ ID NO: 45 and SEQ ID NO: 97, and the CDRH3 comprises an amino acid sequence represented by SEQ ID NO: 46; and

a light chain sequence contains a variable region having CDRL1, CDRL2, and CDRL3, and the CDRL1 comprises an amino acid sequence represented by SEQ ID NO: 47, the CDRL2 comprises an amino acid sequence represented by SEQ ID NO: 48, and the CDRL3 comprises an amino acid sequence represented by SEQ ID NO: 49.

(3) The antibody or a functional fragment of the antibody according to (2), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 41 and a light chain variable region sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 43.

(4) The functional fragment of the antibody according to any one of (1) to (3) which is selected from the group consisting of Fab, F(ab')₂, Fab' and Fv.

(5) The antibody according to any one of (1) to (3), characterized by being an scFv.

(6) The antibody or a functional fragment of the antibody according to any one of (1) to (4), characterized in that the antibody is a chimeric antibody.

(7) The antibody or a functional fragment of the antibody according to (6), characterized by comprising a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 41 and a light chain sequence comprising amino acid residues 21 to 237 of an amino acid sequence represented by SEQ ID NO: 43.

(8) The antibody or a functional fragment of the antibody according to any one of (1) to (4), and (6), characterized in that the antibody is humanized.

(9) The antibody according to (7) or (8), wherein the heavy chain has a constant region of a human immunoglobulin G2 heavy chain and the light chain has a constant region of a human immunoglobulin κ light chain.

(10) An antibody which inhibits osteoclast formation and/or osteoclastic bone resorption or a functional fragment of the antibody, wherein the antibody contains:

(a) a heavy chain variable region selected from the group consisting of the following amino acid sequences:

a1) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 51;

a2) an amino acid sequence comprising amino acid residues

20 to 140 of an amino acid sequence represented by SEQ ID NO:
53;

a3) an amino acid sequence comprising amino acid residues
20 to 140 of an amino acid sequence represented by SEQ ID NO:
55;

a4) an amino acid sequence comprising amino acid residues
20 to 140 of an amino acid sequence represented by SEQ ID NO:
57;

a5) an amino acid sequence comprising amino acid residues
20 to 140 of an amino acid sequence represented by SEQ ID NO:
59;

a6) an amino acid sequence comprising amino acid residues
20 to 140 of an amino acid sequence represented by SEQ ID NO:
99;

a7) an amino acid sequence comprising amino acid residues
20 to 140 of an amino acid sequence represented by SEQ ID NO:
101;

a8) an amino acid sequence having a homology of at least
95% with any one of the amino acid sequences selected from a1)
to a7);

a9) an amino acid sequence having a homology of at least
99% with any one of the amino acid sequences selected from a1)
to a7); and

a10) an amino acid sequence including a substitution,
deletion, or addition of one to several amino acid residues

in any one of the amino acid sequences selected from a1) to a7); and

(b) a light chain variable region selected from the group consisting of the following amino acid sequences:

b1) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 61;

b2) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 63;

b3) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 65;

b4) an amino acid sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 67;

b5) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69;

b6) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 71;

b7) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 103;

b8) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 105;

b9) an amino acid sequence having a homology of at least 95% with any one of the amino acid sequences selected from b1) to b8);

b10) an amino acid sequence having a homology of at least 99% with any one of the amino acid sequences selected from b1) to b8); and

b11) an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in any one of the amino acid sequences selected from b1) to b8).

(11) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 51 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 61.

(12) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region

comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 53 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 63.

(13) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 65.

(14) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 67.

(15) The antibody or a functional fragment of the

antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 57 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69.

(16) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 59 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 71.

(17) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid

sequence represented by SEQ ID NO: 69.

(18) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 101 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69.

(19) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 103.

(20) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light

chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 105.

(21) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 51 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 61.

(22) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 53 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 63.

(23) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 65.

(24) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 55 and a light

chain sequence comprising amino acid residues 21 to 237 of an amino acid sequence represented by SEQ ID NO: 67.

(25) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 57 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 69.

(26) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 59 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 71.

(27) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 69.

(28) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 101 and a light chain sequence comprising amino acid residues 21 to 238 of an

amino acid sequence represented by SEQ ID NO: 69.

(29) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 103.

(30) The antibody or a functional fragment of the antibody according to (10), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 105.

(31) A pharmaceutical composition, characterized by comprising at least one of the antibodies or functional fragments of the antibodies according to (1) to (30).

(32) The pharmaceutical composition according to (31), characterized by being a therapeutic and/or preventive agent for abnormal bone metabolism.

(33) A pharmaceutical composition for treating and/or preventing abnormal bone metabolism, characterized by comprising at least one of the antibodies or functional fragments of the antibodies according to (1) to (30) and at least one member selected from the group consisting of bisphosphonates, active vitamin D₃, calcitonin and derivatives

thereof, hormones such as estradiol, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents, soluble TNF receptors, anti-TNF- α antibodies or functional fragments of the antibodies, anti-PTHrP (parathyroid hormone-related protein) antibodies or functional fragments of the antibodies, IL-1 receptor antagonists, anti-IL-6 receptor antibodies or functional fragments of the antibodies, anti-RANKL antibodies or functional fragments of the antibodies, and OCIF (osteoclastogenesis inhibitory factor).

(34) The pharmaceutical composition according to (32) or (33), wherein the abnormal bone metabolism is selected from the group consisting of osteoporosis, bone destruction accompanying rheumatoid arthritis, cancerous hypercalcemia, bone destruction accompanying multiple myeloma or cancer metastasis to bone, giant cell tumor, osteopenia, tooth loss due to periodontitis, osteolysis around a prosthetic joint, bone destruction in chronic osteomyelitis, Paget's disease of bone, renal osteodystrophy, and osteogenesis imperfecta.

(35) The pharmaceutical composition according to (34), characterized in that the abnormal bone metabolism is osteoporosis, bone destruction accompanying rheumatoid arthritis, or bone destruction accompanying cancer metastasis to bone.

(36) The pharmaceutical composition according to (35), characterized in that the abnormal bone metabolism is osteoporosis.

(37) The pharmaceutical composition according to (36), characterized in that the osteoporosis is postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent such as a steroid or an immunosuppressant, or osteoporosis accompanying rheumatoid arthritis.

(38) A method of treating and/or preventing abnormal bone metabolism, characterized by administering at least one of the antibodies or functional fragments of the antibodies according to (1) to (30).

(39) A method of treating and/or preventing abnormal bone metabolism, characterized by simultaneously or successively administering at least one of the antibodies or functional fragments of the antibodies according to (1) to (30) and at least one member selected from the group consisting of bisphosphonates, active vitamin D₃, calcitonin and derivatives thereof, hormones such as estradiol, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents, soluble TNF receptors, anti-TNF- α antibodies or functional fragments of the antibodies, anti-PTHrP (parathyroid hormone-related protein) antibodies

or functional fragments of the antibodies, IL-1 receptor antagonists, anti-IL-6 receptor antibodies or functional fragments of the antibodies, anti-RANKL antibodies or functional fragments of the antibodies, and OCIF (osteoclastogenesis inhibitory factor).

(40) The treatment and/or prevention method according to (38) or (39), characterized in that the abnormal bone metabolism is osteoporosis, bone destruction accompanying rheumatoid arthritis, or bone destruction accompanying cancer metastasis to bone.

(41) The treatment and/or prevention method according to (40), characterized in that the abnormal bone metabolism is osteoporosis.

(42) The treatment and/or prevention method according to (41), characterized in that the osteoporosis is postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent such as a steroid or an immunosuppressant, or osteoporosis accompanying rheumatoid arthritis.

(43) A polynucleotide encoding the antibody according to any one of (3), (7), and (10) to (30).

(44) The polynucleotide according to (43), characterized by containing a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 40 and a nucleotide sequence comprising nucleotides 61 to 396 of

a nucleotide sequence represented by SEQ ID NO: 42.

(45) The polynucleotide according to (44), characterized by containing a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 40 and a nucleotide sequence comprising nucleotides 61 to 711 of a nucleotide sequence represented by SEQ ID NO: 42.

(46) The polynucleotide according to (43), characterized by containing:

(a) a polynucleotide selected from the group consisting of the following nucleotide sequences:

a1) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 50;

a2) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 52;

a3) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54;

a4) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 56;

a5) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 58;

a6) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98;

a7) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 100;

a8) a nucleotide sequence of a polynucleotide which

hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from a1) to a7) under stringent conditions; and

a9) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in any one of the nucleotide sequences selected from a1) to a7); and

(b) a polynucleotide selected from the group consisting of the following nucleotide sequences:

b1) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 60;

b2) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 62;

b3) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 64;

b4) a nucleotide sequence comprising nucleotides 61 to 396 of a nucleotide sequence represented by SEQ ID NO: 66;

b5) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68;

b6) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 70;

b7) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 102;

b8) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 104;

b9) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from b1) to b8) under stringent conditions; and

b10) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in any one of the nucleotide sequences selected from b1) to b8).

(47) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 50, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 60.

(48) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 52, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 62.

(49) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 64.

(50) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 396 of a nucleotide sequence represented by SEQ ID NO: 66.

(51) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 56, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

(52) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 58, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 70.

(53) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

(54) The polynucleotide according to (46), characterized

by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 100, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

(55) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 102.

(56) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 104.

(57) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 50, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 60.

(58) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide

sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 52, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 62.

(59) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 64.

(60) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 711 of a nucleotide sequence represented by SEQ ID NO: 66.

(61) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 56, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

(62) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide

sequence represented by SEQ ID NO: 58, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 70.

(63) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

(64) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 100, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

(65) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 102.

(66) The polynucleotide according to (46), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide

comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 104.

(67) A vector, comprising any one of the polynucleotides according to (43) to (66).

(68) A transformed host cell, comprising any one of the polynucleotides according to (43) to (66).

(69) A transformed host cell, comprising the vector according to (67).

(70) A method of producing the antibody according to any one of (3), (7), and (10) to (30), comprising culturing the host cell according to (68) or (69), and purifying an antibody from the resulting culture product.

Advantage of the Invention

[0010]

According to the invention, a therapeutic and/or preventive agent for abnormal bone metabolism whose mechanism of action is to inhibit the differentiation and maturation of osteoclasts and the bone resorption activity thereof can be obtained.

Brief Description of the Drawings

[0011]

[Fig. 1] Fig. 1 shows the results of evaluating the purity of mouse Siglec-15-His purified by HisTrap HP column

chromatography and Resource Q column chromatography through SDS-polyacrylamide electrophoresis and silver staining.

[Fig. 2] Fig. 2 shows the results of detecting the characteristics of mouse Siglec-15-His purified by HiTrap HP column chromatography and Resource Q column chromatography through SDS-polyacrylamide electrophoresis and Western blotting using an anti-V5-HRP antibody.

[Fig. 3] Fig. 3 shows the results of evaluating the purity of mouse Siglec-15-Fc purified by HiTrap Protein A column chromatography through SDS-polyacrylamide electrophoresis and silver staining.

[Fig. 4] Fig. 4 shows the results of testing the binding of a rat anti-mouse Siglec-15 monoclonal antibody to a plate having mouse Siglec-15-Fc immobilized thereon by an ELISA method. The symbol (◆) denotes #1A1 antibody, the symbol (■) denotes #3A1 antibody, the symbol (▲) denotes #8A1 antibody, the symbol (×) denotes #24A1 antibody, the symbol (●) denotes #32A1 antibody, the symbol (○) denotes #34A1 antibody, the symbol (+) denotes #39A1 antibody, the symbol (-) denotes #40A1 antibody, the symbol (—) denotes #41B1 antibody, the symbol (◇) denotes #61A1 antibody, and the symbol (□) denotes control IgG.

[Fig. 5] Fig. 5 shows the results of testing the effect of the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#3A1, #8A1, or #32A1) on osteoclast differentiation

(stimulation with RANKL) of mouse bone marrow nonadherent cells. Incidentally, the rat control IgG in the drawing is a negative control common to Figs. 5 and 6.

[Fig. 6] Fig. 6 shows the results of testing the effect of the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#34A1, #39A1, or #40A1) on osteoclast differentiation (stimulation with RANKL) of mouse bone marrow nonadherent cells. Incidentally, the rabbit anti-mouse Siglec-15 polyclonal antibody No. 3 in the drawing is a positive control common to Figs. 5 and 6.

[Fig. 7] Fig. 7 shows the results of evaluating the purity of human Siglec-15-His purified by HisTrap HP column chromatography and Resource Q column chromatography through SDS-polyacrylamide electrophoresis and silver staining.

[Fig. 8] Fig. 8 shows the results of evaluating the purity of human Siglec-15-Fc purified by Protein A column chromatography through SDS-polyacrylamide electrophoresis.

[Fig. 9] Fig. 9 shows the results of testing the binding of a rat anti-mouse Siglec-15 monoclonal antibody to a plate having human Siglec-15-Fc immobilized thereon by an ELISA method. The symbol (◆) denotes #1A1 antibody, the symbol (■) denotes #3A1 antibody, the symbol (▲) denotes #8A1 antibody, the symbol (X) denotes #24A1 antibody, the symbol (●) denotes #32A1 antibody, the symbol (○) denotes #34A1 antibody, the symbol (+) denotes #39A1 antibody, the symbol (-) denotes #40A1

antibody, the symbol (—) denotes #41B1 antibody, the symbol (◇) denotes #61A1 antibody, and the symbol (□) denotes control IgG.

[Fig. 10] Fig. 10 shows photomicrographs depicting, by TRAP staining, the inhibition of giant osteoclast formation from normal human osteoclast precursor cells by the addition of a rat anti-mouse Siglec-15 monoclonal antibody.

[Fig. 11] Fig. 11 shows photomicrographs depicting, by TRAP staining, the inhibition of giant osteoclast formation from normal human osteoclast precursor cells by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody).

[Fig. 12] Fig. 12 is a graph showing the inhibition of the bone resorption activity of normal human osteoclasts by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) (N=6).

[Fig. 13] Fig. 13A is a graph showing the effect of increasing lumbar spine bone mineral density when a rat anti-mouse Siglec-15 monoclonal antibody was administered to ovariectomized rats for 4 weeks; and Fig. 13B is a graph showing the effect of decreasing urinary deoxypyridinoline excretion when a rat anti-mouse Siglec-15 monoclonal antibody was administered to ovariectomized rats for 4 weeks.

[Fig. 14] Fig. 14 is a graph showing that a rat anti-mouse Siglec-15 monoclonal antibody #32A1 binds to the V-set domain

of human Siglec-15 by competitive ELISA.

[Fig. 15] Fig. 15 is a graph showing that a rat #32A1 antibody and a human chimeric antibody thereof have substantially the same affinity for mouse Siglec-15-Fc by competitive ELISA.

[Fig. 16] Fig. 16 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) and a chimeric antibody thereof (N=3).

[Fig. 17] Fig. 17 shows photomicrographs depicting, by TRAP staining, the inhibition of giant osteoclast formation from normal human osteoclast precursor cells by the addition of a human chimeric antibody of a rat anti-mouse Siglec-15 monoclonal antibody #32A1.

[Fig. 18] Fig. 18 is a view in which it was confirmed that 6 types of humanized rat anti-mouse Siglec-15 antibodies bind to mouse Siglec-15 protein in an antibody concentration-dependent manner by an ELISA method using a plate having mouse Siglec-15-Fc immobilized thereon.

[Fig. 19] Fig. 19 is a view in which it was confirmed that 6 types of humanized rat anti-mouse Siglec-15 antibodies bind to human Siglec-15 protein in an antibody concentration-dependent manner by an ELISA method using a plate having human Siglec-15-Fc immobilized thereon.

[Fig. 20] Fig. 20 shows photomicrographs depicting, by TRAP staining, the inhibition of mouse giant osteoclast formation under stimulation with $\text{TNF}\alpha$ by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) or a chimeric antibody thereof.

[Fig. 21] Fig. 21 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation under stimulation with $\text{TNF}\alpha$ by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) or a chimeric antibody thereof (N=3).

[Fig. 22] Fig. 22 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of rat osteoclast formation by the addition of a rat anti-mouse Siglec-15 monoclonal antibody (#8A1 or #32A1 antibody) (N=3).

[Fig. 23] Fig. 23 shows a cloned nucleotide sequence of a rat #32A1 heavy chain and an amino acid sequence thereof.

[Fig. 24] Fig. 24 shows a cloned nucleotide sequence of a rat #32A1 light chain and an amino acid sequence thereof.

[Fig. 25] Fig. 25 shows a nucleotide sequence of a #32A1 human chimeric antibody heavy chain and an amino acid sequence thereof.

[Fig. 26] Fig. 26 shows a nucleotide sequence of a #32A1 human chimeric antibody light chain and an amino acid sequence thereof.

[Fig. 27] Fig. 27 shows a nucleotide sequence of

h#32A1-T1H and an amino acid sequence thereof.

[Fig. 28] Fig. 28 shows a nucleotide sequence of h#32A1-T2H and an amino acid sequence thereof.

[Fig. 29] Fig. 29 shows a nucleotide sequence of h#32A1-T3H and an amino acid sequence thereof.

[Fig. 30] Fig. 30 shows a nucleotide sequence of h#32A1-T5H and an amino acid sequence thereof.

[Fig. 31] Fig. 31 shows a nucleotide sequence of h#32A1-T6H and an amino acid sequence thereof.

[Fig. 32] Fig. 32 shows a nucleotide sequence of h#32A1-T1L and an amino acid sequence thereof.

[Fig. 33] Fig. 33 shows a nucleotide sequence of h#32A1-T2L and an amino acid sequence thereof.

[Fig. 34] Fig. 34 shows a nucleotide sequence of h#32A1-T3L and an amino acid sequence thereof.

[Fig. 35] Fig. 35 shows a nucleotide sequence of h#32A1-T4L and an amino acid sequence thereof.

[Fig. 36] Fig. 36 shows a nucleotide sequence of h#32A1-T5L and an amino acid sequence thereof.

[Fig. 37] Fig. 37 shows a nucleotide sequence of h#32A1-T6L and an amino acid sequence thereof.

[Fig. 38] Fig. 38 shows amino acid sequences of h#32A1-T7H, h#32A1-T8H, and h#32A1-T9H.

[Fig. 39] Fig. 39 shows amino acid sequences of h#32A1-T10H, h#32A1-T11H, and h#32A1-T12H.

[Fig. 40] Fig. 40 shows amino acid sequences of h#32A1-T7L, h#32A1-T8L, h#32A1-T9L, h#32A1-T10L, and h#32A1-T11L.

[Fig. 41] Fig. 41 shows amino acid sequences of h#32A1-T12L, h#32A1-T13L, h#32A1-T14L, h#32A1-T15L, and h#32A1-T16L.

[Fig. 42] Fig. 42 shows amino acid sequences of h#32A1-T17L, h#32A1-T18L, h#32A1-T19L, h#32A1-T20L, and h#32A1-T21L.

[Fig. 43] Fig. 43 shows amino acid sequences of h#32A1-T22L, h#32A1-T23L, h#32A1-T24L, and h#32A1-T25L.

[Fig. 44] Fig. 44 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-T1, h#32A1-T2, or h#32A1-T3). Incidentally, the rat #32A1 antibody in the drawing is a positive control common to Figs. 44 and 45.

[Fig. 45] Fig. 45 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-T4, h#32A1-T5, or h#32A1-T6).

[Fig. 46] Fig. 46 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-H1-1/L5 or h#32A1-H5/L5).

Incidentally, the rat #32A1 antibody and #32A1 human chimeric antibody in the drawing are positive controls common to Figs. 46 and 47.

[Fig. 47] Fig. 47 shows graphs depicting, by the enzymatic activity of TRAP, the inhibition of mouse osteoclast formation by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-H1-1/L2-16 or h#32A1-H-1/L2-15).

[Fig. 48] Fig. 48 shows graphs depicting the inhibition of the bone resorption activity of normal human osteoclasts by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-T1, h#32A1-T2, h#32A1-T3, h#32A1-T4, h#32A1-T5, or h#32A1-T6). Incidentally, the rat #32A1 antibody and #32A1 human chimeric antibody in the drawing are positive controls common to Figs. 48 and 49.

[Fig. 49] Fig. 49 shows graphs depicting the inhibition of the bone resorption activity of normal human osteoclasts by the addition of a humanized rat anti-mouse Siglec-15 antibody (h#32A1-H1-1/L5, h#32A1-H5/L5, h#32A1-H1-1/L2-16, or h#32A1-H1-1/L2-15).

[Fig. 50] Fig. 50 is a thermogram obtained for determining the thermal stability of the h#32A1-H5/L5 antibody.

[Fig. 51] Fig. 51 is a thermogram obtained for determining the thermal stability of the h#32A1-H1-1/L5 antibody.

[Fig. 52] Fig. 52 is a thermogram obtained for determining the thermal stability of the h#32A1-H1-1/L2-15 antibody.

[Fig. 53] Fig. 53 is a thermogram obtained for determining the thermal stability of the h#32A1-H1-1/L2-16 antibody.

[Fig. 54] Fig. 54 shows a nucleotide sequence of h#32A1-H1-1 and the amino acid sequence thereof.

[Fig. 55] Fig. 55 shows a nucleotide sequence of h#32A1-H5 and the amino acid sequence thereof.

[Fig. 56] Fig. 56 shows a nucleotide sequence of h#32A1-L2-15 and the amino acid sequence thereof.

[Fig. 57] Fig. 57 shows a nucleotide sequence of h#32A1-L2-16 and the amino acid sequence thereof.

Mode for Carrying Out the Invention

[0012]

The term "gene" as used herein includes not only DNA, but also mRNA, cDNA, and cRNA.

[0013]

The term "polynucleotide" as used herein is used with the same meaning as a "nucleic acid" and also includes DNA, RNA, probes, oligonucleotides, and primers.

[0014]

The terms "polypeptide" and "protein" as used herein are

used without distinction.

[0015]

The term "RNA fraction" as used herein refers to a fraction containing RNA.

[0016]

The term "cell" as used herein also includes cells in an animal individual and cultured cells.

[0017]

The term "Siglec-15" as used herein is used with the same meaning as "Siglec-15 protein".

[0018]

The term "osteoclast formation" as used herein is used with the same meaning as "osteoclast differentiation" or "osteoclast maturation".

[0019]

The term "functional fragment of an antibody" as used herein refers to a partial fragment of an antibody having an antigen-binding activity and includes Fab, F(ab')₂, Fv, scFv, diabodies, linear antibodies, polyspecific antibodies formed from antibody fragments, and the like. The term also encompasses Fab' which is a monovalent fragment in a variable region of an antibody obtained by treating F(ab')₂ under reducing conditions. However, the term is not limited to these molecules as long as the fragment has a binding affinity for an antigen. Further, these functional fragments include not

only a fragment obtained by treating a full-length molecule of an antibody protein with an appropriate enzyme, but also a protein produced in an appropriate host cell using a genetically modified antibody gene.

[0020]

The term "epitope" as used herein refers to a partial peptide or a partial tertiary structure of Siglec-15 to which a specific anti-Siglec-15 antibody binds. The above-mentioned epitope which is a partial peptide of Siglec-15 can be determined by a method well known to those skilled in the art such as an immunoassay. However, the following method can be employed, for example. Various partial structures of Siglec-15 are produced. In the production of the partial structures, a known oligopeptide synthesis technique can be used. For example, a series of polypeptides having appropriately reduced lengths obtained by sequentially shortening Siglec-15 from the C terminus or N terminus are produced using a genetic recombination technique known to those skilled in the art. Thereafter, the reactivity of an antibody against these polypeptides is examined and a recognition site is roughly determined. Then, peptides having shorter lengths are synthesized and the reactivity with these peptides is examined, whereby the epitope can be determined. Further, the epitope which is a partial tertiary structure of Siglec-15 which binds to a specific Siglec-15 antibody can be determined

by specifying the amino acid residues of Siglec-15 adjacent to the antibody by an X-ray structural analysis. If a second anti-Siglec-15 antibody binds to a partial peptide or a partial tertiary structure to which a first anti-Siglec-15 antibody binds, it can be determined that the first antibody and the second antibody share the same epitope. Further, by confirming that the second anti-Siglec-15 antibody competes with the first anti-Siglec-15 antibody for the binding to Siglec-15 (that is, the second antibody inhibits the binding between Siglec-15 and the first antibody), it can be determined that the first antibody and the second antibody share the same epitope even if the sequence or structure of a specific epitope has not been determined. Further, when the first antibody and the second antibody bind to the same epitope and also the first antibody has a special effect such as an antigen-neutralizing activity, the second antibody can be expected to have the same activity.

[0021]

It is known that each heavy and light chain of an antibody molecule has three complementarity determining regions (CDRs). The complementarity determining region is also called the hypervariable domain, and is present in a variable region of each heavy and light chain of an antibody. It is a site which has unusually high variability in its primary structure, and there are three separate CDRs in the primary structure of each

heavy and light polypeptide chain. In this description, as for the complementarity determining regions of an antibody, the complementarity determining regions of the heavy chain are represented by CDRH1, CDRH2, and CDRH3 from the amino-terminal side of the amino acid sequence of the heavy chain, and the complementarity determining regions of the light chain are represented by CDRL1, CDRL2, and CDRL3 from the amino-terminal side of the amino acid sequence of the light chain. These sites are proximate to one another in the tertiary structure and determine the specificity for an antigen to which the antibody binds.

[0022]

The phrase "hybridization is performed under stringent conditions" as used herein refers to hybridization that is performed under conditions under which identification can be achieved by performing hybridization at 68°C in a commercially available hybridization solution, ExpressHyb Hybridization Solution (manufactured by Clontech, Inc.) or performing hybridization at 68°C in the presence of 0.7 to 1.0 M NaCl using a filter having DNA immobilized thereon, followed by performing washing at 68°C using 0.1 to 2 x SSC solution (1 x SSC solution is composed of 150 mM NaCl and 15 mM sodium citrate) or under conditions equivalent thereto.

[0023]

1. Siglec-15

The present inventors have found that the Siglec-15 gene is specifically expressed in giant cell tumors and have also found that the expression level of the Siglec-15 gene increases when a monocyte-derived cell line differentiates into osteoclasts.

[0024]

As for the Siglec-15 to be used in the invention, Siglec-15 is directly purified from monocytes or bone marrow cells of a human, non-human mammal (such as guinea pig, rat, mouse, rabbit, pig, sheep, cattle, or monkey) or chicken and is used, or a cell membrane fraction of the above-mentioned cells is prepared and can be used. Further, Siglec-15 can be obtained by in vitro synthesis thereof or production thereof in a host cell through genetic engineering. In the genetic engineering production, specifically, Siglec-15 cDNA is integrated into a vector capable of expressing Siglec-15 cDNA, and Siglec-15 is synthesized in a solution containing an enzyme, a substrate, and an energy substance required for transcription and translation, or another prokaryotic or eucaryotic host cell is transformed to express Siglec-15, whereby the protein can be obtained.

[0025]

The nucleotide sequence of human Siglec-15 cDNA has been registered in GenBank with an accession number of NM_213602 and is represented by SEQ ID NO: 1 in the Sequence Listing,

and its amino acid sequence is represented by SEQ ID NO: 2 in the Sequence Listing. The nucleotide sequence of mouse Siglec-15 cDNA has been registered in GenBank with an accession number of XM_884636 and is represented by SEQ ID NO: 3 in the Sequence Listing, and its amino acid sequence is represented by SEQ ID NO: 4 in the Sequence Listing. Mature human Siglec-15 from which the signal sequence has been removed corresponds to an amino acid sequence comprising amino acid residues 21 to 328 of the amino acid sequence represented by SEQ ID NO: 2. Further, mouse Siglec-15 from which the signal sequence has been removed corresponds to an amino acid sequence comprising amino acid residues 21 to 341 of the amino acid sequence represented by SEQ ID NO: 4. Incidentally, Siglec-15 is sometimes called CD33 antigen-like 3, CD33 molecule-like 3, CD33-like 3, or CD33L3, and all of these represent the same molecule.

[0026]

Siglec-15 cDNA can be obtained by, for example, a so-called PCR method in which a polymerase chain reaction (hereinafter referred to as "PCR") is performed using a cDNA library expressing Siglec-15 cDNA as a template and primers which specifically amplify Siglec-15 cDNA (Saiki, R. K., et al., Science, (1988) 239, 487-49).

[0027]

Further, a polynucleotide which hybridizes to a

polynucleotide comprising a nucleotide sequence complementary to at least one nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NOS: 1 and 3 in the Sequence Listing under stringent conditions and encodes a protein having a biological activity comparable to that of Siglec-15 is also regarded as Siglec-15 cDNA. Further, a polynucleotide which is a splicing variant transcribed from the human or mouse Siglec-15 locus or a polynucleotide which hybridizes to a sequence complementary thereto under stringent conditions and encodes a protein having a biological activity comparable to that of Siglec-15 is also regarded as Siglec-15 cDNA.

[0028]

Further, a protein which comprises an amino acid sequence including substitution, deletion or addition of one or several amino acids in at least one amino acid sequence selected from the amino acid sequences represented by SEQ ID NOS: 2 and 4 in the Sequence Listing or an amino acid sequence obtained by removing the signal sequence from any of these sequences and has a biological activity comparable to that of Siglec-15 is also regarded as Siglec-15. Further, a protein which comprises an amino acid sequence encoded by a splicing variant transcribed from the human or mouse Siglec-15 locus or an amino acid sequence including a substitution, deletion or addition of one or several amino acids therein and has a biological

activity comparable to that of Siglec-15 is also regarded as Siglec-15.

[0029]

2. Detection of abnormal bone metabolism

An analysis of the expression level of the Siglec-15 gene in a group of test samples from various human bone tissues showed that the expression level of the gene significantly increases in giant cell tumor (GCT) which is a bone tumor with a large number of osteoclast-like multinucleated giant cells arising and is characterized by clinical findings of osteolytic bone destruction (Bullough et al., Atlas of Orthopedic Pathology 2nd edition, pp. 17.6-17.8, Lippincott Williams & Wilkins Publishers (1992)).

[0030]

It was also found that the expression level of the Siglec-15 gene increases when a monocyte-derived cell line is differentiated into osteoclasts.

[0031]

Accordingly, Siglec-15 is considered to be associated with human pathologies such as GCT in which bone resorption is increased. In other words, measurement of the expression level of the Siglec-15 gene and/or Siglec-15 in each cell and/or each tissue enables determination of the state of abnormal bone metabolism accompanied by overexpression of Siglec-15. The abnormal bone metabolism as used herein refers to a disorder

characterized by net bone loss, and specific examples thereof include, but are not limited to, osteoporosis (postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent such as a steroid or an immunosuppressant, or osteoporosis accompanying rheumatoid arthritis), bone destruction accompanying rheumatoid arthritis, cancerous hypercalcemia, bone destruction accompanying multiple myeloma or cancer metastasis to bone, giant cell tumor, osteopenia, tooth loss due to periodontitis, osteolysis around a prosthetic joint, bone destruction in chronic osteomyelitis, Paget's disease of bone, renal osteodystrophy, and osteogenesis imperfecta.

[0032]

In the invention, the "test sample" to be used for examining the expression level of the Siglec-15 gene and/or Siglec-15 refers to a sample from a tissue of bone marrow, bone, prostate, testis, penis, bladder, kidney, oral cavity, pharynx, lip, tongue, gingiva, nasopharynx, esophagus, stomach, small intestine, large intestine, colon, liver, gallbladder, pancreas, nose, lung, soft tissue, skin, breast, uterus, ovary, brain, thyroid, lymph node, muscle, fat tissue or the like, or blood, a body fluid, an excretion, or the like obtained from a test subject, a clinical specimen, etc., however, in the invention, blood or bone marrow is more preferred.

[0033]

As for RANKL which is known to be associated with osteoclast differentiation, a knockout mouse has been produced, and the phenotype when the function of RANKL has been lost has been analyzed (Young-Yun Kong, et. al., Nature (1999) 397, pp. 315-323). By producing a knockout mouse devoid of Siglec-15 in the same manner as above, the phenotype when the function of Siglec-15 has been lost can be analyzed.

[0034]

3. Production of anti-Siglec-15 antibody

The antibody of the invention, which is against Siglec-15, can be obtained by immunizing an animal with Siglec-15 or an arbitrary polypeptide selected from the amino acid sequence of Siglec-15, and collecting and purifying the antibody produced in vivo according to a common procedure. The biological species of Siglec-15 to be used as an antigen is not limited to being human, and an animal can be immunized with Siglec-15 derived from an animal other than humans such as a mouse or a rat. In this case, by examining the cross-reactivity between an antibody binding to the obtained heterologous Siglec-15 and human Siglec-15, an antibody applicable to a human disease can be selected.

[0035]

Further, a monoclonal antibody can be obtained by fusing antibody-producing cells which produce an antibody against Siglec-15 with myeloma cells to establish a hybridoma according

to a known method (for example, Kohler and Milstein, Nature, (1975) 256, pp. 495-497; Kennet, R. ed., Monoclonal Antibodies, pp. 365-367, Plenum Press, N.Y. (1980)).

[0036]

Incidentally, Siglec-15 to be used as an antigen can be obtained by genetic engineering to cause a host cell to produce the Siglec-15 gene.

[0037]

Specifically, a vector capable of expressing Siglec-15 gene is produced, and the resulting vector is transfected into a host cell to express the gene, and then the expressed Siglec-15 is purified. Hereinafter, a method of obtaining an antibody against Siglec-15 will be specifically described.

(1) Preparation of antigen

Examples of the antigen to be used for producing the anti-Siglec-15 antibody include Siglec-15, a polypeptide comprising a partial amino acid sequence containing at least 6 consecutive amino acids of Siglec-15, and a derivative obtained by adding a given amino acid sequence or carrier thereto.

[0038]

Siglec-15 can be purified directly from human tumor tissues or tumor cells and used. Further, Siglec-15 can be obtained by synthesizing it in vitro or by causing a host cell to produce it by genetic engineering.

[0039]

With respect to the genetic engineering, specifically, Siglec-15 cDNA is integrated into a vector capable of expressing Siglec-15 cDNA and Siglec-15 is synthesized in a solution containing an enzyme, a substrate, and an energy substance required for transcription and translation, or another prokaryotic or eucaryotic host cell is transformed to express Siglec-15, whereby the antigen can be obtained.

[0040]

Further, the antigen can also be obtained as a secretory protein by expressing a fusion protein obtained by ligating the extracellular domain of Siglec-15, which is a membrane protein, to the constant region of an antibody in an appropriate host-vector system.

[0041]

Siglec-15 cDNA can be obtained by, for example, a so-called PCR method in which a polymerase chain reaction (hereinafter referred to as "PCR") is performed using a cDNA library expressing Siglec-15 cDNA as a template and primers which specifically amplify Siglec-15 cDNA (see Saiki, R. K., et al., Science, (1988) 239, pp. 487-489).

[0042]

As the in vitro synthesis of the polypeptide, for example, Rapid Translation System (RTS) manufactured by Roche Diagnostics, Inc. can be exemplified, but it is not limited

thereto.

[0043]

Examples of the prokaryotic host include *Escherichia coli* and *Bacillus subtilis*. In order to transform the host cell with a target gene, the host cell is transformed using a plasmid vector containing a replicon, i.e., a replication origin derived from a species compatible with the host, and a regulatory sequence. Further, the vector preferably has a sequence capable of imposing phenotypic selectivity on the transformed cell.

[0044]

Examples of the eucaryotic host cell include vertebrate cells, insect cells, and yeast cells. As the vertebrate cells, for example, dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., *Proc. Natl. Acad. Sci. USA* (1980) 77, pp. 4126-4220) of simian COS cells (Gluzman, Y., *Cell*, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61); and the like are often used, however, they are not limited thereto.

[0045]

The thus obtained transformant can be cultured according to a common procedure, and by the culturing of the transformant, a target polypeptide is produced intracellularly or extracellularly.

[0046]

A suitable medium to be used for the culturing can be selected from various commonly used culture media depending on the employed host cell. If *Escherichia coli* is employed, for example, an LB medium supplemented with an antibiotic such as ampicillin or IPMG as needed can be used.

[0047]

A recombinant protein produced intracellularly or extracellularly by the transformant through such culturing can be separated and purified by any of various known separation methods utilizing the physical or chemical property of the protein.

[0048]

Specific examples of the methods include treatment with a common protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, and affinity chromatography, dialysis, and a combination thereof.

[0049]

Further, by attaching a tag of six histidine residues to a recombinant protein to be expressed, the protein can be efficiently purified with a nickel affinity column. Alternatively, by attaching a IgG Fc region to a recombinant protein to be expressed, the protein can be efficiently

purified with a protein A column.

By combining the above-mentioned methods, a large amount of a target polypeptide can be easily produced in high yield and high purity.

[0050]

(2) Production of anti-Siglec-15 monoclonal antibody

Examples of the antibody specifically binding to Siglec-15 include a monoclonal antibody specifically binding to Siglec-15, and a method of obtaining the antibody are as described below.

[0051]

The production of a monoclonal antibody generally requires the following operational steps of:

- (a) purifying a biopolymer to be used as an antigen;
- (b) preparing antibody-producing cells by immunizing an animal by injection of the antigen, collecting the blood, assaying its antibody titer to determine when the spleen is to be excised;
- (c) preparing myeloma cells (hereinafter referred to as "myeloma");
- (d) fusing the antibody-producing cells with the myeloma;
- (e) screening a group of hybridomas producing a target antibody;
- (f) dividing the hybridomas into single cell clones

(cloning);

(g) optionally, culturing the hybridoma or rearing an animal implanted with the hybridoma for producing a large amount of a monoclonal antibody;

(h) examining the thus produced monoclonal antibody for biological activity and binding specificity, or assaying the same for properties as a labeled reagent; and the like.

[0052]

Hereinafter, the method of producing a monoclonal antibody will be described in detail following the above steps, however, the method is not limited thereto, and, for example, antibody-producing cells and myeloma can be used other than spleen cells.

[0053]

(a) Purification of antigen

As the antigen, Siglec-15 prepared by the method as described above or a partial peptide thereof can be used.

[0054]

Further, a membrane fraction prepared from recombinant cells expressing Siglec-15 or the recombinant cells expressing Siglec-15 themselves, and also a partial peptide of the protein of the invention chemically synthesized by a method known to those skilled in the art can also be used as the antigen.

[0055]

(b) Preparation of antibody-producing cells

The antigen obtained in step (a) is mixed with an adjuvant such as Freund's complete or incomplete adjuvant, or aluminum potassium sulfate and the resulting mixture is used as an immunogen to immunize an experimental animal. As the experimental animal, any animal used in a known hybridoma production method can be used without any trouble. Specifically, for example, a mouse, a rat, a goat, sheep, cattle, a horse, or the like can be used. However, from the viewpoint of ease of availability of myeloma cells to be fused with the extracted antibody-producing cells, a mouse or a rat is preferably used as the animal to be immunized.

[0056]

Further, the strain of mouse or rat to be used is not particularly limited, and in the case of a mouse, for example, various strains such as A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BL, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, R III, SJL, SWR, WB, and 129 can be used, and in the case of a rat, for example, Wistar, Low, Lewis, Sprague, Dawley, ACI, BN, Fischer, and the like can be used.

[0057]

These mice and rats are commercially available from breeders/distributors of experimental animals, for example, CLEA Japan, Inc. and Charles River Laboratories Japan, Inc.

[0058]

Among these, in consideration of compatibility of fusing

with myeloma cells as described below, in the case of a mouse, BALB/c strain, and in the case of a rat, Wistar and Low strains are particularly preferred as the animal to be immunized.

[0059]

Further, in consideration of antigenic homology between humans and mice, it is also preferred to use a mouse having decreased biological function to remove autoantibodies, that is, a mouse with an autoimmune disease.

[0060]

The age of the mouse or rat at the time of immunization is preferably 5 to 12 weeks of age, more preferably 6 to 8 weeks of age.

[0061]

In order to immunize an animal with Siglec-15 or a recombinant thereof, for example, a known method described in detail in, for example, Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher Springfield, Illinois (1964), or the like can be used.

[0062]

Among these immunization methods, a preferred specific method in the invention is, for example, as follows.

[0063]

That is, first, a membrane protein fraction serving as

the antigen or cells caused to express the antigen is/are intradermally or intraperitoneally administered to an animal.

[0064]

However, the combination of both routes of administration is preferred for increasing the immunization efficiency, and when intradermal administration is performed in the first half and intraperitoneal administration is performed in the latter half or only at the last dosing, the immunization efficiency can be particularly increased.

[0065]

The administration schedule of the antigen varies depending on the type of animal to be immunized, individual differences, or the like. However, in general, an administration schedule in which the frequency of administration of the antigen is 3 to 6 times and the dosing interval is 2 to 6 weeks is preferred, and an administration schedule in which the frequency of administration of the antigen is 3 to 4 times and the dosing interval is 2 to 4 weeks is more preferred.

[0066]

Further, the dose of the antigen varies depending on the type of animal to be immunized, individual differences, or the like, however, the dose is generally set to 0.05 to 5 mg, preferably about 0.1 to 0.5 mg.

[0067]

A booster immunization is performed 1 to 6 weeks, preferably 2 to 4 weeks, more preferably 2 to 3 weeks after the administration of the antigen as described above.

[0068]

The dose of the antigen at the time of performing the booster immunization varies depending on the type or size of animal or the like, however, in the case of a mouse, the dose is generally set to 0.05 to 5 mg, preferably 0.1 to 0.5 mg, more preferably about 0.1 to 0.2 mg.

[0069]

Spleen cells or lymphocytes including antibody-producing cells are aseptically removed from the immunized animal 1 to 10 days, preferably 2 to 5 days, more preferably 2 to 3 days after the booster immunization.

[0070]

At this time, the antibody titer is measured, and if an animal having a sufficiently increased antibody titer is used as a supply source of the antibody-producing cells, the subsequent procedure can be carried out more efficiently.

[0071]

Examples of the method of measuring the antibody titer to be used here include an RIA method and an ELISA method, but the method is not limited thereto.

[0072]

For example, if an ELISA method is employed, the

measurement of the antibody titer in the invention can be carried out according to the procedures as described below.

[0073]

First, a purified or partially purified antigen is adsorbed onto the surface of a solid phase such as a 96-well plate for ELISA, and the surface of the solid phase having no antigen adsorbed thereto is covered with a protein unrelated to the antigen such as bovine serum albumin (hereinafter referred to as "BSA"). After washing the surface, the surface is brought into contact with a serially-diluted sample (for example, mouse serum) as a primary antibody to allow the antibody in the sample to bind to the antigen.

[0074]

Further, as a secondary antibody, an antibody labeled with an enzyme against a mouse antibody is added and is allowed to bind to the mouse antibody. After washing, a substrate for the enzyme is added and a change in absorbance which occurs due to color development induced by degradation of the substrate or the like is measured and the antibody titer is calculated based on the measurement.

[0075]

The separation of the antibody-producing cells from the spleen cells or lymphocytes can be carried out according to a known method (for example, Kohler et al., *Nature* (1975), 256, p. 495; Kohler et al., *Eur. J. Immunol.* (1977), 6, p. 511;

Milstein et al., Nature (1977), 266, p. 550; Walsh, Nature (1977), 266, p. 495).

[0076]

For example, in the case of spleen cells, a general method in which the antibody-producing cells are separated by homogenizing the spleen to obtain cells through filtration with a stainless steel mesh and suspending the cells in Eagle's Minimum Essential Medium (MEM) can be employed.

[0077]

(c) Preparation of myeloma cells (hereinafter referred to as "myeloma")

The myeloma cells to be used for cell fusion are not particularly limited and suitable cells can be selected from known cell lines. However, in consideration of convenience when a hybridoma is selected from fused cells, it is preferred to use an HGPRT (hypoxanthine-guanine phosphoribosyl transferase) deficient strain whose selection procedure has been established.

[0078]

More specifically, examples of the HGPRT-deficient strain include X63-Ag8(X63), NS1-ANS/1(NS1), P3X63-Ag8.U1(P3U1), X63-Ag8.653(X63.653), SP2/0-Ag14(SP2/0), MPC11-45.6TG1.7(45.6TG), F0, S149/5XX0, and BU.1 derived from mice; 210.RSY3.Ag.1.2.3(Y3) derived from rats; and U266AR(SKO-007), GM1500-GTG-A12(GM1500), UC729-6,

LICR-LOW-HMy2(HMy2) and 8226AR/NIP4-1(NP41) derived from humans.

[0079]

These HGPRT-deficient strains are available from, for example, the American Type Culture Collection (ATCC) or the like.

[0080]

These cell strains are subcultured in an appropriate medium such as an 8-azaguanine medium [a medium obtained by adding 8-azaguanine to an RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, and fetal calf serum (hereinafter referred to as "FCS")]; Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM"), or Dulbecco's Modified Eagle Medium (hereinafter referred to as "DMEM"). In this case, 3 to 4 days before performing cell fusion, the cells are subcultured in a normal medium [for example, an ASF104 medium (manufactured by Ajinomoto Co., Ltd.) containing 10% FCS] to obtain not less than 2×10^7 cells on the day of cell fusion.

[0081]

(d) Cell fusion

Fusion between the antibody-producing cells and the myeloma cells can be appropriately performed according to a known method (Weir, D. M. Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford

(1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher, Springfield, Illinois (1964), etc.), under conditions such that the survival rate of cells is not excessively reduced.

[0082]

As such a method, a chemical method in which the antibody-producing cells and the myeloma cells are mixed in a solution containing a polymer such as polyethylene glycol at a high concentration, a physical method using electric stimulation, or the like can be used.

[0083]

Among these methods, a specific example of the chemical method is as described below.

That is, in the case where polyethylene glycol is used in the solution containing a polymer at a high concentration, the antibody-producing cells and the myeloma cells are mixed in a solution of polyethylene glycol having a molecular weight of 1500 to 6000, more preferably 2000 to 4000 at a temperature of from 30 to 40°C, preferably from 35 to 38°C for 1 to 10 minutes, preferably 5 to 8 minutes.

[0084]

(e) Selection of a group of hybridomas

The method of selecting hybridomas obtained by the above-mentioned cell fusion is not particularly limited. Usually, an HAT (hypoxanthine, aminopterin, thymidine)

selection method (Kohler et al., Nature (1975), 256, p. 495; Milstein et al., Nature (1977), 266, p. 550) is used.

[0085]

This method is effective when hybridomas are obtained using the myeloma cells of an HGPRT-deficient strain which cannot survive in the presence of aminopterin.

[0086]

That is, by culturing unfused cells and hybridomas in an HAT medium, only hybridomas resistant to aminopterin are selectively allowed to survive and proliferate.

[0087]

(f) Division into single cell clone (cloning)

As a cloning method for hybridomas, a known method such as a methylcellulose method, a soft agarose method, or a limiting dilution method can be used (see, for example, Barbara, B. M. and Stanley, M. S.: Selected Methods in Cellular Immunology, W. H. Freeman and Company, San Francisco (1980)). Among these methods, particularly, a limiting dilution method is preferred.

[0088]

In this method, a fibroblast cell strain derived from a rat fetus or feeder cells such as normal mouse spleen cells, thymus gland cells, or ascites cells are seeded on a microplate.

[0089]

Meanwhile, hybridomas are diluted in a medium to give

a cell density of 0.2 to 0.5 cells per 0.2 ml. A 0.1 ml aliquot of the diluted hybridoma suspension is added to each well and culturing is continued for about 2 weeks while replacing about 1/3 of the culture solution with fresh medium at predetermined time intervals (for example, every 3 days), whereby hybridoma clones can be proliferated.

[0090]

The hybridomas in wells for which the antibody titer has been confirmed are subjected to, for example, cloning by the limiting dilution method repeatedly 2 to 4 times. A hybridoma which has been confirmed to have a stable antibody titer is selected as an anti-Siglec-15 monoclonal antibody-producing hybridoma strain.

[0091]

Examples of the hybridoma strain thus cloned include hybridoma #32A1 described in WO 09/48072. The hybridoma #32A1 was deposited at the International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology (located at Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on August 28, 2008, and has been given an accession number of FERM BP-10999 under the name of anti-Siglec-15 Hybridoma #32A1. Incidentally, in this description, the antibody produced by the hybridoma #32A1 is referred to as "#32A1 antibody" or simply "#32A1". Further, antibodies obtained in the Examples of this description other

than the #32A1 antibody are also represented by the antibody names in the same manner. A partial fragment containing the heavy chain variable region of the #32A1 antibody has an amino acid sequence comprising amino acid residues 20 to 167 of SEQ ID NO: 28 in the Sequence Listing. Further, a partial fragment containing the light chain variable region sequence of the #32A1 antibody has an amino acid sequence comprising amino acid residues 21 to 139 of SEQ ID NO: 30 in the Sequence Listing.

[0092]

(g) Preparation of monoclonal antibody by culturing hybridoma

By culturing the thus selected hybridoma, a monoclonal antibody can be efficiently obtained. However, prior to culturing, it is preferred to perform screening of a hybridoma which produces a target monoclonal antibody.

[0093]

In such screening, a known method can be employed.

[0094]

The measurement of the antibody titer in the invention can be carried out by, for example, an ELISA method explained in item (b) described above.

[0095]

The hybridoma obtained by the method described above can be stored in a frozen state in liquid nitrogen or in a freezer at -80°C or below.

[0096]

After completion of cloning, the medium is changed from an HT medium to a normal medium, and the hybridoma is cultured.

[0097]

Large-scale culture is performed by rotation culture using a large culture bottle or by spinner culture.

From the supernatant obtained by the large-scale culture, a monoclonal antibody which specifically binds to the protein of the invention can be obtained by purification using a method known to those skilled in the art such as gel filtration.

[0098]

Further, the hybridoma is injected into the abdominal cavity of a mouse of the same strain as the hybridoma (for example, the above-mentioned BALB/c) or a Nu/Nu mouse to proliferate the hybridoma, whereby the ascites containing a large amount of the monoclonal antibody of the invention can be obtained.

[0099]

In the case where the hybridoma is administered in the abdominal cavity, if a mineral oil such as 2,6,10,14-tetramethyl pentadecane (pristane) is administered 3 to 7 days prior thereto, a larger amount of the ascites can be obtained.

[0100]

For example, an immunosuppressant is previously injected

into the abdominal cavity of a mouse of the same strain as the hybridoma to inactivate T cells. 20 days thereafter, 10^6 to 10^7 hybridoma clone cells are suspended in a serum-free medium (0.5 ml), and the suspension is injected into the abdominal cavity of the mouse. In general, when the abdomen is expanded and filled with the ascites, the ascites is collected from the mouse.

[0101]

By this method, the monoclonal antibody can be obtained at a concentration which is about 100 times or more higher than that in the culture solution.

[0102]

The monoclonal antibody obtained by the above-mentioned method can be purified by a method described in, for example, Weir, D. M.: Handbook of Experimental Immunology Vol. I, II, III, Blackwell Scientific Publications, Oxford (1978).

[0103]

That is, examples of the method include an ammonium sulfate precipitation method, gel filtration, ion exchange chromatography, and affinity chromatography.

[0104]

As a simple purification method, a commercially available monoclonal antibody purification kit (for example, MAbTrap GII Kit manufactured by Pharmacia, Inc.) or the like can also be used.

[0105]

The thus obtained monoclonal antibody has high antigen specificity for Siglec-15.

[0106]

(h) Assay of monoclonal antibody

The isotype and subclass of the thus obtained monoclonal antibody can be determined as follows.

[0107]

First, examples of the identification method include an Ouchterlony method, an ELISA method and an RIA method.

[0108]

An Ouchterlony method is simple, but when the concentration of the monoclonal antibody is low, a condensation operation is required.

[0109]

On the other hand, when an ELISA method or an RIA method is used, by directly reacting the culture supernatant with an antigen-adsorbed solid phase and using antibodies corresponding to various types of immunoglobulin isotypes and subclasses as secondary antibodies, the isotype and subclass of the monoclonal antibody can be identified.

[0110]

In addition, as a simpler method, a commercially available identification kit (for example, Mouse Typer Kit manufactured by Bio-Rad Laboratories, Inc.) or the like can

also be used.

[0111]

Further, the quantitative determination of a protein can be performed by the Folin Lowry method and a method of calculation based on the absorbance at 280 nm [1.4 (OD 280) = Immunoglobulin 1 mg/ml].

[0112]

(3) Other antibodies

The antibody of the invention includes not only the above-mentioned monoclonal antibody against Siglec-15 but also a recombinant antibody obtained by artificial modification for the purpose of decreasing heterologous antigenicity to human such as a chimeric antibody, a humanized antibody and a human antibody. These antibodies can be produced using a known method.

[0113]

As the chimeric antibody, an antibody in which antibody variable and constant regions are derived from different species, for example, a chimeric antibody in which a mouse- or rat-derived antibody variable region is connected to a human-derived constant region can be exemplified (see Proc. Natl. Acad. Sci. USA, 81, 6851-6855, (1984)). As one example of the chimeric antibody derived from a rat anti-mouse antibody #32A1, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ

ID NO: 41 in the Sequence Listing and a light chain having an amino acid sequence comprising amino acid residues 21 to 237 of SEQ ID NO: 43 in the Sequence Listing can be exemplified. [0114]

As the humanized antibody, an antibody obtained by integrating only a complementarity determining region (CDR) into a human-derived antibody (see Nature (1986) 321, pp. 522-525), and an antibody obtained by grafting a part of the amino acid residues of the framework as well as the CDR sequence to a human antibody by a CDR-grafting method (WO 90/07861) can be exemplified. As an example of the humanized antibody of a rat antibody #32A1, an arbitrary combination of a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 51 in the Sequence Listing, amino acid residues 20 to 140 of SEQ ID NO: 53, amino acid residues 20 to 140 of SEQ ID NO: 55, amino acid residues 20 to 140 of SEQ ID NO: 57, amino acid residues 20 to 140 of SEQ ID NO: 59, amino acid residues 20 to 140 of SEQ ID NO: 99, amino acid residues 20 to 140 of SEQ ID NO: 101, or amino acid residues 1 to 121 of any one of amino acid sequences represented by SEQ ID NOS: 72 to 77 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 61, amino acid residues 21 to 133 of SEQ ID NO: 63, amino acid residues 21 to 133 of SEQ

ID NO: 65, amino acid residues 21 to 132 of SEQ ID NO: 67, amino acid residues 21 to 133 of SEQ ID NO: 69, amino acid residues 21 to 133 of SEQ ID NO: 71, amino acid residues 21 to 133 of SEQ ID NO: 103, amino acid residues 21 to 133 of SEQ ID NO: 105, or amino acid residues 1 to 113 of any one of amino acid sequences represented by SEQ ID NOS: 78 to 96 can be exemplified.

[0115]

As a preferred combination, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 51 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 61, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 53 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 63, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 55 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 65, an antibody comprising a heavy chain containing a

heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 55 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 132 of SEQ ID NO: 67, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 57 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 69, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 59 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 71, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 99 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 69, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 101 and a light chain containing a light chain variable region comprising an amino acid sequence

comprising amino acid residues 21 to 133 of SEQ ID NO: 69, an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 99 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 103, or an antibody comprising a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of SEQ ID NO: 99 and a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of SEQ ID NO: 105 can be exemplified.

[0116]

As a more preferred combination, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 51 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 61, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 53 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 63, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 55 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 65, an

antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 55 and a light chain having an amino acid sequence comprising amino acid residues 21 to 237 of SEQ ID NO: 67, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 57 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 69, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 470 of SEQ ID NO: 59 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 71, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 466 of SEQ ID NO: 99 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 69, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 466 of SEQ ID NO: 101 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 69, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 466 of SEQ ID NO: 99 and a light chain having an amino acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 103, or an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 466 of SEQ ID NO: 99 and a light chain having an amino

acid sequence comprising amino acid residues 21 to 238 of SEQ ID NO: 105 can be exemplified.

[0117]

However, the humanized antibody derived from the #32A1 antibody is not limited to the above-mentioned humanized antibodies as long as the humanized antibody has all 6 types of CDR sequences of #32A1 and has an activity of inhibiting osteoclast formation. Incidentally, the heavy chain variable region of the #32A1 antibody has CDRH1 (DYFMN) comprising an amino acid sequence represented by SEQ ID NO: 44, either one of CDRH2 (QIRNKIYTYATFYAESLEG) comprising an amino acid sequence represented by SEQ ID NO: 45 and CDRH2 (QIRNKIYTYATFYA) represented by SEQ ID NO: 97, and CDRH3 (SLTGGDYFDY) comprising an amino acid sequence represented by SEQ ID NO: 46. The CDRH2 represented by SEQ ID NO: 45 is in accordance with the Kabat definition (SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST VOL. I, FIFTH EDITION (1991)). The CDRH2 represented by SEQ ID NO: 97 is made shorter by five residues at the C terminus than the Kabat definition. In the heavy chain sequence containing this CDRH2, the CDR sequence derived from a rat is made shorter and more of a human framework sequence is incorporated, and therefore, when it is administered to humans, it is much less likely to be recognized as a heterologous antigen. Further, the light chain variable region of the #32A1 antibody has CDRL1 (RASQSVTISGYSFIH)

comprising an amino acid sequence represented by SEQ ID NO: 47, CDRL2 (RASNLAS) comprising an amino acid sequence represented by SEQ ID NO: 48, and CDRL3 (QQSRKSPWT) comprising an amino acid sequence represented by SEQ ID NO: 49.

[0118]

Further, the antibody of the invention includes a human antibody. A human anti-Siglec-15 antibody refers to a human antibody having only a gene sequence of an antibody derived from a human chromosome. The human anti-Siglec-15 antibody can be obtained by a method using a human antibody-producing mouse having a human chromosome fragment containing heavy and light chain genes of a human antibody (see Tomizuka, K. et al., *Nature Genetics* (1997) 16, pp. 133-143; Kuroiwa, Y. et al., *Nucl. Acids Res.* (1998) 26, pp. 3447-3448; Yoshida, H. et al., *Animal Cell Technology: Basic and Applied Aspects* vol. 10, pp. 69-73 (Kitagawa, Y., Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999; Tomizuka, K. et al., *Proc. Natl. Acad. Sci. USA* (2000) 97, pp. 722-727, etc.).

[0119]

Such a human antibody-producing mouse can be created specifically as follows. A genetically modified animal in which endogenous immunoglobulin heavy and light chain gene loci have been disrupted, and instead, human immunoglobulin heavy and light chain gene loci have been introduced via a yeast artificial chromosome (YAC) vector or the like is created by

producing a knockout animal and a transgenic animal and mating these animals.

[0120]

Further, according to a genetic engineering technique, by using cDNAs encoding such a heavy chain and a light chain of a human antibody, respectively, and preferably a vector containing the cDNAs, eukaryotic cells are transformed, and a transformant which produces a recombinant human monoclonal antibody is cultured, whereby the antibody can also be obtained from the culture supernatant.

[0121]

Here, as the host, for example, eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes or myeloma cells can be used.

[0122]

Further, a method of obtaining a phage display-derived human antibody screened from a human antibody library (see Wormstone, I. M. et al., *Investigative Ophthalmology & Visual Science*. (2002) 43 (7), pp. 2301-2308; Carmen, S. et al., *Briefings in Functional Genomics and Proteomics* (2002), 1 (2), pp. 189-203; Siriwardena, D. et al., *Ophthalmology* (2002) 109 (3), pp. 427-431, etc.) is also known.

[0123]

For example, a phage display method in which a variable region of a human antibody is expressed on the surface of a

phage as a single-chain antibody (scFv), and a phage which binds to an antigen is selected (Nature Biotechnology (2005), 23, (9), pp. 1105-1116) can be used.

[0124]

By analyzing the gene of the phage selected based on the binding to an antigen, a DNA sequence encoding the variable region of a human antibody which binds to an antigen can be determined.

[0125]

If the DNA sequence of scFv which binds to an antigen is determined, a human antibody can be obtained by preparing an expression vector having the sequence and introducing the vector into an appropriate host to express it (WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, WO 95/15388, Annu. Rev. Immunol (1994) 12, pp. 433-455, Nature Biotechnology (2005) 23 (9), pp. 1105-1116).

[0126]

The antibodies obtained by the above method are evaluated for the property of binding to an antigen by the method described in Example 25 or the like, and a preferred antibody can be selected. As one example of another index when the properties of antibodies are compared, the stability of antibodies can be exemplified. The differential scanning calorimetry (DSC) shown in Example 33 is an instrument capable of rapidly and accurately measuring a thermal denaturation

midpoint temperature (T_m) which is a favorable index of relative structural stability of proteins. By measuring T_m values using DSC and comparing the values, a difference in thermal stability can be compared. It is known that the storage stability of antibodies shows some correlation with the thermal stability of antibodies (Lori Burton, et. al., *Pharmaceutical Development and Technology* (2007) 12, pp. 265-273), and a preferred antibody can be selected by using thermal stability as an index. Examples of other indices for selecting antibodies include as follows: the yield in an appropriate host cell is high and the aggregability in an aqueous solution is low. For example, an antibody which shows the highest yield does not always show high thermal stability, and therefore, it is necessary to select an antibody most suitable for administering to humans by making comprehensive evaluation based on the above-mentioned indices.

[0127]

Further, a method in which the full-length heavy and light chain sequences of an antibody are ligated using an appropriate linker, whereby a single-chain immunoglobulin is obtained is also known (Lee, H-S, et. al., *Molecular Immunology* (1999) 36, pp. 61-71; Shirrmann, T. et. al., *mAbs* (2010), 2, (1) pp. 1-4). By dimerizing such a single-chain immunoglobulin, the resulting dimer can have a structure and an activity similar to those of an antibody which is a tetramer

itself. Further, the antibody of the invention may be an antibody which has a single heavy chain variable region and does not have a light chain sequence. Such an antibody is called a single domain antibody (sdAb) or a nanobody, and in fact, it is observed in camels and llamas and has been reported to have an antigen-binding affinity (Muyldemans S. et. al., Protein Eng. (1994) 7(9), 1129-35, Hamers-Casterman C. et. al., Nature (1993) 363 (6428) 446-8). It can be understood that the above-mentioned antibodies are types of functional fragments of the antibody according to the invention.

[0128]

Further, by controlling glycosylation in which a glycan is bound to the antibody of the invention, it is possible to enhance antibody-dependent cellular cytotoxic activity. As regards techniques for controlling the glycosylation of antibodies, WO 99/54342, WO 00/61739, WO 02/31140, etc. are known. However, the techniques are not limited thereto.

[0129]

In cases where an antibody is produced by first isolating an antibody gene and then introducing the gene into an appropriate host, a combination of an appropriate host and an appropriate expression vector can be used. Specific examples of the antibody gene include a combination of a gene encoding a heavy chain sequence of an antibody described in this description and a gene encoding a light chain sequence thereof.

When a host cell is transformed, it is possible to insert the heavy chain sequence gene and the light chain sequence gene into the same expression vector, and also into different expression vectors separately. In cases where eukaryotic cells are used as the host, animal cells, plant cells, and eukaryotic microorganisms can be used. As the animal cells, (1) mammalian cells, for example, dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61); can be exemplified. Further, in cases where prokaryotic cells are used, for example, *Escherichia coli* and *Bacillus subtilis* can be exemplified. By introducing a target antibody gene into these cells through transformation, and culturing the thus transformed cells in vitro, the antibody can be obtained. In the above-mentioned culture method, the yield may sometimes vary depending on the sequence of the antibody, and therefore, it is possible to select one which is easily produced as a pharmaceutical by using the yield as an index among the antibodies having a comparable binding activity.

[0130]

There is no limitation on isotype of the antibody of the invention, and examples thereof include IgG (IgG1, IgG2, IgG3,

IgG4), IgM, IgA (IgA1, IgA2), IgD, and IgE, and preferred examples thereof include IgG and IgM, and further, more preferred examples thereof include IgG1 and IgG2.

[0131]

Further, the antibody of the invention may be a functional fragment of the antibody having an antigen-binding site of the antibody or a modified fragment thereof. The fragment of the antibody can be obtained by treating the antibody with a protease such as papain or pepsin, or modifying the antibody gene according to a genetic engineering technique and expressing the modified gene in suitable cultured cells. Among these antibody fragments, a fragment having all or part of the functions of the full-length molecule of the antibody can be called a functional fragment of the antibody. As the functions of the antibody, generally an antigen-binding activity, an activity of neutralizing the activity of an antigen, an activity of increasing the activity of an antigen, an antibody-dependent cytotoxic activity, a complement-dependent cytotoxic activity, and a complement-dependent cellular cytotoxic activity can be exemplified. The function of the functional fragment of the antibody according to the invention is a binding activity to Siglec-15, preferably an activity of inhibiting the formation of osteoclasts, more preferably an activity of inhibiting the process of cell fusion of osteoclasts.

[0132]

Examples of the fragment of the antibody include Fab, F(ab')₂, Fv, single-chain Fv (scFv) in which Fv molecules of the heavy chain and the light chain are ligated via an appropriate linker, a diabody (diabodies), a linear antibody, and a polyspecific antibody composed of the antibody fragment. Further, Fab' which is a monovalent fragment in a variable region of an antibody obtained by treating F(ab')₂ under reducing conditions is also regarded as a fragment of the antibody.

[0133]

Further, the antibody of the invention may be a polyspecific antibody with specificity for at least two different antigens. In general, such a molecule binds to two antigens (that is, a bispecific antibody), however, the "polyspecific antibody" as used herein includes an antibody having specificity for two or more (for example, three) antigens.

[0134]

The polyspecific antibody of the invention may be a full-length antibody or a fragment of such an antibody (for example, a F(ab')₂ bispecific antibody). The bispecific antibody can be produced by ligating the heavy and light chains (HL pairs) of two types of antibodies, or can also be produced by fusing hybridomas which produce different monoclonal

antibodies to prepare bispecific antibody-producing fused cells (Millstein et al., Nature (1983) 305, pp. 537-539).

[0135]

The antibody of the invention may be a single-chain antibody (also referred to as scFv). The single-chain antibody can be obtained by ligating the heavy chain variable region and the light chain variable region of an antibody via a polypeptide linker (Pluckthun, The Pharmacology of Monoclonal Antibodies, 113 (edited by Rosenberg and Moore, Springer Verlag, New York, pp. 269-315 (1994), Nature Biotechnology (2005), 23, pp. 1126-1136). Further, a BiscFv fragment produced by ligating two scFv molecules via a polypeptide linker can also be used as the bispecific antibody.

[0136]

The method of producing a single-chain antibody is known in this technical field (see, for example, US patent Nos. 4,946,778, 5,260,203, 5,091,513, 5,455,030, etc.). In this scFv, the heavy chain variable region and the light chain variable region are ligated via a linker which does not form a conjugate, preferably via a polypeptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. USA (1988), 85, pp. 5879-5883). In the scFv, the heavy chain variable region and the light chain variable region may be derived from the same antibody or different antibodies.

As the polypeptide linker to be used for ligating the

variable regions, for example, a given single-chain peptide composed of 12 to 19 residues is used.

[0137]

DNA encoding scFv can be obtained by performing amplification using a DNA encoding the entire amino acid sequence, or a desired partial amino acid sequence, selected from the heavy chain or the heavy chain variable region of the above-mentioned antibody and the light chain or the light chain variable region thereof as a template by a PCR method using a primer pair that defines both ends thereof, and further performing amplification by combining a DNA encoding a polypeptide linker portion and a primer pair that defines both ends thereof so as to ligate both of the ends to the heavy chain and the light chain, respectively.

[0138]

Further, once DNA encoding scFv is produced, an expression vector containing the same and a host transformed by the expression vector can be obtained according to common procedures. Further, by using the resulting host, scFv can be obtained according to common procedures. An antibody fragment thereof can be produced in a host by obtaining a gene and expressing the gene in the same manner as described above.

[0139]

The antibody of the invention may be multimerized to increase its affinity for an antigen. The antibody to be

multimerized may be one type of antibody or a plurality of antibodies which recognize a plurality of epitopes of the same antigen. As a method of multimerization of the antibody, binding of the IgG CH3 domain to two scFv molecules, binding to streptavidin, introduction of a helix-turn-helix motif and the like can be exemplified.

[0140]

The antibody of the invention may be a polyclonal antibody which is a mixture of plural types of anti-Siglec-15 antibodies having different amino acid sequences. As one example of the polyclonal antibody, a mixture of plural types of antibodies having different CDR can be exemplified. As such a polyclonal antibody, a mixture of cells which produce different antibodies is cultured, and an antibody purified from the resulting culture can be used (see WO 2004/061104).

[0141]

As a modified antibody, an antibody bound to any of various types of molecules such as polyethylene glycol (PEG) can also be used.

[0142]

Further, the antibody of the invention may be in the form of a conjugate formed between any of these antibodies and another medicinal agent (immunoconjugate). Examples of such an antibody include one in which the antibody is conjugated to a radioactive material or a compound having a

pharmacological action (Nature Biotechnology (2005) 23, pp. 1137-1146).

[0143]

The obtained antibody can be purified to homogeneity. The separation and purification of the antibody can be performed employing a conventional protein separation and purification method. For example, the antibody can be separated and purified by appropriately selecting and combining column chromatography, filter filtration, ultrafiltration, salt precipitation, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing electrophoresis, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the method is not limited thereto.

[0144]

Examples of such chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and adsorption chromatography.

[0145]

Such chromatography can be performed employing liquid chromatography such as HPLC or FPLC.

[0146]

As a column to be used in affinity chromatography, a Protein A column and a Protein G column can be exemplified.

[0147]

For example, as a column using a Protein A column, Hyper D, POROS, Sepharose FF (Pharmacia) and the like can be exemplified.

Further, by using a carrier having an antigen immobilized thereon, the antibody can also be purified utilizing the binding property of the antibody to the antigen.

[0148]

4. Pharmaceutical containing anti-Siglec-15 antibody

From the anti-Siglec-15 antibodies obtained by the method described in the above item "3. Production of anti-Siglec-15 antibody", an antibody which neutralizes the biological activity of Siglec-15 can be obtained. Such an antibody which neutralizes the biological activity of Siglec-15 inhibits the biological activity of Siglec-15 in vivo, i.e., the differentiation and/or maturation of osteoclasts, and therefore can be used as a therapeutic and/or preventive agent for abnormal bone metabolism caused by abnormal differentiation and/or maturation of osteoclasts as a pharmaceutical. The abnormal bone metabolism may be any disorder characterized by net bone loss (osteopenia or osteolysis). In general, the treatment and/or prevention by

the anti-Siglec-15 antibody are/is applied to a case where inhibition of bone resorption is required. Examples of the abnormal bone metabolism which can be treated and/or prevented by the anti-Siglec-15 antibody include osteoporosis (postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent such as a steroid or an immunosuppressant, or osteoporosis accompanying rheumatoid arthritis), bone destruction accompanying rheumatoid arthritis, cancerous hypercalcemia, bone destruction accompanying multiple myeloma or cancer metastasis to bone, giant cell tumor, osteopenia, tooth loss due to periodontitis, osteolysis around a prosthetic joint, bone destruction in chronic osteomyelitis, Paget's disease of bone, renal osteodystrophy, and osteogenesis imperfecta, however, the abnormal bone metabolism is not limited thereto as long as it is a disease accompanied by net bone loss caused by osteoclasts. Examples of the anti-Siglec-15 antibody to be used as the above-mentioned pharmaceutical include a chimeric antibody and a humanized antibody produced from the #32A1 antibody by the method described in item 3. "(3) Other antibodies". Further, a chimeric antibody, a humanized antibody, and a human antibody sharing the same epitope as the #32A1 antibody can also be used as a pharmaceutical. Whether a certain anti-Siglec-15 antibody shares the same epitope as the #32A1 antibody can be confirmed by observing whether or

not these antibodies bind to the same specific partial peptide of Siglec-15. Further, it can also be determined that if a certain anti-Siglec-15 antibody competes with the #32A1 antibody for binding to Siglec-15, these antibodies share the same epitope.

[0149]

The in vitro activity of the anti-Siglec-15 antibody of neutralizing the biological activity of Siglec-15 can be determined by, for example, the activity of inhibiting the differentiation of the cells which overexpress Siglec-15 into osteoclasts. For example, the anti-Siglec-15 antibody is added to RAW 264.7 cells or RAW 264 cells which are a mouse monocyte-derived cell line at various concentrations, and the activity of inhibiting the differentiation into osteoclasts by stimulation with RANKL or TNF- α can be determined. Further, the anti-Siglec-15 antibody is added to bone marrow-derived primary cultured cells at various concentrations, and the activity of inhibiting the differentiation into osteoclasts by stimulation with RANKL, TNF- α , or active vitamin D₃ can be determined. Further, the anti-Siglec-15 antibody is added to normal human osteoclast precursor cells (Normal Human Natural Osteoclast Precursor Cells, available from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) at various concentrations, and the activity of inhibiting the differentiation into osteoclasts by stimulation with RANKL or M-CSF can be determined. Such

an inhibitory effect on osteoclast differentiation can be determined by using the inhibition of tartrate-resistant acid phosphatase (TRAP) activity of osteoclasts as an index. Further, the inhibitory effect on osteoclast differentiation can also be determined by using the inhibition of formation of TRAP-positive multinucleated osteoclasts, i.e., the inhibition of cell fusion of osteoclasts as an index. Further, in an experiment utilising a pit assay (Takada et al., Bone and Mineral, (1992) 17, 347-359) using femur- and/or tibia-derived cells, the in vitro activity of inhibiting the bone resorption by osteoclasts can be determined by adding the anti-Siglec-15 antibody to femur- and/or tibia-derived cells at various concentrations, and observing pit formation on a dentine slice. As a system for determining the in vitro activity of inhibiting the bone resorption by osteoclasts, it is also possible to use a plate coated with human collagen conjugated to europium. The in vivo therapeutic or preventive effect of the anti-Siglec-15 antibody on abnormal bone metabolism using an experimental animal can be confirmed by administering the anti-Siglec-15 antibody to an animal model of osteoporosis or a transgenic animal which overexpresses Siglec-15 and measuring any change in osteoclasts.

[0150]

The thus obtained antibody which neutralizes the biological activity of Siglec-15 is useful as a pharmaceutical,

particularly as a pharmaceutical composition for treating or preventing abnormal bone metabolism such as osteoporosis, bone destruction accompanying rheumatoid arthritis, or bone destruction accompanying cancer metastasis to bone, or as an antibody for immunological diagnosis of such a disease.

[0151]

In the treatment of rheumatoid arthritis (RA), a major problem is bone loss accompanying the occurrence of the disease. It has been reported that in this bone loss accompanying RA, osteoclasts play a primary role. The cytokines considered to be most important for osteoclast induction (differentiation and maturation) and activation and as the cause of bone destruction in RA are RANKL and TNF- α (Romas E. et al., Bone 30, pp. 340-346, 2002). OCIF/OPG which is a decoy receptor for RANKL can inhibit osteoclast formation induced by RANKL but does not inhibit osteoclast formation induced by TNF- α . On the other hand, the anti-Siglec-15 antibody according to the invention effectively inhibited osteoclast formation induced by both RANKL and TNF- α . Therefore, it is expected that the anti-Siglec-15 antibody of the invention can inhibit bone loss and bone destruction induced by TNF- α in RA or the like more strongly than an RANKL blocker (OCIF/OPG, an anti-RANKL antibody, or the like).

[0152]

As one example, for the treatment or prevention of

abnormal bone metabolism, the anti-Siglec-15 antibody can be administered alone or in combination with at least one other therapeutic agent for a bone-related disease. As another example, the anti-Siglec-15 antibody can be administered in combination with a therapeutically effective amount of a therapeutic agent for abnormal bone metabolism. Examples of the therapeutic agent which can be administered in combination with the anti-Siglec-15 antibody include, but are not limited to: bisphosphonates (for example, alendronate, etidronate, ibandronate, incadronate, pamidronate, risedronate, and zoledronate), active vitamin D₃, calcitonin and derivatives thereof, hormones such as estradiol, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents (for example, celecoxib and rofecoxib), soluble TNF receptors (for example, etanercept), anti-TNF- α antibodies or functional fragments of the antibodies (for example, infliximab), anti-PTHrP (parathyroid hormone-related protein) antibodies or functional fragments of the antibodies, IL-1 receptor antagonists (for example, anakinra), anti-IL-6 receptor antibodies or functional fragments of the antibodies (for example, tocilizumab), anti-RANKL antibodies or functional fragments of the antibodies (for example, denosumab), and OCIF (osteoclastogenesis inhibitory factor). Depending on the

state of abnormal bone metabolism or the intended degree of the treatment and/or prevention, two or three, or more types of medicinal agents can be administered, and these medicinal agents can be supplied all together by encapsulating them in the same preparation. These medicinal agents and the anti-Siglec-15 antibody can be supplied all together by encapsulating them in the same preparation. Further, these medicinal agents can be supplied all together by encapsulating them as a kit to be used for treatment and/or prevention. Further, these medicinal agents and the anti-Siglec-15 antibody can be supplied separately. In the case of administration in gene therapy, a gene of a proteinous therapeutic agent for a bone disease and a gene of the anti-Siglec-15 antibody can be inserted downstream of the same promoter region or different promoter regions, and can be introduced into the same vector or different vectors.

[0153]

By conjugating a therapeutic agent for a bone disease to the anti-Siglec-15 antibody or a fragment thereof, a targeted drug conjugate as described in M. C. Garnet "Targeted drug conjugates: principles and progress", Advanced Drug Delivery Reviews, (2001) 53, 171-216 can be produced. For achieving this purpose, other than the antibody molecule, any antibody fragment can be applied as long as it does not completely lose the ability to recognize osteoclasts, and

examples thereof include fragments such as Fab, F(ab')₂, and Fv. In the invention, the antibody and the fragment can be used in the same manner. The conjugate formed by the anti-Siglec-15 antibody or a fragment thereof and a therapeutic agent for a bone disease can be any of various forms described in M. C. Garnet "Targeted drug conjugates: principles and progress", *Advanced Drug Delivery Reviews*, (2001) 53, 171-216, G. T. Hermanson "Bioconjugate Techniques" Academic Press, California (1996), Putnam and J. Kopecek "Polymer Conjugates with Anticancer Activity" *Advances in Polymer Science* (1995) 122, 55-123 and the like. That is, a conjugate in which the anti-Siglec-15 antibody and a therapeutic agent for a bone disease are conjugated to each other chemically and directly or via a spacer such as an oligopeptide and a conjugate formed via an appropriate drug carrier can be exemplified. Examples of the drug carrier include a liposome and a water-soluble polymer. More specific examples of the conjugate formed via such a drug carrier include a conjugate in which the antibody and a therapeutic agent for a bone disease are incorporated in a liposome and the liposome and the antibody are conjugated to each other, and a conjugate in which a therapeutic agent for a bone disease is conjugated to a water-soluble polymer (a compound having a molecular weight of from about 1000 to 100000) chemically and directly or via a spacer such as an oligopeptide and the antibody is conjugated to the

water-soluble polymer. The conjugation of the antibody (or a fragment thereof) to a therapeutic agent for a bone disease or a drug carrier such as a liposome or a water-soluble polymer can be effected by a method known to those skilled in the art such as the method described in G. T. Hermanson "Bioconjugate Techniques" Academic Press, California (1996), Putnam and J. Kopecek "Polymer Conjugates with Anticancer Activity" Advances in Polymer Science (1995) 122, 55-123. The incorporation of a therapeutic agent for a bone disease in a liposome can be effected by a method known to those skilled in the art such as the method described in D. D. Lasic "Liposomes: From Physics to Applications" Elsevier Science Publishers B. V., Amsterdam (1993) or the like. The conjugation of a therapeutic agent for a bone disease to a water-soluble polymer can be effected by a method known to those skilled in the art such as the method described in D. Putnam and J. Kopecek "Polymer Conjugates with Anticancer Activity" Advances in Polymer Science (1995) 122, 55-123. A conjugate of the antibody (or a fragment thereof) and a proteinous therapeutic agent for a bone disease (or a fragment thereof) can be produced by methods known to those skilled in the art through genetic engineering other than the above-mentioned method.

[0154]

The invention also provides a pharmaceutical composition

containing a therapeutically and/or preventively effective amount of the anti-Siglec-15 antibody and a pharmaceutically acceptable diluent, carrier, solubilizing agent, emulsifying agent, preservative, and/or adjuvant.

[0155]

The invention also provides a pharmaceutical composition containing a therapeutically and/or preventively effective amount of the anti-Siglec-15 antibody, a therapeutically and/or preventively effective amount of at least one therapeutic agent for a bone disease, and a pharmaceutically acceptable diluent, carrier, solubilizing agent, emulsifying agent, preservative, and/or adjuvant. Examples of the therapeutic agent for a bone disease include, but are not limited to, bisphosphonates (for example, alendronate, etidronate, ibandronate, incadronate, pamidronate, risedronate, and zoledronate), active vitamin D₃, calcitonin and derivatives thereof, hormones such as estradiol, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents (for example, celecoxib and rofecoxib), soluble TNF receptors (for example, etanercept), anti-TNF- α antibodies or functional fragments of the antibodies (for example, infliximab), anti-PTHrP (parathyroid hormone-related protein) antibodies or functional fragments of the antibodies,

IL-1 receptor antagonists (for example, anakinra), anti-IL-6 receptor antibodies or functional fragments of the antibodies (for example, tocilizumab), anti-RANKL antibodies or functional fragments of the antibodies (for example, denosumab) and OCIF (osteoclastogenesis inhibitory factor).
[0156]

A substance to be used in a preparation acceptable in the pharmaceutical composition according to the invention is preferably non-toxic to a person to whom the pharmaceutical composition is to be administered in terms of the dose and concentration.

[0157]

The pharmaceutical composition of the invention can contain a substance for pharmaceutical use which is capable of changing or maintaining the pH, osmotic pressure, viscosity, transparency, color, isotonicity, aseptic condition, stability, solubility, release rate, absorption rate, and permeability thereof. Examples of such a substance for pharmaceutical use include, but are not limited to, amino acids such as glycine, alanine, glutamine, asparagine, arginine, and lysine; antimicrobial agents; antioxidants such as ascorbic acid, sodium sulfate, and sodium hydrogen sulfite; buffers such as phosphate, citrate, borate buffers, sodium hydrogen carbonate, and Tris-HCl solutions; fillers such as mannitol and glycine; chelating agents such as ethylenediamine

tetraacetate (EDTA); complexing agents such as caffeine, polyvinylpyrrolidone, β -cyclodextrin, and hydroxypropyl- β -cyclodextrin; expanders such as glucose, mannose, and dextrin; other carbohydrates such as monosaccharides and disaccharides; coloring agents; flavors; diluents; emulsifying agents; hydrophilic polymers such as polyvinylpyrrolidone; preservatives such as low molecular weight polypeptides, salt forming counter ions, benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, and hydrogen peroxide; solvents such as glycerin, propylene glycol, and polyethylene glycol; sugar alcohols such as mannitol and sorbitol; suspending agents; surfactants such as sorbitan ester, polysorbates including polysorbate 20 and polysorbate 80, Triton, tromethamine, lecithin, and cholesterol; stability enhancing agents such as sucrose and sorbitol; elasticity enhancing agents such as sodium chloride, potassium chloride, and mannitol and sorbitol; transport agents; excipients; and/or pharmaceutical adjuvants. The amount of these substances added for pharmaceutical use is preferably from 0.01 to 100 times, particularly preferably from 0.1 to 10 times the weight of the anti-Siglec-15 antibody. Those skilled in the art can appropriately determine a preferred formulation of the pharmaceutical composition in a preparation depending on the disease to which the composition

is applied, the route of administration to be applied, or the like.

[0158]

The excipient or carrier in the pharmaceutical composition may be in the form of a liquid or a solid. An appropriate excipient or carrier may be injectable water, physiological saline, an artificial cerebral spinal fluid, or other substance commonly used for parenteral administration. Further, neutral physiological saline or physiological saline containing serum albumin can also be used as a carrier. The pharmaceutical composition may contain a Tris buffer of pH 7.0 to 8.5, an acetate buffer of pH 4.0 to 5.5, or a citrate buffer of pH 3.0 to 6.2. Further, such a buffer may be supplemented with sorbitol or another compound. Examples of the pharmaceutical composition of the invention include a pharmaceutical composition containing the anti-Siglec-15 antibody and a pharmaceutical composition containing the anti-Siglec-15 antibody and at least one therapeutic agent for a bone disease. The pharmaceutical composition of the invention is prepared in the form of a lyophilized product or a liquid as a medicinal agent having a selected composition and a required purity. The pharmaceutical composition containing the anti-Siglec-15 antibody and the pharmaceutical composition containing the anti-Siglec-15 antibody and at least one therapeutic agent for abnormal bone metabolism can

also be formed into a lyophilized product using an appropriate excipient such as sucrose.

[0159]

The pharmaceutical composition of the invention can be prepared for parenteral administration or for gastrointestinal absorption through oral administration. The composition and concentration of a preparation can be determined depending on the administration method. The higher the affinity of the anti-Siglec-15 antibody contained in the pharmaceutical composition of the invention is for Siglec-15, that is, the lower the dissociation constant (K_d value) thereof is for Siglec-15, the more the anti-Siglec-15 antibody can exhibit its drug efficacy even when decreasing the dose for humans. Hence, the dose of the pharmaceutical composition of the invention for humans can also be determined based on this consideration. As for the dose, in the case where a human anti-Siglec-15 antibody is administered to humans, the antibody may be administered at a dose of from about 0.1 to 100 mg/kg once per one to 180 days.

[0160]

Examples of the dosage form of the pharmaceutical composition of the invention include injections including infusions, suppositories, transnasal agents, sublingual agents, and percutaneous absorbents.

Examples

Hereinafter, the invention will be more specifically described with reference to the Examples, however, the invention is not limited thereto. Note that the respective operations regarding gene manipulation in the following Examples were performed according to the methods described in "Molecular Cloning" (written by Sambrook, J., Fritsch, E. F. and Maniatis, T., published by Cold Spring Harbor Laboratory Press in 1989), or in the case of using commercially available reagents or kits, they are used according to the protocols attached thereto unless otherwise stated.

Example 1

[0161]

Production of soluble mouse Siglec-15 protein expression construct

A partial nucleotide sequence encoding the extracellular domain of mouse Siglec-15 protein is represented by SEQ ID NO: 5 in the Sequence Listing and an amino acid sequence thereof is represented by SEQ ID NO: 6 in the Sequence Listing. By utilizing such a partial sequence, soluble mouse Siglec-15 protein can be produced in a culture supernatant of an animal cell or the like.

[0162]

a) Amplification of soluble mouse Siglec-15 gene by PCR

As primers for amplifying the mouse Siglec-15 extracellular domain cDNA by PCR, an oligonucleotide having a sequence of 5'-ggggacaagt ttgtacaaaa aagcaggctt caccATGGAG GGGTCCCTCC AACTC-3' (mSiglec-15-ECD-F: SEQ ID NO: 7 in the Sequence Listing); and an oligonucleotide having a sequence of 5'-ggggaccact ttgtacaaga aagctgggtc TCCGGGGGCG CCGTGGAAGC GGAAC-3' (mSiglec-15-ECD-R: SEQ ID NO: 8 in the Sequence Listing) were synthesized according to a common procedure. Incidentally, these primers were designed as amplification primers for producing a gateway entry clone, such that an attB1 sequence is added to mSiglec-15-ECD-F and an attB2 sequence is added to mSiglec-15-ECD-R. The PCR was performed using this combination of primers and a polynucleotide containing an open reading frame sequence of the mouse Siglec-15 as a template according to a common procedure. The conditions for a thermal cyclers were set as follows: after heating at 94°C for 5 minutes, a temperature cycle of "94°C for 0.5 minutes, 55°C for 0.5 minutes, and 68°C for 1.5 minutes" was repeated 15 times, followed by heating at 68°C for 5 minutes and incubating at 4°C.

[0163]

b) Production of entry clone by Gateway BP reaction

An entry clone into which the mouse Siglec-15 extracellular domain cDNA was integrated by the Gateway technology (Invitrogen, Inc.) employing a lambda phage

site-specific recombination system was produced by the following method. First, a BP reaction using BP Clonase was performed between the PCR product having an attB sequence at both ends produced in a) and pDNOR221 (manufactured by Invitrogen, Inc.) which is a donor vector having an attP sequence. By using this reaction solution, *Escherichia coli* DH10B was transformed, colony PCR was performed for drug-resistant clones, and the size of inserts was confirmed. Then, for a clone confirmed to have an insert with the correct size, a sequence analysis of the total DNA sequence of the insert was performed. As a result, an entry clone which is completely identical to the target nucleotide sequence (SEQ ID NO: 5 in the Sequence Listing) encoding the extracellular domain of the mouse Siglec-15 protein was obtained.

[0164]

c) Production of expression clone by Gateway LR reaction

An expression clone into which the mouse Siglec-15 extracellular domain cDNA was integrated by the Gateway technology (Invitrogen, Inc.) employing a lambda phage site-specific recombination system was produced by the following method. The entry clone produced in b) contains an insert having an attL sequence at both ends. An LR reaction using LR Clonase was performed between this entry clone and two types of destination vectors having an attR sequence. Incidentally, as the destination vectors, two types of

destination vectors: pDONM designed such that a V5 epitope tag and a 6 x His tag are added to the C terminus of the insert; and pHgFc designed such that a human Fc tag is added to the C terminus of the insert were used. By using the reaction solution obtained by the LR reaction, *Escherichia coli* DH10B was transformed, and colony PCR was performed for the obtained drug-resistant clones, and the size of inserts was confirmed. Then, for a clone confirmed to have an insert with the correct size, a sequence analysis of both ends from the insert side to the vector side was performed.

[0165]

Primer sequences for sequence analysis:

5'-tgcggtgaagg tgcagggcag-3' (mSiglec-15-ECD-seq-upstm: SEQ ID NO: 9 in the Sequence Listing)

and

5'-cctcgccctgg tcgggtc-3' (mSiglec-15-ECD-seq-dnstm: SEQ ID NO: 10 in the Sequence Listing)

As a result of the sequence analysis, expression clones (soluble mouse Siglec-15/pDONM and soluble mouse Siglec-15/pHgFc) in which correct recombination occurred for both pDONM and pHgFc, respectively, were obtained. By transfecting the soluble mouse Siglec-15/pDONM into an animal cell or the like, mRNA having a base sequence represented by SEQ ID NO: 11 in the Sequence Listing is transcribed and translated into a protein (mouse Siglec-15-His) having an amino

acid sequence represented by SEQ ID NO: 12 in the Sequence Listing. Further, by transfecting the soluble mouse Siglec-15/phIgFc into an animal cell or the like, mRNA having a base sequence represented by SEQ ID NO: 13 in the Sequence Listing is transcribed and translated into a protein (mouse Siglec-15-Fc) having an amino acid sequence represented by SEQ ID NO: 14 in the Sequence Listing.

Example 2

[0166]

Large-scale preparation of culture solution containing soluble mouse Siglec-15 protein using 293-F cells

The two types of expression vectors (soluble mouse Siglec-15/pDONM and soluble mouse Siglec-15/phIgFc) obtained in Example 1 were prepared in an amount of about 5 mg, respectively. Incidentally, in the purification of plasmids from *Escherichia coli* cultured on a large scale, Invitrogen PureLink HiPure Plasmid Gigaprep Kit (manufactured by Invitrogen, Inc.) was used. The thus prepared plasmid was mixed with Opti-MEM (manufactured by Invitrogen, Inc.), the resulting mixture was sterilized by filtration, 10 ml of a transfection reagent 293fectin (manufactured by Invitrogen, Inc.) was added thereto, and the resulting mixture was incubated at room temperature for 20 minutes. This mixture was added to FreeStyle 293-F cells (manufactured by Invitrogen,

Inc.) cultured in Erlenmeyer flasks such that the cell density reached 1.1×10^6 cells/ml $\times$ 5 L (1 L/flask $\times$ 5 flasks) in FreeStyle 293 Expression Medium (manufactured by Invitrogen, Inc.). After the cells had been subjected to culture with agitation (125 rotations/min) at a CO₂ concentration of 8.0% for 96 hours (4 days) at 37°C, the culture solution was collected and centrifuged to prepare a culture supernatant. It is considered that in the thus prepared culture supernatant, a protein in which a V5 epitope tag and a 6 x His tag have been added to the C-terminal side of the mouse Siglec-15 extracellular domain (mouse Siglec-15-His) and a protein in which a human Fc tag has been added to the C-terminal side of the mouse Siglec-15 extracellular domain (mouse Siglec-15-Fc) are expressed, respectively.

Example 3

[0167]

Purification of mouse Siglec-15-His

a) HisTrap HP column chromatography

To 2 L of the culture solution of mouse Siglec-15-His-expressing 293F cells prepared in Example 2, 225 mL of 10 x buffer (500 mM Tris, 1.5 M NaCl, 200 mM imidazole, pH 8.0) was added, and the resulting mixture was stirred well and filtered through a Sterivex-GV filter (manufactured by Millipore Co., Ltd.). This culture solution was applied at a flow rate of

2 ml/min to a column which comprised three HisTrap HP 5 ml columns (manufactured by Amersham Biosciences, Inc.) connected in series and was previously treated with a pyrogen removing agent PyroCLEAN (manufactured by ALerCHEK, Inc.) and washed with distilled water for injection. After the column was washed with 60 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl at a flow rate of 1 ml/min, a protein adsorbed to the column was eluted with 50 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 500 mM imidazole at a flow rate of 1 ml/min. The eluate was fractionated at 1 ml per fraction into mini-sorp tubes (manufactured by Nunc, Inc.) to which 10 μ l of 10% Tween 20 had previously been added. After about 20 ml of a solution obtained by combining the fractions (fractions 14 to 20) and containing the eluted protein was concentrated to 2.5 ml with a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.), the concentrate was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with phosphate-buffered saline containing 0.01% Tween 20 (T-PBS), followed by elution with T-PBS, whereby 3.5 ml of a sample whose solvent was replaced with T-PBS was obtained.

[0168]

b) Resource Q column chromatography

To 3.5 ml of the sample which was purified by HisTrap

HP column chromatography and whose solvent was replaced with T-PBS, 22.5 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS was added and the resulting mixture was stirred. Then, the mixture was centrifuged at 4°C for 30 minutes at 3,000 rpm and the precipitate was removed. After the resulting supernatant was filtered through a Millex-GV filter (manufactured by Millipore Co., Ltd.), the filtrate was applied at a flow rate of 1 ml/min to a Resource Q 6 ml column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS. Thereafter, the column was washed with this buffer at a flow rate of 1 ml/min and a protein fraction which was not adsorbed to the column was collected. A protein adsorbed to the column was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS and 1 M NaCl at a flow rate of 1 ml/min. After 26.5 ml of the fraction which was not adsorbed to the column was concentrated to 2.0 ml with a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.), the concentrate was centrifuged at 4°C for 10 minutes at 3,000 rpm and the precipitate was removed. The supernatant after centrifugation was cryopreserved at -80°C until use. The above-mentioned purification procedure (HisTrap HP column chromatography and Resource Q column chromatography) was performed by repeating it twice.

[0169]

c) Detection and purity assay of purified mouse Siglec-15-His

By using a sample prepared by the above-mentioned purification procedure (HisTrap HP column chromatography and Resource Q column chromatography), SDS-polyacrylamide electrophoresis under reducing conditions and silver staining were performed. That is, to 5 μ l of each of the samples purified by the respective purification steps, an equivalent amount of an SDS-treatment solution was added, and the resulting mixture was thermally treated at 95°C for 10 minutes. 0.3 μ l of each of the thermally treated samples was used for SDS-polyacrylamide electrophoresis. As a gel for electrophoresis, an 8-25% polyacrylamide gradient gel (manufactured by Amersham Biosciences, Inc.) was used, and the electrophoresis was performed using PhastSystem (manufactured by Amersham Biosciences, Inc.). Further, as molecular weight markers, Rainbow Molecular Weight Markers (manufactured by Amersham Biosciences, Inc.) were used. After completion of the electrophoresis, silver staining was performed using PhastGel Silver Kit (manufactured by Amersham Biosciences, Inc.) and PhastSystem. The results are shown in Fig. 1. It was shown that a protein having a molecular weight of about 35 kDa (mouse Siglec-15-His) was efficiently purified and concentrated in the protein fraction which was not adsorbed to the Resource Q column.

[0170]

Electrophoresis was performed under the same conditions except that ECL DualVue Western Blotting Markers (manufactured by Amersham Biosciences, Inc.) were used as the molecular weight markers, and the protein in the gel was transferred (blotted) to a PVDF membrane (Hybond-P, manufactured by Amersham Biosciences, Inc.) using a PhastTransfer Semi-dry Transfer Kit (manufactured by Amersham Biosciences, Inc.) and PhastSystem. This PVDF membrane was gently shaken in 10 ml of a blocking agent (BlockAce, manufactured by Snow Brand Milk Products, Co., Ltd.) containing 0.1% Tween 20 at room temperature for 1 hour. To this blocking solution, 10 μ l of S-protein HRP (manufactured by Amersham Biosciences, Inc.) and 10 μ l of an anti-V5-HRP antibody (Monoclonal Antibody to Pk-TAG-HRP, manufactured by Acris Antibodies) were added and the membrane in the solution was gently shaken at room temperature for an additional 1 hour. The PVDF membrane was washed 4 times by gently shaking it in 50 mL of phosphate-buffered saline (PBS) containing 0.01% Tween 20 for 5 minutes. After washing, the PVDF membrane was treated according to the protocol accompanying an ECL detection kit (manufactured by Amersham Biosciences, Inc.) to develop the color of the band of the protein, and the developed color was detected using an ECL Mini-Camera (manufactured by Amersham Biosciences, Inc.) and Polaroid film (Polapan 3200B,

manufactured by Polaroid, Inc.). The results are shown in Fig. 2. Also from these results, it could be confirmed that a protein which has a molecular weight of about 35 kDa (mouse Siglec-15-His) and reacts with an anti-V5-HRP antibody was efficiently purified and concentrated in the protein fraction which was not adsorbed to the Resource Q column.

[0171]

d) Measurement of protein concentration of purified mouse Siglec-15-His

For the purified mouse Siglec-15-His (the protein fraction which was not adsorbed to the Resource Q column), the protein concentration was measured with a DC protein assay kit (manufactured by Bio-Rad Laboratories, Inc.) using bovine serum albumin as a standard sample. As shown in Table 1, a total of 1.66 mg of purified mouse Siglec-15-His protein was obtained by performing the purification procedure twice.

[0172]

[Table 1]

	Protein Conc. (mg/ml)	Sample Vol. (ml)	Total protein (mg)
1st	0.475	2.0	0.95
2nd	0.354	2.0	0.71
Total			1.66

Example 4

[0173]

Purification of mouse Siglec-15-Fc

a) HiTrap Protein A column chromatography

1.8 L of the culture solution of mouse Siglec-15-Fc-expressing 293F cells prepared in Example 2 was filtered through a Sterivex-GV filter (manufactured by Millipore Co., Ltd.), and then the filtrate was applied to a HiTrap Protein A 5 ml column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with Dulbecco's PBS (D-PBS, manufactured by Invitrogen, Inc.) at a flow rate of 5 ml/min. After the column was washed with D-PBS at a flow rate of 5 ml/min, a protein adsorbed to the column was eluted with 50 ml of 0.1 M sodium citrate buffer (pH 3.0) at a flow rate of 5 ml/min. The eluate was fractionated at 5 ml per fraction into mini-sorp tubes (manufactured by Nunc, Inc.), and immediately thereafter, 1.3 ml of 1 M Tris was added thereto to neutralize the eluate. After a solution obtained by combining the fractions (fractions 1 and 2) in which the eluted protein was detected was concentrated to 2.5 ml with a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.), the concentrate was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with Otsuka Physiological Saline for Injection (TO-SS, manufactured by Otsuka Pharmaceutical Co., Ltd.) containing 0.01% Tween 20, followed by elution with TO-SS, whereby 3.5 ml of a sample whose solvent was replaced with TO-SS was

obtained. This sample was cryopreserved at -80°C until use. By using 2.9 L of a culture solution of 293F cells, the same purification procedure was performed by repeating it once more. [0174]

b) Detection and purity assay of purified mouse Siglec-15-Fc

By using a sample prepared by the above-mentioned purification procedure, SDS-polyacrylamide electrophoresis under reducing conditions and silver staining were performed. That is, to 5 μl of each of the samples purified by the respective purification steps, an equivalent amount of an SDS-treatment solution was added, and the resulting mixture was heated at 95°C for 10 minutes. 0.3 μl of a sample obtained by diluting each of the thermally treated samples to 1/300 or 1/900 with a half concentration of the SDS-treatment solution was used for SDS-polyacrylamide electrophoresis. The electrophoresis and silver staining were performed in the same manner as the purity assay of mouse Siglec-15-His described in c) of Example 3. The results are shown in Fig. 3 along with the results of examining preliminary purification conditions on a small scale (the pH of the applied culture solution was 8.9 or 7.0). It was shown that a protein having a molecular weight of about 55 kDa (mouse Siglec-15-Fc) was efficiently purified and concentrated in the protein fraction which was eluted from the HiTrap Protein A column.

[0175]

c) Measurement of protein concentration of purified mouse Siglec-15-Fc

For the purified mouse Siglec-15-Fc (the protein fraction eluted from the PD-10 desalting column), the protein concentration was measured with a DC protein assay kit (manufactured by Bio-Rad Laboratories, Inc.) using bovine serum albumin as a standard sample. As shown in Table 2, a total of 92 mg of purified mouse Siglec-15-Fc protein was obtained by performing the purification procedure twice.

[0176]

[Table 2]

	Protein Conc. (mg/ml)	Sample Vol. (ml)	Total protein (mg)
1st	8.0	3.5	28
2nd	18.5	3.5	64
Total			92

Example 5

[0177]

Establishment of rat anti-mouse Siglec-15 monoclonal antibody-producing hybridoma

a) Preparation of antigen

The mouse Siglec-15-His protein produced in Example 3 was prepared at 100 µg/0.5 ml, and an equivalent amount of an adjuvant was added thereto and an emulsion was produced using a glass syringe. As the adjuvant, Freund's complete adjuvant

(FCA, Manufactured by Difco Laboratories, Inc.) was used only for the first immunization, and Freund's incomplete adjuvant (FICA, Manufactured by Difco Laboratories, Inc.) was used for the second immunization and thereafter.

[0178]

b) Immunization of rat

Four rats (Wistar, female, 6 weeks of age, purchased from CLEA Japan, Inc.) were used as immunized animals. The emulsion obtained in a) was injected subcutaneously and intradermally using a 27 G injection needle such that the amount of the antigen was 50 μ g per rat. Immunization was performed a total of 4 times every 7 days after the first immunization. A small amount (200 μ l) of the blood was collected from the tail vein after 7 days from the date of the 4th immunization, and an antiserum was prepared. In order to confirm the antibody titer of the antiserum, ELISA using the mouse Siglec-15-His protein that had been used as the antigen, the mouse Siglec-15-Fc protein produced in Example 4, or bovine serum albumin (BSA), each of which was immobilized, was performed. As a result, the reactivity with the mouse Siglec-15-His protein and the mouse Siglec-15-Fc protein was observed in all four rats (rat Nos. 1 to 4). On the other hand, the reactivity with BSA was not observed. From these results, it was confirmed that the antibody titer in the serum of each of the immunized rats increased, and therefore, the No. 2 rat which showed the highest

antibody titer was subjected to a cell fusion procedure.

[0179]

c) Cell fusion

Cell fusion was performed according to a common method of fusing mouse (rat) spleen cells with myeloma cells. The whole blood was collected from the heart of the rat under ether anesthesia and the rat was euthanized, and then the spleen was excised. The collected spleen cells and P3X63Ag8.653 cells (ATCC CRL 1580) which are mouse myeloma cells were subjected to cell fusion using polyethylene glycol (PEG). The resulting cells were seeded in a 96-well plate, and a medium containing hypoxanthine (H), aminopterin (A) and thymidine (T) (HAT selection medium) was added thereto, and then, the cells were cultured for 7 to 10 days. The culture supernatant was collected from 61 wells in which the survival of hybridomas obtained by cell fusion was confirmed. Then, the antibody titer was evaluated by ELISA using the mouse Siglec-15-His protein that had been used as the antigen, the mouse Siglec-15-Fc protein produced in Example 4, or BSA, each of which was immobilized, and anti-mouse Siglec-15 monoclonal antibody-producing hybridomas were screened. From the results of the screening, 12 wells showing a high antibody titer were selected and the hybridomas contained in the wells were subjected to a cloning procedure.

[0180]

d) Cloning of hybridoma

For the thus selected hybridomas, a first cloning step was performed by a limiting dilution method. After the limiting dilution, the hybridomas were cultured for 2 weeks, and the antibody titer in the culture supernatant was confirmed by ELISA using the mouse Siglec-15-Fc protein produced in Example 4 or BSA, each of which was immobilized. For 11 clones which were confirmed to be positive clones, a second cloning step was performed (in the same manner as the first cloning step), whereby 10 clones of the anti-mouse Siglec-15 monoclonal antibody-producing hybridomas (#1A1, #3A1, #8A1, #24A1, #32A1, #34A1, #39A1, #40A1, #41B1, #61A1) were established in the end. Incidentally, the hybridoma #32A1 was deposited at the International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology (located at Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on August 28, 2008, and has been given an accession number of FERM BP-10999 under the name of anti-Siglec-15 Hybridoma #32A1.

Example 6

[0181]

Preparation of rat anti-mouse Siglec-15 monoclonal antibody

a) Preparation of nude mouse ascites

The hybridomas established in Example 5 were cultured using TIL Media I (manufactured by Immuno-biological Laboratories Co., Ltd.) medium containing 10% FCS. Subculturing of the cells was carried out by performing a procedure in which the culture solution was diluted to about one-fourth every two to three days by using the time point when the cells were grown to about 5×10^5 cells/ml as an index. Each of the thus cultured hybridomas was intraperitoneally implanted in a nude mouse to which pristane had been intraperitoneally administered (0.2 ml/mouse) previously at 1×10^7 cells per mouse. In the implantation, three nude mice were used for each of the 10 clones of hybridomas. After the implantation, the ascites was collected when sufficient accumulation of ascites was observed, which was combined with those collected from the other two mice implanted with the same hybridoma, the amount of the ascites thus combined was measured, and the ascites was cryopreserved until purification of the antibody. The amounts of the collected ascites for the respective hybridomas were summarized in Table 3.

[0182]

[Table 3]

Hybridoma	Amount of collected ascites (ml)	Hybridoma	Amount of collected ascites (ml)
#1A1	12.5	#34A1	8.2
#3A1	8.0	#39A1	14.5
#8A1	6.0	#40A1	20.3
#24A1	7.8	#41B1	10.5
#32A1	5.5	#61A1	12.3

[0183]

b) Purification of antibody

The total amount of the collected ascites was subjected to IgG purification using a 20 ml Protein G column (manufactured by GE Healthcare, Co., Ltd.). The purified IgG was assayed for purity by gel filtration analysis (Superdex 200 column chromatography), and some of the antibodies were subjected to centrifugal membrane concentration. That is, among the purified anti-mouse Siglec-15 monoclonal antibodies, 9 types of antibodies except for the #24A1 antibody were concentrated to about one-sixth to one-eighth of the original volume by centrifuging the antibodies at 3,000 rpm for 30 to 60 minutes at 4°C using a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.). Subsequently, for the #24A1 antibody and the other concentrated 9 types of antibodies, the protein concentration was measured with a DC protein assay kit (manufactured by Bio-Rad Laboratories, Inc.) using bovine serum albumin (BSA) as a standard sample. By the above-mentioned procedure, the anti-mouse Siglec-15 monoclonal antibody was prepared.

Example 7

[0184]

Evaluation of property of rat anti-mouse Siglec-15 monoclonal antibody binding to mouse Siglec-15 protein

The property of the rat anti-mouse Siglec-15 monoclonal antibody binding to mouse Siglec-15 protein was evaluated by an ELISA method. The mouse Siglec-15-Fc protein produced in Example 4 was diluted to 5 µg/ml with 0.1 M sodium carbonate buffer (pH 9.5), and the resulting solution was added to a 96-well plate (manufactured by Nalge Nunc International, Inc., Cat. No. 430341) at 100 µl/well. After the plate was left at room temperature for 1 hour, the solution was removed and a washing buffer (phosphate-buffered saline containing 0.05% Tween 20) was added at 300 µl/well and then removed. After this washing procedure was performed one more time, phosphate-buffered saline containing 25% BlockAce (manufactured by Dainippon Sumitomo Pharma Co., Ltd.) was added at 200 µl/well, and the plate was left at room temperature for 1 hour, whereby blocking was performed. The liquid was removed, and the plate was washed twice with 300 µl/well of the washing buffer. Then, each of the rat anti-mouse Siglec-15 monoclonal antibodies prepared in Example 6 or rat control IgG (manufactured by R&D Systems, Inc.) was diluted to a final concentration of from 1.28 to 20,000 ng/ml (5-fold dilution

series) with an ELISA buffer (phosphate-buffered saline containing 12.5% BlockAce and 0.05% Tween 20), and the resulting diluted antibody solution was added to the plate at 100 μ l/well. After the plate was left at room temperature for 1 hour, the liquid was removed, and the plate was washed 3 times with 300 μ l/well of the washing buffer. Subsequently, an HRP (horseradish peroxidase)-labeled goat anti-rat IgG antibody (manufactured by Beckman Coulter, Inc.) diluted to 1,000-fold with an ELISA buffer was added at 100 μ l/well, and the plate was left at room temperature for 1 hour. The liquid was removed and the plate was washed 3 times with 300 μ l/well of the washing buffer, and then, by using a color developing kit for peroxidase (manufactured by Sumitomo Bakelite Co., Ltd.), the color was developed according to the protocol accompanying the kit. After developing the color, an absorbance at 492 nm was measured using a microplate reader (manufactured by Nihon Molecular Devices Corporation). As a result, it was confirmed that all of the examined 10 test samples of the rat anti-mouse Siglec-15 monoclonal antibodies bind to the mouse Siglec-15 protein in an antibody concentration-dependent manner (Fig. 4). On the other hand, in the case of the rat control IgG, binding to the mouse Siglec-15 protein was not observed.

Example 8

[0185]

Preparation of mouse bone marrow nonadherent cells

The femur and tibia were excised from a male ddY mouse at the age of 5 to 8 weeks and soft tissues were removed. Both ends of the femur or tibia were cut off, and D-PBS was injected using a syringe with a 25-gauge injection needle to push out bone marrow cells, which were collected in a centrifugal tube. Centrifugation was performed at room temperature for 5 minutes at 100 g, and the supernatant was removed. To the resulting cell pellet, 1 ml of a hemolytic buffer (Red Blood Cell Lysing Buffer, manufactured by Sigma Co., Ltd.) was added to suspend it, and the resulting suspension was left at room temperature for 5 minutes. 20 ml of D-PBS was added thereto, and the suspension was centrifuged at room temperature for 5 minutes at 100 g, and the supernatant was removed. To the resulting cell pellet, 10 ml of MEM- α medium (manufactured by Invitrogen, Inc.) containing 5 ng/ml of M-CSF (manufactured by R&D Systems, Inc.) and 10% fetal bovine serum (FBS) was added to suspend it. Then, the resulting suspension was passed through a cell strainer (40 μ m Nylon, manufactured by BD Falcon) to remove aggregates. The resulting cells were transferred to a 75 cm² T-flask (for the attachment of adherent cells) and cultured overnight in a CO₂ incubator. After the overnight culture, the cells which did not adhere to the T-flask were recovered and used as mouse bone marrow nonadherent cells.

Example 9

[0186]

Evaluation of biological activity of rat anti-mouse Siglec-15 monoclonal antibody based on test for mouse osteoclast formation

By using all of the 10 test samples of the anti-mouse Siglec-15 monoclonal antibodies produced in Example 6, an effect on osteoclast differentiation of mouse bone marrow nonadherent cells was examined. The mouse bone marrow nonadherent cells prepared by the method of Example 8 were prepared at 1.5×10^5 cells/ml in α -MEM medium containing 10% FBS and 10 ng/ml of M-CSF (manufactured by R&D Systems, Inc.), and the resulting cell preparation was seeded in each well of a 96-well plate in an amount of 200 μ l and the cells were cultured for 2 days in a CO₂ incubator. The old culture solution in the 96-well plate was removed, and 100 μ l of MEM- α medium was added to each well, the 100 μ l of MEM- α medium containing 10% FBS to which human RANKL (RANKL, manufactured by Peprotech, Inc.) and M-CSF had been added to give final concentrations of 20 ng/ml and 10 ng/ml, respectively. To the cell culture solution, each of the rat anti-mouse Siglec-15 monoclonal antibodies produced in Example 6, a sample obtained by removing sodium azide from commercially available rat control IgG (purified rat IgG, manufactured by R&D Systems, Inc.), or a separately produced rabbit anti-mouse Siglec-15 polyclonal antibody (No.

3) was added at a concentration of from 32 to 1,000 ng/ml, and the cells were cultured for an additional 3 days in a CO₂ incubator. Incidentally, the polyclonal antibody (No. 3) is an antibody which has already been confirmed to inhibit osteoclast formation in the experimental system described in this Example. After completion of the culturing, the activity of tartrate-resistant acid phosphatase (TRAP) of the formed osteoclasts was measured by a procedure described below. The culture solution in each well of the 96-well plate was removed by suction, and 50 µl of 50 mM sodium citrate buffer (pH 6.1) containing 1% Triton X-100 was added to each well. Then, the plate was shaken for 5 minutes on a plate shaker to lyse the cells. To each well, 50 µl of a substrate solution (50 mM sodium citrate buffer (pH 6.1) containing 5 mg/ml p-nitrophenyl phosphate and 0.46% sodium tartrate) was added, and the plate was incubated at room temperature for 5 minutes. After the incubation, 50 µl of a 1 N sodium hydroxide solution was added to each well of the 96-well plate to stop the enzymatic reaction. After stopping the enzymatic reaction, an absorbance of each well at 405 nm was measured, and the obtained measurement was used as an index of TRAP activity. The results are shown in Figs. 5 and 6. A significant inhibition of TRAP activity was not observed in the case of the commercially available rat control IgG. On the other hand, a significant inhibition of TRAP activity was observed in the cases of the #32A1 antibody

added in the range of from 32 ng/ml to 1000 ng/ml, and the #8A1 antibody and the affinity-purified rabbit polyclonal No. 3 antibody added in the range of from 63 ng/ml to 1000 ng/ml. Also in the cases of the #3A1 antibody, #34A1 antibody, and #39A1 antibody, a dose-dependent inhibition of TRAP activity was observed at a relatively high concentration of 500 ng/ml or higher. The inhibition of mouse osteoclast formation by the other antibodies was not observed. From the above results, antibodies which strongly inhibit mouse osteoclast formation (osteoclast differentiation and maturation) were found among the prepared rat anti-mouse Siglec-15 monoclonal antibodies. Further, as a property common to the #3A1 antibody, #8A1 antibody, #32A1 antibody, #34A1 antibody, and #39A1 antibody, the activity of inhibiting osteoclast formation at a concentration of 1000 ng/ml, i.e., 1 µg/ml or less can be exemplified.

Example 10

[0187]

Production of soluble human Siglec-15 protein expression construct

A partial nucleotide sequence encoding the extracellular domain of human Siglec-15 protein is represented by SEQ ID NO: 15 in the Sequence Listing and the amino acid sequence thereof is represented by SEQ ID NO: 16 in the Sequence Listing. By

utilizing such a partial sequence, soluble human Siglec-15 protein can be produced in a culture supernatant of an animal cell or the like.

[0188]

a) Amplification of soluble human Siglec-15 gene by PCR

As primers for amplifying the human Siglec-15 extracellular domain cDNA by PCR, an oligonucleotide having a sequence of 5'-ggggacaagt ttgtacaaaa aagcaggctt caccATGGAA AAGTCCATCT GGCTGC-3' (hSiglec-15-ECD-F: SEQ ID NO: 17 in the Sequence Listing); and an oligonucleotide having a sequence of 5'-ggggaccact ttgtacaaga aagctgggtc CCCGCTGGCG CCATGGAAGC GG-3' (hSiglec-15-ECD-R: SEQ ID NO: 18 in the Sequence Listing) were synthesized according to a common procedure. Incidentally, these primers were designed, as amplification primers for producing a gateway entry clone, such that an attB1 sequence is added to hSiglec-15-ECD-F and an attB2 sequence is added to hSiglec-15-ECD-R. The PCR was performed using this combination of primers and a polynucleotide containing an open reading sequence of the human Siglec-15 as a template according to a common procedure. The resulting PCR reaction solution was purified using PureLink PCR Purification Kit (manufactured by Invitrogen, Inc.).

[0189]

b) Production of entry clone by Gateway BP reaction

An entry clone into which the human Siglec-15

extracellular domain cDNA was integrated by the Gateway technology (Invitrogen, Inc.) employing a lambda phage site-specific recombination system was produced by the following method. First, a BP reaction using BP Clonase was performed between the PCR product having an attB sequence at both ends produced in a) and pDNOR221 (manufactured by Invitrogen, Inc.) which is a donor vector having an attP sequence. By using this reaction solution, *Escherichia coli* TOP10 was transformed, colony PCR was performed for drug-resistant clones, and the size of inserts was confirmed. Then, for a clone confirmed to have an insert with the correct size, a sequence analysis of the total DNA sequence of the insert was performed. As a result, an entry clone which is completely identical to the target nucleotide sequence (SEQ ID NO: 15 in the Sequence Listing) encoding the extracellular domain of the human Siglec-15 protein was obtained.

[0190]

c) Production of expression clone by Gateway LR reaction

An expression clone into which the human Siglec-15 extracellular domain cDNA was integrated by the Gateway technology (Invitrogen, Inc.) employing a lambda phage site-specific recombination system was produced by the following method. The entry clone produced in b) contains an insert having an attL sequence at both ends. An LR reaction using LR Clonase was performed between this entry clone and

two types of destination vectors having an attR sequence. Incidentally, as the destination vectors, two types of destination vectors: pDONM designed such that a V5 epitope tag and a 6 x His tag are added to the C terminus of the insert; and pHgFc designed such that a human Fc tag is added to the C terminus of the insert, were used. By using the reaction solution obtained by the LR reaction, *Escherichia coli* TOP10 was transformed, and a sequence analysis was performed for the resulting drug-resistant clones to confirm whether correct recombination occurred.

As a result of the sequence analysis, expression clones (soluble human Siglec-15/pDONM and soluble human Siglec-15/pHgFc) in which correct recombination occurred for both pDONM and pHgFc, respectively, were obtained. By transfecting the soluble human Siglec-15/pDONM into an animal cell or the like, mRNA having a base sequence represented by SEQ ID NO: 19 in the Sequence Listing is transcribed and translated into a protein (human Siglec-15-His) having an amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing. Further, by transfecting the soluble human Siglec-15/pHgFc into an animal cell or the like, mRNA having a base sequence represented by SEQ ID NO: 21 in the Sequence Listing is transcribed and translated into a protein (human Siglec-15-Fc) having an amino acid sequence represented by SEQ ID NO: 22 in the Sequence Listing.

Example 11

[0191]

Large-scale preparation of culture solution containing soluble human Siglec-15 protein using 293-F cells

a) Preparation of culture solution containing human Siglec-15-His

The soluble human Siglec-15/pDONM obtained in Example 10 was prepared in an amount of about 25 mg. Incidentally, in the purification of plasmids from *Escherichia coli* cultured on a large scale, Invitrogen PureLink HiPure Plasmid Gigaprep Kit (manufactured by Invitrogen, Inc.) was used. The thus prepared plasmid was mixed with Opti-MEM (manufactured by Invitrogen, Inc.), 50 ml of transfection reagent 293fectin (manufactured by Invitrogen, Inc.) was added thereto, and the resulting mixture was incubated at room temperature for 20 minutes. This mixture was added to FreeStyle 293-F cells (manufactured by Invitrogen, Inc.) cultured in FreeStyle 293 Expression Medium (manufactured by Invitrogen, Inc.) containing 1% penicillin-streptomycin such that the cell density reached 1.0 to 3.4×10^6 cells/ml using a 25 L bioprocess culture apparatus (WAVE Bioreactor). After the cells had been subjected to culture with agitation (30 rotations/min) at a CO₂ concentration of from 6 to 12% for 96 hours (4 days) at 37°C, the culture solution was collected and centrifuged to prepare

a culture supernatant. It is observed that in the thus prepared culture supernatant, a protein in which a V5 epitope tag and a 6 x His tag have been added to the C-terminal side of the human Siglec-15 extracellular domain (human Siglec-15-His) is expressed.

[0192]

b) Preparation of culture solution containing human Siglec-15-Fc

The soluble human Siglec-15/phIgFc obtained in Example 10 was prepared in an amount of about 5 mg. Incidentally, in the purification of plasmids from *Escherichia coli* cultured on a large scale, Invitrogen PureLink HiPure Plasmid Gigaprep Kit (manufactured by Invitrogen, Inc.) was used. The thus prepared plasmid was mixed with Opti-MEM (manufactured by Invitrogen, Inc.), followed by filter sterilization. Then, 10 ml of a transfection reagent 293fectin (manufactured by Invitrogen, Inc.) was added thereto, and the resulting mixture was incubated at room temperature for 20 minutes. This mixture was added to FreeStyle 293-F cells (manufactured by Invitrogen, Inc.) cultured in Erlenmeyer flasks such that the cell density reached 1.0 to 3.0×10^6 cells/ml x 5 L (1 L/flask x 5 flasks) in FreeStyle 293 Expression Medium (manufactured by Invitrogen, Inc.). After the cells had been subjected to culture with agitation (125 rotations/min) at a CO₂ concentration of 8.0% for 96 hours (4 days) at 37°C, the culture solution was collected

and centrifuged to prepare a culture supernatant. It is observed that in the thus prepared culture supernatant, a protein in which a human Fc tag has been added to the C-terminal side of the human Siglec-15 extracellular domain (human Siglec-15-Fc) is expressed.

Example 12

[0193]

Purification of soluble human Siglec-15 protein

a) Purification of soluble human Siglec-15-His

a-i) HisTrap HP column chromatography

To 12 L of the culture solution of 293F cells expressing human Siglec-15-His prepared in a) of Example 11, 1350 mL of 10 x buffer (500 mM Tris, 1.5 M NaCl, 200 mM imidazole, pH 8.0) was added, and the resulting mixture was stirred well and filtered through a MilliPak-60 filter (manufactured by Millipore Co., Ltd.). This culture solution was applied at a flow rate of 10 ml/min to a Ni-Sepharose HP (manufactured by Amersham Biosciences, Inc.) 100 ml column which had previously been washed with pure water (Milli-Q water). After the column had been washed with 400 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl at a flow rate of 8 mL/min, a protein adsorbed to the column was eluted with 200 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 500 mM imidazole at a flow rate of 2.5 ml/min, and the eluate was

fractionated into mini-sorp tubes (manufactured by Nunc, Inc.). In order to prevent precipitation of the protein, 8 ml of a 5 M NaCl solution was added to about 40 ml of a fraction containing the eluted protein, followed by stirring, and then the resulting mixture was concentrated to about 20 ml with a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.). Insoluble matter generated during the concentration was removed by centrifugation at 3000 rpm for 30 minutes at 4°C, and 2.5 ml of the resulting supernatant was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with phosphate-buffered saline containing 1 M NaCl (N-PBS), followed by elution with N-PBS, whereby 3.5 ml of a sample whose solvent was replaced with N-PBS was obtained. This procedure was performed by repeating it 7 more times, and about 28 ml of a solution of partially purified human Siglec-15-His was obtained.

[0194]

a-ii) Resource Q column chromatography

12 ml of the sample which had been purified by Ni-Sepharose HP column chromatography and whose solvent had been replaced with N-PBS was dialyzed overnight at 4°C against 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS (1 L, 3 times) and the resulting dialysate was centrifuged at 3,000 rpm for 30 minutes at 4°C, and the precipitate was removed.

After the resulting supernatant had been filtered through a Millex-GV filter (manufactured by Millipore Co., Ltd.), the filtrate was applied at a flow rate of 1 ml/min to a Resource Q 6 ml column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS. Thereafter, the column was washed with this buffer at a flow rate of 1 ml/min and a protein fraction which was not adsorbed to the column was collected. A protein adsorbed to the column was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS and 1 M NaCl at a flow rate of 1 ml/min. After 26.5 ml of the fraction which had not been adsorbed to the column was concentrated to 3.0 ml with a centrifugal membrane concentrator Amicon Ultra-15 (manufactured by Millipore Co., Ltd.), the concentrate was centrifuged at 3,000 rpm for 10 minutes at 4°C and the precipitate was removed. 2.5 ml of the resulting supernatant was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with phosphate-buffered saline containing 50 mM arginine hydrochloride (pH 7.0, A-PBS), followed by elution with A-PBS, whereby 3.5 ml of a sample whose solvent was replaced with A-PBS was obtained. The arginine hydrochloride in the solvent of the prepared sample was added in order to prevent soluble human Siglec-15-His from precipitating. The supernatant after centrifugation was cryopreserved at -80°C

until use. The above-mentioned purification procedure (Resource Q column chromatography) was performed by repeating it twice.

[0195]

a-iii) Detection and purity assay of purified human Siglec-15-His

By using a sample prepared by the above-mentioned purification procedure (Ni-Sepharose HP column chromatography and Resource Q column chromatography), SDS-polyacrylamide electrophoresis under reducing conditions and silver staining were performed. That is, to 5 μ l of each of the samples purified by the respective purification steps, an equivalent amount of an SDS-treatment solution was added, and the resulting mixture was thermally treated at 95°C for 10 minutes. 0.3 μ l of each of the thermally treated samples was used for SDS-polyacrylamide electrophoresis. The electrophoresis procedure was performed in the same manner as in Example 3 except that Rainbow Molecular Weight Markers (manufactured by Amersham Biosciences, Inc.) were used as the molecular weight markers. After completion of the electrophoresis, silver staining was performed using a PhastGel Silver Kit (manufactured by Amersham Biosciences, Inc.) and PhastSystem. The results are shown in Fig. 7. It was shown that a protein having a molecular weight of about 35 kDa (human Siglec-15-His) was efficiently purified and concentrated in the protein

fraction which was not adsorbed to the Resource Q column.

[0196]

a-iv) Measurement of protein concentration of purified human Siglec-15-His

For the purified human Siglec-15-His (the protein fraction which was not adsorbed to the Resource Q column), the protein concentration was measured with a DC protein assay kit (manufactured by Bio-Rad Laboratories, Inc.) using bovine serum albumin as a standard sample. By performing the purification procedure twice, a total of 1.66 mg of purified human Siglec-15-His was obtained.

[0197]

b) Purification of soluble human Siglec-15-Fc

b-i) HiTrap Protein A column chromatography

1.5 L of the culture solution of 293F cells expressing human Siglec-15-Fc prepared in b) of Example 11 was filtered through a Sterivex-GV filter (manufactured by Millipore Co., Ltd.), and then the filtrate was applied at a flow rate of 5 ml/min to a HiTrap Protein A 5 ml column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with Dulbecco's PBS (D-PBS, manufactured by Invitrogen, Inc.). After the column had been washed with 70 ml of D-PBS at a flow rate of 5 ml/min, a protein adsorbed to the column was eluted with 24 ml of 0.1 M sodium citrate buffer (pH 3.0) at a flow rate of 1.2 ml/min. The eluate was

fractionated at 1.2 ml per fraction into mini-sorp tubes (manufactured by Nunc, Inc.), and immediately thereafter, 0.31 ml of 1 M Tris was added thereto to neutralize the eluate. A 2.5 ml aliquot of a solution (about 7.5 ml) obtained by combining the eluted protein fractions (fractions 5 to 9) was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with phosphate-buffered saline containing 50 mM arginine hydrochloride (pH 7.0, A-PBS), followed by elution with A-PBS, whereby 3.5 ml of a sample whose solvent was replaced with A-PBS was obtained. This procedure was performed by repeating it twice. The arginine hydrochloride in the solvent was added in order to prevent soluble human Siglec-15-Fc from precipitating. 2.5 ml of the remaining solution of the eluted protein fractions (fractions 5 to 9) was applied to a PD-10 desalting column (manufactured by Amersham Biosciences, Inc.) which had previously been equilibrated with phosphate-buffered saline containing 1 M NaCl (pH 6.7, N-PBS), followed by elution with N-PBS, whereby 3.5 ml of a sample whose solvent was replaced with N-PBS was obtained. NaCl in the solvent in the prepared sample was added in order to prevent soluble human Siglec-15-Fc from precipitating without adding an amino group-containing compound such as arginine. The samples prepared by the above-mentioned procedure were cryopreserved at -80°C until use.

[0198]

b-ii) Detection and purity assay of purified human Siglec-15-Fc

By using the samples prepared by the above-mentioned purification procedure, SDS-polyacrylamide electrophoresis under reducing conditions and silver staining were performed. That is, to 5 μ l of each of the samples purified by the respective purification steps, an equivalent amount of an SDS-treatment solution was added, and the resulting mixture was heated at 95°C for 10 minutes. 0.3 μ l of a sample obtained by diluting each of the thermally treated samples to 1/100 or 1/300 with a half concentration of the SDS-treatment solution was used for SDS-polyacrylamide electrophoresis. The electrophoresis and silver staining were performed in the same manner as the purity assay of human Siglec-15-His described in a-iii). The results are shown in Fig. 8. It was shown that a protein having a molecular weight of about 55 kDa (human Siglec-15-Fc) was efficiently purified and concentrated in the protein fraction which was eluted from the HiTrap Protein A column.

[0199]

b-iii) Measurement of protein concentration of purified human Siglec-15-Fc

For the purified human Siglec-15-Fc (the protein fraction eluted from the PD-10 desalting column), the protein concentration was measured with a DC protein assay kit

(manufactured by Bio-Rad Laboratories, Inc.) using bovine serum albumin as a standard sample. As shown in Table 4, a total of 25.2 mg of purified human Siglec-15-Fc was obtained by performing the purification procedure twice.

[0200]

[Table 4]

	Protein Conc. (mg/ml)	Sample Vol. (ml)	Total protein (mg)
Arg-containing solvent	2.3	7.0	16.1
NaCl-containing solvent	2.6	3.5	9.1
Total			25.2

Example 13

[0201]

Evaluation of property of rat anti-mouse Siglec-15 monoclonal antibody binding to human Siglec-15 protein

The property of the rat anti-mouse Siglec-15 monoclonal antibody binding to human Siglec-15 protein was evaluated by an ELISA method. The human Siglec-15-Fc protein (subjected to replacement with A-PBS) produced in b) of Example 12 was diluted to 5 µg/ml with 0.1 M sodium carbonate buffer (pH 9.5), and the resulting solution was added to a 96-well plate (manufactured by Nalge Nunc International, Inc., Cat. No. 430341) at 100 µl/well. After the plate had been left at room temperature for 1 hour, the solution was removed and a washing buffer (phosphate-buffered saline containing 0.05% Tween 20) was added at 300 µl/well and then removed. After this washing

procedure had been performed one more time, phosphate-buffered saline containing 25% BlockAce (manufactured by Dainippon Sumitomo Pharma Co., Ltd.) was added at 200 μ l/well, and the plate was left at room temperature for 1 hour, whereby blocking was performed. The liquid was removed, and the plate was washed twice with 300 μ l/well of the washing buffer. Then, each of the rat anti-mouse Siglec-15 monoclonal antibodies prepared in Example 6 or rat control IgG (manufactured by R&D Systems, Inc.) was diluted to a final concentration of from 1.28 to 20,000 ng/ml (5-fold dilution series) with an ELISA buffer (phosphate-buffered saline containing 12.5% BlockAce and 0.05% Tween 20), and the resulting diluted antibody solution was added to the plate at 100 μ l/well. After the plate was left at room temperature for 1 hour, the liquid was removed, and the plate was washed 3 times with 300 μ l/well of the washing buffer. Subsequently, an HRP (horseradish peroxidase)-labeled goat anti-rat IgG antibody (manufactured by Beckman Coulter, Inc.) diluted to 1,000-fold with an ELISA buffer was added at 100 μ l/well, and the plate was left at room temperature for 1 hour. The liquid was removed and the plate was washed 3 times with 300 μ l/well of the washing buffer, and then, by using a color developing kit for peroxidase (manufactured by Sumitomo Bakelite Co., Ltd.), the color was developed according to the protocol accompanying the kit. After developing the color, an absorbance at 492 nm was measured

using a microplate reader (manufactured by Nihon Molecular Devices Corporation). As a result, it was confirmed that all of the examined 10 test samples of the rat anti-mouse Siglec-15 monoclonal antibodies bind to the human Siglec-15 protein in an antibody concentration-dependent manner (Fig. 9). In particular, the binding activity of 5 test samples, namely #1A1, #3A1, #24A1, #32A1, and #61A1, was high, and the binding activity of 3 test samples, namely #8A1, #34A1, and #39A1, was relatively low. On the other hand, in the case of the rat control IgG, binding to the human Siglec-15 protein was not observed. From the above results, it was shown that the rat anti-mouse Siglec-15 monoclonal antibodies prepared in Example 6 bind not only to mouse Siglec-15, but also to human Siglec-15, and moreover, it was found that some antibodies strongly bind to human Siglec-15.

Example 14

[0202]

Effect of addition of rat anti-mouse Siglec-15 monoclonal antibody on cell fusion and bone resorption activity of normal human osteoclast precursor cells (evaluation of in vitro biological activity)

Since it was confirmed that the rat anti-mouse Siglec-15 monoclonal antibodies bind also to human Siglec-15 in Example 13, the effects of these antibodies on human osteoclast

formation and bone resorption activity were examined.

[0203]

a) Effect of addition of rat anti-mouse Siglec-15 monoclonal antibody on cell fusion of osteoclasts from normal human osteoclast precursor cells (TRAP Staining)

Normal human osteoclast precursor cells (Normal Human Natural Osteoclast Precursor Cells, purchased from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) were seeded in a 96-well plate at 1×10^4 cells/well according to the protocol accompanying the cells. As the medium, a minimal essential medium for osteoclast precursor cells (OPBM, purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-8201) supplemented with an OPGM supplement set (purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-9501) containing fetal bovine serum (final concentration: 10%), human RANKL (final concentration: 66 ng/ml), human M-CSF (final concentration: 33 ng/ml), and the like was used. To the resulting culture supernatant, each of the rat anti-mouse Siglec-15 monoclonal antibodies prepared in Example 6 or rat control IgG (manufactured by R&D Systems, Inc.) was added to give a final concentration of 30 $\mu\text{g/ml}$, and the cells were cultured for 4 days in a CO_2 incubator. After the culturing, the supernatant was removed, and 10% neutral formalin was added to fix the cells. After fixing the cells, the cells were washed twice with distilled water, a TRAP staining solution (0.27 mM naphthol AS-MX phosphate

(manufactured by Sigma Co., Ltd.), 1.6 mM fast red violet LB salt (manufactured by Sigma Co., Ltd.), 1% dimethylformamide, 50 mM sodium tartrate, 0.1 M sodium acetate buffer (pH 5.0)) was added at 100 μ l/well, and a reaction was allowed to proceed at room temperature for 5 minutes. After the reaction, the cells were washed twice with distilled water, and then observed by microscopy (Fig. 10). As a result, the formation of giant osteoclasts resulting from a high degree of cell fusion was almost completely inhibited by the addition of the #32A1 antibody. Further, in the case of the #41B1 antibody, the formation of giant osteoclasts resulting from a high degree of cell fusion was also significantly inhibited. On the other hand, in the case of the other rat anti-mouse Siglec-15 monoclonal antibodies (the #1A1 antibody and others) and the rat control IgG, such a significant inhibition of osteoclast cell fusion was not observed. In this manner, it was revealed that multinucleation and cell fusion of TRAP-positive osteoclasts from normal human osteoclast precursor cells are inhibited by monoclonal antibodies specifically binding to the Siglec-15 protein.

[0204]

b) Effect of addition of rat anti-mouse Siglec-15 monoclonal antibody (#32A1) on cell fusion of osteoclasts from normal human osteoclast precursor cells (TRAP Staining)

Normal human osteoclast precursor cells (Normal Human

Natural Osteoclast Precursor Cells, purchased from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) were seeded in a 96-well plate at 1×10^4 cells/well according to the protocol accompanying the cells. As the medium, a minimal essential medium for osteoclast precursor cells (OPBM, purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-8201) supplemented with an OPGM supplement set (purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-9501) containing fetal bovine serum (final concentration: 10%), human RANKL (final concentration: 68.4 ng/ml), human M-CSF (final concentration: 33 ng/ml), and the like was used. To the resulting culture supernatant, the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) prepared in Example 6 was added to give a final concentration of 0.1, 0.3, 1, or 3 $\mu\text{g/ml}$, and the cells were cultured for 3 days in a CO_2 incubator. After the culturing, the supernatant was removed, and 10% neutral formalin was added to fix the cells. After fixing the cells, the cells were washed twice with distilled water, and a TRAP staining solution (0.27 mM naphthol AS-MX phosphate (manufactured by Sigma Co., Ltd.), 1.6 mM fast red violet LB salt (manufactured by Sigma Co., Ltd.), 1% dimethylformamide, 50 mM sodium tartrate, 0.1 M sodium acetate buffer (pH 5.0)) was added at 100 $\mu\text{l/well}$, and a reaction was allowed to proceed at room temperature for 5 minutes. After the reaction, the cells were washed twice with distilled water, and then, observed by microscopy (Fig. 11). As a result, the

formation of TRAP-positive multinucleated osteoclasts was inhibited in a #32A1 antibody concentration-dependent manner within the range of from 0.3 µg/ml to 3 µg/ml.

[0205]

c) Effect of addition of rat anti-mouse Siglec-15 monoclonal antibody (#32A1) on bone resorption activity of normal human osteoclast precursor cells (Evaluation using collagen-coated plate)

It is known that osteoclasts release a protease such as cathepsin K and degrade type I collagen which is a constitutional component of bone tissue. The OsteoLyse Assay Kit (manufactured by Lonza, Inc., Cat. No. PA-1500) provides a 96-well plate coated with europium-conjugated human collagen (96-well OsteoLyse cell culture plate), and it is possible to evaluate the bone resorption activity of osteoclasts in vitro by measuring the amount of fluorescent collagen fragments released into the supernatant when osteoclasts are cultured on the plate. Normal human osteoclast precursor cells (Normal Human Natural Osteoclast Precursor Cells, purchased from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) were seeded in a 96-well OsteoLyse cell culture plate at 1×10^4 cells/well according to the protocol accompanying the cells. As the medium, a minimal essential medium for osteoclast precursor cells (OPBM, purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-8201) supplemented with an OPGM supplement set (purchased from Sanko

Junyaku Co., Ltd., Cat. No. PT-9501) containing fetal bovine serum (final concentration: 10%), human RANKL (final concentration: 68.4 ng/ml), human M-CSF (final concentration: 33 ng/ml), and the like was used. To the resulting culture supernatant, the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) prepared in Example 6 was added to give a final concentration of 0.1, 0.3, 1, or 3 µg/ml, and the cells were cultured for 3 days in a CO₂ incubator. A 10 µl aliquot of the culture supernatant was collected, and 200 µl of Fluorophore Releasing Reagent included in the OsteoLyse Assay Kit was added thereto, and a fluorescence intensity was measured (Excitation: 340 nm, Emission: 615 nm) using a fluorescence plate reader (ARVO MX, manufactured by Perkin Elmer Inc.), whereby the amount of free fluorescent collagen fragments released in the culture supernatant was determined (Fig. 12). As a result, the amount of fluorescent collagen fragments increased by the addition of RANKL was reduced by the #32A1 antibody in a concentration-dependent manner within the range of from 0.3 µg/ml to 3 µg/ml. From this result, it was revealed that the bone resorption activity of human osteoclasts is inhibited by the studied monoclonal antibody specifically binding to the Siglec-15 protein.

Example 15

[0206]

Evaluation of biological activity of rat anti-mouse
Siglec-15 monoclonal antibody using ovariectomized rats

a) Protocol of animal experiment

The ovaries on both sides were removed from female F344 rats (obtained from Charles River Laboratories Japan, Inc.) at the age of 12 weeks, and the rats were divided into three groups: a vehicle administration group; a rat anti-mouse Siglec-15 monoclonal antibody #8A1 administration group; and a rat anti-mouse Siglec-15 monoclonal antibody #32A1 administration group. Further, one group was prepared as a sham operation group. In the antibody administration groups, the rat anti-mouse Siglec-15 monoclonal antibody #8A1 or the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 6 was intraperitoneally administered at a dose of 1 mg/kg three times a week repeatedly for 4 weeks from the next day of the operation. In the vehicle administration group and the sham operation group, PBS containing 0.01% Tween 20 was intraperitoneally administered as the vehicle. At 4 weeks after the initiation of administration, urine was collected for 24 hours under fasting conditions, and the urine samples were stored at -80°C until measurement. After completion of the urine collection, the rats were euthanized, and the lumbar spine was excised from each rat.

[0207]

b) Measurement of lumbar spine bone mineral density

Soft tissues adhered to the excised lumbar spine were removed, and the 4th to 6th lumbar vertebrae were extracted. The extracted lumbar vertebrae were degreased and dehydrated by being shaken in ethanol and then air-dried, and the bone mineral density was measured using a bone densitometer (DCS-600EX, manufactured by Aloka Co., Ltd.). The results are shown in Fig. 13(A). A significant decrease in lumbar spine bone mineral density was observed in the ovariectomized group as compared with the sham operation group, however, in the #8A1 and #32A1 antibody administration groups, a decrease in bone mineral density due to ovariectomy was significantly inhibited.

[0208]

c) Measurement of urinary deoxypyridinoline excretion

A variety of type I collagen crosslinked metabolites sharply reflect bone metabolic turnover, particularly bone resorption. Above all, deoxypyridinoline is localized mainly in bone collagen, and therefore, is considered to be highly reliable as an index of bone resorption.

[0209]

The cryopreserved urine sample was thawed, and insoluble matter was precipitated by a centrifugal operation, whereby a supernatant was obtained. The amount of deoxypyridinoline contained in this supernatant was measured using Osteolinks "DPD" (manufactured by DS Pharma Biomedical Co., Ltd.).

Further, by using Creatinine Test Wako (manufactured by Wako Pure Chemical Industries, Ltd.), the content of creatinine in the supernatant was also measured, and the amount of deoxypyridinoline corrected for creatinine was calculated. The results are shown in Fig. 13(B). The urinary deoxypyridinoline excretion was significantly increased in the ovariectomized group as compared with the sham group, and therefore, it was indicated that in the ovariectomized rats, osteoclastic bone resorption is increased. On the other hand, in the #8A1 and #32A1 antibody administration groups, an increase in deoxypyridinoline excretion due to ovariectomy was inhibited such that the level of deoxypyridinoline excretion was comparable to that of the sham operation group. From this result, it was also confirmed in the animal models that the studied monoclonal antibodies specifically binding to Siglec-15 inhibit osteoclastic bone resorption, and it was strongly indicated that due to the inhibitory effect on bone resorption, a decrease in lumbar spine bone mineral density in the ovariectomized rats was inhibited.

Example 16

[0210]

Determination of binding site (epitope) of rat anti-mouse Siglec-15 monoclonal antibody

a) Expression and purification of human Siglec-15 V-set

domain

A DNA encoding a protein in which a His tag and a thrombin recognition sequence were attached to the N-terminal side of a human Siglec-15 V-set domain (a polypeptide comprising 39 to 165 amino acid residues of an amino acid sequence in the NCBI Protein database with the accession number of NP_998767 or an amino acid sequence represented by SEQ ID NO: 2 in the Sequence Listing) was integrated into a vector pDEST14 (Invitrogen, Inc., Cat. No. 11801-016). By using this plasmid, *Escherichia coli* Rosetta-gamiB (DE3) (Novagen, Inc., Cat. No. 71136-4) was transformed, and cultured in TB medium (Invitrogen, Inc., Cat. No. 22711-022). After culturing, the bacterial cells were homogenized by ultrasound, the resulting homogenate was centrifuged, and the supernatant was purified with a HisTrap HP column (GE Healthcare, Co., Ltd., Cat. No. 17-5247-01). Thereafter, the His tag was cleaved with thrombin, and then the human Siglec-15 V-set domain was purified using a Mono S5/50 GL column (GE Healthcare, Co., Ltd., Cat. No. 17-5168-01) and a Superdex 75 10/300 column (GE Healthcare, Co., Ltd., Cat. No. 17-5174-01) until a single band with a molecular weight of 14 kDa was obtained by electrophoresis.

[0211]

b) Purification of soluble human Siglec-15-Fc

The soluble human Siglec-15-Fc was purified by the method

described in Example 12.

[0212]

c) Competitive ELISA of human V-set domain and rat anti-mouse Siglec-15 monoclonal antibody #32A1

In a 96-well maxi-sorp plate (manufactured by Nunc, Inc., model number: 442404), 100 μ l of a goat anti-human Fc antibody (Jackson ImmunoResearch, Inc., model number: 109-005-098) (1.25 μ g/ml) was added to each well and immobilized overnight at 4°C. After the 96-well maxi-sorp plate was washed twice with PBS, 100 μ l of the soluble human Siglec-15-Fc (1 μ g/ml) was added to each well and immobilized at room temperature for 1 hour. Thereafter, 300 μ l of a 5% skim milk/PBS solution was added to each well, and blocking of the wells was performed at room temperature for 3 hours. In the meantime, 2 μ g/ml of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 was mixed with an equivalent amount of 0, 0.032, 0.16, 0.8, 4, or 20 μ g/ml of the human Siglec-15 V-set domain, and a reaction was allowed to proceed at room temperature for 1.5 hours. After the 96-well maxi-sorp plate had been washed twice with PBS, 100 μ l of the mixed solution of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 and the human Siglec-15 V-set domain was added thereto, and a reaction was allowed to proceed at room temperature for 1 hour. After the 96-well maxi-sorp plate had been washed 5 times with a 0.05% Tween 20 (Bio-Rad Laboratories, Inc., Cat. No. 170-6531)/PBS solution

(hereinafter referred to as "PBST solution"), 100 μ l of an HRP-labeled goat anti-rat IgG antibody (Beckman Coulter, Inc., Cat. No. 732664, diluted to 2000-fold) was added thereto, and a reaction was allowed to proceed at room temperature for 1 hour. After the plate had been washed 5 times with the PBST solution, 100 μ l of a color developing liquid of ELISA POD Substrate ABTS Kit (Nacalai Tesque Co., Ltd., Cat. No. 14351-80) was added thereto, and a reaction was allowed to proceed for 30 minutes. Then, 100 μ l of a reaction stopping solution was added thereto, and an absorbance at 405 nm was measured. The results of competitive ELISA showed that the binding of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 to the immobilized human Siglec-15 is inhibited in a human V-set domain concentration-dependent manner. Accordingly, it was shown that the epitope for the rat anti-mouse Siglec-15 monoclonal antibody #32A1 is the human Siglec-15 V-set domain (a domain comprising 39 to 165 amino acid residues of an amino acid sequence in the NCBI Protein database with the accession number of NP_998767 or an amino acid sequence represented by SEQ ID NO: 2 in the Sequence Listing) (Fig. 14).

Example 17

[0213]

Amplification of cDNA encoding variable region of rat anti-mouse Siglec-15 monoclonal antibody #32A1 and base

sequence analysis thereof

a) Preparation of mRNA

The hybridoma #32A1 was cultured according to a) of Example 6. From 4×10^7 cells of the hybridoma #32A1, about 65 μ g of mRNA was prepared using a QuickPrep mRNA purification kit (GE Healthcare, Co., Ltd.).

[0214]

b) Synthesis of cDNA (5'-RACE-Ready cDNA)

The synthesis of cDNA (5'-RACE-Ready cDNA) was performed using 0.3 μ g of the mRNA prepared in a) with PrimeScript Reverse Transcriptase (TaKaRa Bio, Inc.) and SMART RACE cDNA Amplification Kit (Clontech Co., Ltd.).

[0215]

c) Amplification of cDNA of #32A1 gene heavy chain variable region by 5'-RACE PCR

As primers for amplifying the cDNA of the #32A1 heavy chain gene variable region by PCR, UPM (Universal Primer A Mix, attached to the SMART RACE cDNA Amplification Kit) and an oligonucleotide having a sequence of 5'-GGCCGGGTGGGCTACGTTGCAGGTGACGGTCTG-3' (RG2AR2: SEQ ID NO: 23 in the Sequence Listing) were used. As the UPM, one attached to the SMART RACE cDNA Amplification Kit (Clontech Co., Ltd.) was used, and the RG2AR2 was designed from the sequence of the rat heavy chain (IgG2a) constant region in the database and synthesized according to a common procedure.

The cDNA of the #32A1 heavy chain gene variable region was amplified by 5'-RACE PCR using this combination of primers and the cDNA synthesized in b) (5'-RACE-Ready cDNA) as a template. The PCR was performed using Advantage 2 PCR Kit (Clontech Co., Ltd.), and the conditions for a thermal cycler were set as follows: after heating at 94°C for 1 minute, a temperature cycle of "94°C for 0.5 minutes and 72°C for 3 minutes" was repeated 5 times, then, a temperature cycle of "94°C for 0.5 minutes, 70°C for 0.5 minutes, and 72°C for 3 minutes" was repeated 5 times, and further, a temperature cycle of "94°C for 0.5 minutes, 68°C for 0.5 minutes, and 72°C for 3 minutes" was repeated 20 times, followed by incubating at 4°C.

[0216]

d) Amplification of cDNA of #32A1 light chain gene variable region by 5'-RACE PCR

As primers for amplifying the cDNA of the #32A1 light chain gene variable region by PCR, UPM (Universal Primer A Mix, attached to the SMART RACE cDNA Amplification Kit) and an oligonucleotide having a sequence of 5'-CATGCTGTACGTGCTGTCTTTGCTGTCCTGATCAG-3' (RKR2: SEQ ID NO: 24 in the Sequence Listing) were used. As the UPM, one attached to the SMART RACE cDNA Amplification Kit (Clontech Co., Ltd.) was used, and the RKR2 was designed from the sequence of the rat light chain (κ chain) constant region in the database and

synthesized according to a common procedure.

The cDNA of the #32A1 light chain gene variable region was amplified by 5'-RACE PCR using this combination of primers and the cDNA synthesized in b) (5'-RACE-Ready cDNA) as a template. The PCR was performed using Advantage 2 PCR Kit (Clontech Co., Ltd.), and the conditions for a thermal cycler were set as follows: after heating at 94°C for 1 minute, a temperature cycle of "94°C for 0.5 minutes and 72°C for 3 minutes" was repeated 5 times, then, a temperature cycle of "94°C for 0.5 minutes, 70°C for 0.5 minutes, and 72°C for 3 minutes" was repeated 5 times, and further, a temperature cycle of "94°C for 0.5 minutes, 68°C for 0.5 minutes, and 72°C for 3 minutes" was repeated 20 times, followed by incubating at 4°C.

[0217]

e) Determination of base sequences of cDNAs of heavy and light chain variable regions

The cDNA of the heavy chain variable region amplified in c) was purified using MinElute PCR Purification Kit (QIAGEN Inc.), and then the sequence analysis of the DNA sequence was performed. As a sequencing primer, an oligonucleotide having a sequence of 5'-CTCCAGAGTTCCAGGTCACGGTGACTGGC-3' (RG2AR3: SEQ ID NO: 25 in the Sequence Listing) was designed from the sequence of the rat heavy chain (IgG2a) constant region in the database and synthesized according to a common procedure.

[0218]

The cDNA of the light chain variable region amplified in d) was purified using MinElute PCR Purification Kit (QIAGEN Inc.), and then the sequence analysis of the DNA sequence was performed. As a sequencing primer, an oligonucleotide having a sequence of 5'-TCCAGTTGCTAACTGTTCCG-3' (sqRK: SEQ ID NO: 26 in the Sequence Listing) was designed from the sequence of the rat light chain (κ chain) constant region in the database and synthesized according to a common procedure.

[0219]

The cDNA containing the heavy chain variable region obtained by the sequence analysis has a base sequence represented by SEQ ID NO: 27 in the Sequence Listing and encodes the amino acid sequence represented by SEQ ID NO: 28 in the Sequence Listing. The amino acid sequence represented by amino acid numbers 1 to 19 of SEQ ID NO: 28 corresponds to a secretory signal, the amino acid sequence represented by amino acid numbers 20 to 140 thereof corresponds to a heavy chain variable region, and the amino acid sequence represented by amino acid numbers 141 to 167 thereof corresponds to a heavy chain constant region (partial). The above-mentioned heavy chain variable region contains CDRH1 (DYFMN) comprising the amino acid sequence represented by SEQ ID NO: 44, CDRH2 (QIRNKIYTYATFYAESLEG) comprising the amino acid sequence represented by SEQ ID NO: 45, and CDRH3 (SLTGGDYFDY) comprising

the amino acid sequence represented by SEQ ID NO: 46. Further, the cDNA containing the light chain variable region has a base sequence represented by SEQ ID NO: 29 in the Sequence Listing and encodes the amino acid sequence represented by SEQ ID NO: 30 in the Sequence Listing. The amino acid sequence represented by amino acid numbers 1 to 20 of SEQ ID NO: 30 corresponds to a secretory signal, the amino acid sequence represented by amino acid numbers 21 to 132 thereof corresponds to a light chain variable region, and the amino acid sequence represented by amino acid numbers 133 to 139 thereof corresponds to a light chain constant region (partial). The above-mentioned light chain variable region contains CDRL1 (RASQSVTISGYSTIH) comprising the amino acid sequence represented by SEQ ID NO: 47, CDRL2 (RASNLAS) comprising the amino acid sequence represented by SEQ ID NO: 48, and CDRL3 (QQSRKSPWT) comprising the amino acid sequence represented by SEQ ID NO: 49.

Example 18

[0220]

Production of gene expression construct of human chimeric antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

a) Production of universal expression vectors pEF1/FCCU-1 and pEF6KCL

a)-i) Construction of human light chain expression vector pEF6KCL

By performing PCR using a plasmid pEF6/V5-HisB (Invitrogen) as a template and also using the following primers, a DNA fragment from immediately downstream of BGHpA (2174) to SmaI (2958) (a DNA fragment containing fl origin of replication and SV40 promoter and origin, hereinafter referred to as "fragment A") was obtained.

5'-CCACGCGCCCTGTAGCGGCGCATTAAGC-3' (primer EFF1: SEQ ID NO: 31 in the Sequence Listing)

5'-AAACCCGGGAGCTTTTGC AAAAGCCTAGG-3' (primer EFsmaR: SEQ ID NO: 32)

The obtained fragment A and a DNA fragment (SEQ ID NO: 33, hereinafter referred to as "fragment B") containing a DNA sequence encoding a human κ chain secretory signal, a human κ chain constant region, and a human poly-A additional signal were ligated by overlapping PCR. The thus obtained DNA fragment in which the fragment A and the fragment B were ligated (hereinafter referred to as "fragment A+B") was digested with the restriction enzymes KpnI and SmaI, which was ligated to a plasmid pEF6/V5-HisB (Invitrogen) which was digested with the restriction enzymes KpnI and SmaI, whereby a human light chain expression vector pEF6KCL having a signal sequence, a cloning site, a human κ chain constant region, and a human poly-A additional signal sequence downstream of the EF1

promoter was constructed.

[0221]

a)-ii) Construction of pEF1/KCL

A DNA fragment obtained by cleaving the pEF6KCL obtained by the above-mentioned method with the restriction enzymes KpnI and SmaI was ligated to pEF1/myc-HisB (Invitrogen, Inc.) which was digested with KpnI and SmaI, whereby a plasmid pEF1/KCL was constructed.

[0222]

a)-iii) Construction of human heavy chain expression vector pEF1/FCCU-1

A DNA fragment (SEQ ID NO: 34 in the Sequence Listing) containing a DNA sequence encoding a human IgG1 signal sequence and a constant region amino acid sequence was digested with the restriction enzymes NheI and PmeI and was ligated to the plasmid pEF1/KCL which was digested with NheI and PmeI, whereby a human heavy chain expression vector pEF1/FCCU-1 having a signal sequence, a cloning site, a human heavy chain constant region, and a human poly-A additional signal sequence downstream of the EF1 promoter was constructed.

[0223]

b) Production of #32A1 human chimeric antibody heavy chain expression construct

As primers for amplifying the cDNA of the #32A1 heavy chain variable region by PCR, an oligonucleotide having a

sequence of 5'-aaagctgagcGAGGTGCAAATTTTGGAGACTGGAGGAGGC-3' (32A1HF: SEQ ID NO: 35 in the Sequence Listing) and an oligonucleotide having a sequence of 5'-aaagctgagctGACTGTGACCATGACTCCTTGGCCCCAG-3' (32A1HR: SEQ ID NO: 36 in the Sequence Listing) were synthesized according to a common procedure.

[0224]

Incidentally, in order to integrate a PCR product into pEF1/FCCU-1, these primers were designed such that a recognition sequence for the restriction enzyme BspI was added. The PCR was performed according to a common procedure using this combination of primers and the cDNA of the heavy chain variable region purified in e) of Example 17 as a template. The resulting PCR product was purified using MinElute PCR Purification Kit (QIAGEN, Inc.), and then a DNA fragment obtained by digesting the PCR product with the restriction enzyme BspI (NEW ENGLAND BIOLABS, Inc.) was inserted into the pEF1/FCCU-1 at the site cleaved with the restriction enzyme BspI, whereby a #32A1 human chimeric antibody heavy chain expression construct was constructed. The sequence of the insert was confirmed by a sequence analysis. As a primer for sequencing, an oligonucleotide having a sequence of 5'-TAATACGACTCACTATAGGG-3' (F11: SEQ ID NO: 37 in the Sequence Listing) was synthesized according to a common procedure. An expression vector in which the insert could be correctly

inserted was named "32A1H/pEF1/FCCU".

[0225]

c) Production of #32A1 human chimeric antibody light chain expression construct

As primers for amplifying the cDNA of the #32A1 light chain variable region by PCR, an oligonucleotide having a sequence of 5'-aaacatatggcGACATTGTCTTGACCCAGTCTCCTGCTTTGG-3' (32A1LF: SEQ ID NO: 38 in the Sequence Listing) and an oligonucleotide having a sequence of 5'-aaacgtacgTCTCAATTCCAGCTTGGTGCCTCCAGCG-3' (32A1LR SEQ ID NO: 39 in the Sequence Listing) were synthesized according to a common procedure.

[0226]

Incidentally, in order to integrate a PCR product into pEF6KCL, these primers were designed such that a recognition sequence for the restriction enzyme NdeI was added to 32A1LF, and a recognition sequence for the restriction enzyme BsiWI was added to 32A1LR. The PCR was performed according to a common procedure using this combination of primers and the cDNA of the light chain variable region purified in e) of Example 17 as a template. The resulting PCR product was purified using MinElute PCR Purification Kit (QIAGEN, Inc.), and then, a DNA fragment obtained by digesting the PCR product with the restriction enzymes NdeI (TaKaRa Bio, Inc.) and BsiWI (NEW

ENGLAND BIOLABS, Inc.) was inserted into the pEF6KCL at the site cleaved with the restriction enzymes NdeI (TaKaRa Bio, Inc.) and BsiWI (NEW ENGLAND BIOLABS, Inc.), whereby a #32A1 human chimeric antibody light chain expression construct was constructed. The sequence of the insert was confirmed by a sequence analysis. As a primer for sequencing, a primer F11 represented by SEQ ID NO: 37 in the Sequence Listing was used. An expression vector in which the insert could be correctly inserted was named "32A1L/pEF6KCL".

[0227]

The #32A1 human chimeric antibody heavy chain gene produced in b) has the nucleotide sequence represented by SEQ ID NO: 40 in the Sequence Listing and encodes the amino acid sequence represented by SEQ ID NO: 41 in the Sequence Listing. The amino acid sequence represented by amino acid numbers 1 to 19 of SEQ ID NO: 41 corresponds to a secretory signal, the amino acid sequence represented by amino acid numbers 20 to 140 thereof corresponds to a heavy chain variable region, and the amino acid sequence represented by amino acid numbers 141 to 470 thereof corresponds to a heavy chain constant region. Further, the #32A1 human chimeric antibody light chain gene produced in c) has the nucleotide sequence represented by SEQ ID NO: 42 in the Sequence Listing and encodes the amino acid sequence represented by SEQ ID NO: 43 in the Sequence Listing. The amino acid sequence represented by amino acid numbers 1

to 20 of SEQ ID NO: 43 corresponds to a secretory signal, the amino acid sequence represented by amino acid numbers 21 to 132 thereof corresponds to a light chain variable region, and the amino acid sequence represented by amino acid numbers 133 to 237 thereof corresponds to a light chain constant region.

Example 19

[0228]

Preparation of human chimeric antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

a) Production of human chimeric antibody

3×10^7 cells of 293 FreeStyle cells in logarithmic growth phase were seeded in 30 ml of fresh FreeStyle 293 Expression Medium (Invitrogen, Inc.) (four lots were prepared, in which the 30-ml culture was taken as one lot) and subjected to shaking culture (125 rpm) at 37°C in an 8% CO₂ incubator. 300 µg of polyethyleneimine (manufactured by Polyscience, Inc. #24765) was dissolved in 1 ml of Opti-Pro SFM medium (manufactured by Invitrogen, Inc.), and the resulting solution was left at room temperature for 5 minutes. The #32A1 human chimeric antibody heavy chain expression vector 32A1H/pEF1/FCCU and the #32A1 human chimeric antibody light chain expression vector 32A1L/pEF6KCL produced in Example 18 were prepared using PureLink HiPure Plasmid Kit (Invitrogen, Inc.). The 32A1H/pEF1/FCCU (15 µg) and the 32A1L/pEF6KCL (45 µg) were

suspended in 1 ml of Opti-Pro SFM medium (Invitrogen, Inc.), and the resulting suspension was added to 1 ml of the polyethyleneimine/Opti-Pro SFM mixed liquid which had been left at room temperature for 5 minutes, and the resulting mixture was left at room temperature for an additional 5 minutes. Subsequently, 2 ml of the polyethyleneimine/the expression vectors/Opti-Pro SFM mixed liquid was added to each lot of the 293 FreeStyle cell suspension, and the shaking culture was continued. After the cells were cultured for 7 days at 37°C in 8% CO₂, the culture supernatant was collected from each lot. [0229]

b) Purification of human chimeric antibody

90 ml of the culture supernatant (for three lots) obtained above was filtered through a filter (manufactured by NALGENE, Inc., #295-4545), and 0.5 ml of MabSelect SuRe (manufactured by GE Healthcare Bio-science Co., Ltd., #17-5438-01) equilibrated with PBS was added to the filtrate, and the resulting mixture was shaken overnight at 80 rpm and 10°C. On the next day, the carrier was collected and washed with PBS, and thereafter, the antibody was eluted with 1 M arginine solution (pH 4.0). The eluate was applied to a PD-10 column (manufactured by GE Healthcare Bio-science Co., Ltd., #17-0851-01) to replace the liquid with PBS, and then concentrated with Amicon Ultra-4 (manufactured by Millipore Co., Ltd., #UFC805008), whereby 1.2 ml of a human chimeric

antibody (0.98 mg/ml) was obtained. The concentration of the antibody was calculated from the measurement at 280 nm obtained using Hitachi Diode Array BioPhotometer U-0080D.

Example 20

[0230]

Evaluation of property of human chimeric antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 binding to mouse Siglec-15 protein

The competitive inhibition of the rat #32A1 antibody against the purified human chimeric antibody prepared in Example 19 was determined by the following method. The mouse Siglec-15-Fc purified in Example 4 was diluted to 1 μ g/ml with PBS, the diluted solution was dispensed at 100 μ l/well onto an immunoplate (manufactured by Nunc, Inc., #437111), and the plate was left to stand overnight at 4°C, whereby the protein was adsorbed to the plate. On the next day, each well was washed twice with a PBS-T solution (PBS, 0.05% (v/v) Tween 20), a solution obtained by diluting skim milk (manufactured by Morinaga Milk Industry Co., Ltd.) to 5% with PBS was dispensed at 350 μ l/well, and the plate was left to stand at room temperature for 2 hours. The liquid in each well was removed, and each well was washed 3 times with the PBS-T solution. Thereafter, a mixed solution (a PBS solution containing skim milk at a final concentration of 0.5%) containing the human

chimeric antibody at 0.25 $\mu\text{g/ml}$ and the #32A1 antibody or a rat control IgG antibody (manufactured by R&D Systems, Inc., #6-001-A) at a different concentration (0 $\mu\text{g/ml}$, 0.016 $\mu\text{g/ml}$, 0.05 $\mu\text{g/ml}$, 0.16 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, 1.66 $\mu\text{g/ml}$, or 5 $\mu\text{g/ml}$) was dispensed at 100 $\mu\text{l/well}$, and the plate was left to stand at room temperature for 2 hours. After each well was washed 3 times with the PBS-T solution, alkaline phosphatase-conjugated AffiniPure goat anti-human IgG (manufactured by Jackson ImmunoResearch, Inc., #109-055-097) diluted to 2500-fold with a TBS-T solution (TBS, 0.05% (v/v) Tween 20) was added at 100 $\mu\text{l/well}$, and the plate was left to stand at room temperature for 1 hour. The liquid in each well was removed, and each well was washed 5 times with the TBS-T solution. Thereafter, a fluorescent substrate solution (manufactured by Roche Co., Ltd., #11681982001) was added at 100 $\mu\text{l/well}$, and a fluorescence reaction was allowed to proceed. At 10 minutes after the addition of the fluorescent substrate solution, the fluorescence intensity was measured using a plate reader. As a result, it was shown that the #32A1 antibody inhibits the binding of the human chimeric antibody to the mouse Siglec-15-Fc in a concentration-dependent manner. Further, when the #32A1 antibody and the human chimeric antibody were mixed at the same concentration (1:1), about 40% competitive inhibition was exhibited. Therefore, it is considered that the #32A1 antibody and the human chimeric antibody have

substantially the same affinity for the mouse Siglec-15-Fc. On the other hand, the rat control IgG did not show competitive inhibition (Fig. 15).

Example 21

[0231]

Evaluation of biological activity of human chimeric antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 based on test for mouse osteoclast formation and test for human osteoclast formation

a) Test for mouse osteoclast formation

By using the human chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 19, the effect on osteoclast differentiation of mouse bone marrow nonadherent cells was examined. Mouse bone marrow nonadherent cells prepared by the method of Example 8 were prepared at 1.5×10^5 cells/ml in α -MEM medium containing 10% FBS and 10 ng/ml of M-CSF (manufactured by R&D Systems, Inc.), the resulting cell preparation was seeded in each well of a 96-well plate in an amount of 200 μ l and the cells were cultured for 2 days in a CO₂ incubator. The old culture solution in the 96-well plate was removed, and 100 μ l of MEM- α medium was added to each well, the 100 μ l of MEM- α medium containing 10% FBS to which human RANKL (RANKL, manufactured by Peprotech, Inc.) and M-CSF had been added to give final concentrations of 20

ng/ml and 10 ng/ml, respectively. To the cell culture solution, the rat anti-mouse Siglec-15 monoclonal antibody #32A1 produced in Example 6 or the human chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 19 was added at a concentration of from 3 to 100 ng/ml, and the cells were cultured for an additional 3 days in a CO₂ incubator. After completion of the culturing, the activity of tartrate-resistant acid phosphatase (TRAP) of the formed osteoclasts was measured by the method described in Example 9. After stopping the enzymatic reaction, an absorbance of each well at 405 nm was measured, and the obtained measurement was used as an index of TRAP activity. The results are shown in Fig. 16. A dose-dependent inhibition of TRAP activity was observed at a concentration of 50 ng/ml or higher in the case of the #32A1 antibody and the human chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1. From this result, it was shown that the human chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 has an activity of inhibiting osteoclast formation (osteoclast differentiation and maturation) substantially comparable to that of the rat #32A1 antibody.

[0232]

b) Evaluation using normal human osteoclast precursor cells (TRAP Staining)

Normal human osteoclast precursor cells (Normal Human

Natural Osteoclast Precursor Cells, purchased from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) were seeded in a 96-well plate at 1×10^4 cells/well according to the protocol attached to the cells. As the medium, a minimal essential medium for osteoclast precursor cells (OPBM, purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-8201) supplemented with an OPGM supplement set (purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-9501) containing fetal bovine serum (final concentration: 10%), human RANKL (final concentration: 66 ng/ml), human M-CSF (final concentration: 33 ng/ml), and the like was used. To the resulting culture supernatant, the human chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 19 was added at a final concentration of 0.3, 1, 3, or 10 $\mu\text{g/ml}$, and the cells were cultured for 3 days in a CO_2 incubator. After the culturing, the supernatant was removed, and 10% neutral formalin was added to fix the cells. After fixing the cells, the cells were washed twice with distilled water, a TRAP staining solution (0.27 mM naphthol AS-MX phosphate (manufactured by Sigma Co., Ltd.), 1.6 mM fast red violet LB salt (manufactured by Sigma Co., Ltd.), 1% dimethylformamide, 50 mM sodium tartrate, 0.1 M sodium acetate buffer (pH 5.0)) was added at 100 $\mu\text{l/well}$, and a reaction was allowed to proceed at room temperature for 5 minutes. After the reaction, the cells were washed twice with distilled water, and then observed by microscopy (Fig. 17).

As a result, the formation of TRAP-positive multinucleated osteoclasts was inhibited by the addition of the human chimeric antibody of #32A1 within the range of from 0.3 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$.

Example 22

[0233]

Designing of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 (1)

a) Designing of humanized version of #32A1

a)-i) Molecular modeling of variable region of #32A1

The molecular modeling of the variable region of #32A1 was performed by a method generally known as homology modeling (Methods in Enzymology, 203, 121-153, (1991)). The primary sequences (three-dimensional structures derived from the X-ray crystal structures are available) of the variable regions of human immunoglobulin registered in Protein Data Bank (Nuc. Acid Res. 35, D301-D303 (2007)) were compared with the variable region of #32A1 determined above. As a result, 1LK3 was selected as having the highest sequence homology with the variable region of the light chain of #32A1 among antibodies having a similar deletion in the framework. Further, 1AD0 was selected as having the highest sequence homology with the variable region of the heavy chain of #32A1. The three-dimensional structure of the framework region was generated by obtaining the "framework model" by combining the

coordinates of 1LK3 and 1AD0 corresponding to the light and heavy chains of #32A1. The CDRs of #32A1 were classified according to the classification of Thornton et al. (J. Mol. Biol., 263, 800-815, (1996)) as follows: CDRL1, CDRL2, CDRL3, CDRH1, and CDRH2 were assigned to clusters 15A, 7A, 9A, 10A, and 12B, respectively. CDRH3 was classified into k(8)C according to the H3-rules (FEBS letter 399, 1-8 (1996)). Then, the representative conformations of the respective CDRs were integrated into the framework model.

[0234]

Finally, in order to obtain a probable molecular model of the variable region of #32A1 in terms of energy, an energy calculation was performed for excluding disadvantageous interatomic contact. The above procedure was performed using the commercially available three-dimensional protein structure prediction program Prime and the coordinate search program MacroModel (Schrodinger, LLC).

[0235]

a)-ii) Designing of amino acid sequence of humanized #32A1

A humanized #32A1 antibody was constructed according to a method generally known as CDR grafting (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). An acceptor antibody was selected in two ways based on the amino acid homology within the framework region. The sequence of the framework region

of #32A1 was compared with the sequences of all human frameworks in the Kabat Database (Nuc. Acid Res. 29, 205-206 (2001)) involving antibody amino acid sequences. As a result, an M37G037'CL antibody was selected as an acceptor based on a sequence homology of 73% in the framework region. The amino acid residues in the framework region of M37G037'CL were aligned with the amino acid residues of #32A1, and the positions where different amino acids were used were identified. The positions of these residues were analyzed using the three-dimensional model of #32A1 constructed above. Then, donor residues to be grafted onto the acceptor were selected according to the criteria provided by Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)).

[0236]

The sequence of the framework region of #32A1 was compared with the sequences of all human frameworks in the IgBLAST (Nuc. Acid Res. 36, D25-D30 (2007)). As a result, for the L-chain, AAB34430 was selected as an acceptor based on a sequence homology of 79% in the framework region. As for the H-chain, CAF31288 was selected as an acceptor based on a sequence homology of 78% in the framework region. In respect of the L-chain, the amino acid residues in the framework region of AAB34430, and in respect of the H-chain, the amino acid residues in the framework region of CAF31288, were aligned with the amino acid residues of #32A1, and the positions where

different amino acids were used were identified. The positions of these residues were analyzed using the three-dimensional model of #32A1 constructed above. Then, donor residues to be grafted on the acceptor were selected according to the criteria provided by Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). In these methods, by transferring some selected donor residues to the acceptor antibody, humanized #32A1 sequences were constructed as described in the following Examples.

[0237]

b) Humanization of #32A1 heavy chain

b)-i) h#32A1-T1H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 61 (glutamic acid), 89 (valine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 105 (serine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, glycine, phenylalanine, asparagine, alanine, lysine, asparagine, leucine, methionine, asparagine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T1H-type heavy chain".

[0238]

The amino acid sequence of the h#32A1-T1H-type heavy chain is represented by SEQ ID NO: 51 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 51, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 470 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 51 is represented by SEQ ID NO: 50 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 50, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1410 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 50 and the amino acid sequence of SEQ ID NO: 51 are also shown in Fig. 27.

[0239]

b)-ii) h#32A1-T2H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 114 (isoleucine), 118 (threonine), 134 (valine), 135

(methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, asparagine, alanine, lysine, asparagine, leucine, methionine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T2H-type heavy chain".

[0240]

The amino acid sequence of the h#32A1-T2H-type heavy chain is represented by SEQ ID NO: 53 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 53, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 470 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 53 is represented by SEQ ID NO: 52 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 52, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1410 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 52 and the amino acid sequence of SEQ ID NO: 53 are also shown in Fig.

[0241]

b)-iii) h#32A1-T3H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 24 (leucine), 26 (threonine), 32 (lysine), 104 (valine), 114 (isoleucine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with valine, serine, glutamine, methionine, valine, threonine, leucine, and serine, respectively, was named "h#32A1-T3H-type heavy chain".

[0242]

The amino acid sequence of the h#32A1-T3H-type heavy chain is represented by SEQ ID NO: 55 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 55, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 470 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. The nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 55 is represented by SEQ ID NO: 54 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 54, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1410 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence,

respectively. The nucleotide sequence of SEQ ID NO: 54 and the amino acid sequence of SEQ ID NO: 55 are also shown in Fig. 29.

[0243]

b)-iv) h#32A1-T5H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 61 (glutamic acid), 89 (valine), 95 (aspartic acid), 97 (glutamic acid), 99 (serine), 104 (valine), 105 (serine), 114 (isoleucine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with valine, valine, serine, glutamine, glycine, phenylalanine, asparagine, lysine, threonine, methionine, asparagine, valine, threonine, leucine, and serine, respectively, was named "h#32A1-T5H-type heavy chain".

[0244]

The amino acid sequence of the h#32A1-T5H-type heavy chain is represented by SEQ ID NO: 57 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 57, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 470 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence

encoding the amino acid sequence of SEQ ID NO: 57 is represented by SEQ ID NO: 56 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 56, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1410 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 56 and the amino acid sequence of SEQ ID NO: 57 are also shown in Fig. 30.

[0245]

b)-v) h#32A1-T6H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 95 (aspartic acid), 97 (glutamic acid), 99 (serine), 104 (valine), 114 (isoleucine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with valine, valine, serine, glutamine, asparagine, lysine, threonine, methionine, valine, threonine, leucine, and serine, respectively, was named "h#32A1-T6H-type heavy chain".

[0246]

The amino acid sequence of the h#32A1-T6H-type heavy chain is represented by SEQ ID NO: 59 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino

acid sequence of SEQ ID NO: 59, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 470 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 59 is represented by SEQ ID NO: 58 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 58, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1410 thereof encode a signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 58 and the amino acid sequence of SEQ ID NO: 59 are also shown in Fig. 31.

[0247]

b)-vi) h#32A1-T7H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 89 (valine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 105 (serine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine,

phenylalanine, asparagine, alanine, lysine, asparagine, leucine, methionine, asparagine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T7H-type heavy chain".

[0248]

The amino acid sequence of the h#32A1-T7H-type heavy chain is represented by SEQ ID NO: 72 in the Sequence Listing. The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 72 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 72 is also shown in Fig. 38.

[0249]

b)-vii) h#32A1-T8H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 61 (glutamic acid), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 105 (serine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, glycine, asparagine, alanine, lysine, asparagine, leucine, methionine, asparagine, valine, alanine, threonine,

leucine, and serine, respectively, was named "h#32A1-T8H-type heavy chain".

[0250]

The amino acid sequence of the h#32A1-T8H-type heavy chain is represented by SEQ ID NO: 73 in the Sequence Listing. The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 73 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 73 is also shown in Fig. 38.

[0251]

b-viii) h#32A1-T9H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 61 (glutamic acid), 89 (valine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, glycine, phenylalanine, asparagine, alanine, lysine, asparagine, leucine, methionine, valine, alanine, threonine, leucine, and serine respectively, was named "h#32A1-T9H-type heavy chain".

[0252]

The amino acid sequence of the h#32A1-T9H-type heavy chain is represented by SEQ ID NO: 74 in the Sequence Listing. The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 74 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 74 is also shown in Fig. 38.

[0253]

b)-ix) h#32A1-T10H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 105 (serine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, asparagine, alanine, lysine, asparagine, leucine, methionine, asparagine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T10H-type heavy chain".

[0254]

The amino acid sequence of the h#32A1-T10H-type heavy chain is represented by SEQ ID NO: 75 in the Sequence Listing.

The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 75 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 75 is also shown in Fig. 39.

[0255]

b)-x) h#32A1-T11H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 89 (valine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, phenylalanine, asparagine, alanine, lysine, asparagine, leucine, methionine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T11H-type heavy chain".

[0256]

The amino acid sequence of the h#32A1-T11H-type heavy chain is represented by SEQ ID NO: 76 in the Sequence Listing. The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 76 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy

chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 76 is also shown in Fig. 39.

[0257]

b)-xi) h#32A1-T12H-type heavy chain:

A humanized #32A1 heavy chain designed by replacing amino acid residues 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 61 (glutamic acid), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine), 100 (valine), 104 (valine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of the #32A1 heavy chain represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, glycine, asparagine, alanine, lysine, asparagine, leucine, methionine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-T12H-type heavy chain".

[0258]

The amino acid sequence of the h#32A1-T12H-type heavy chain is represented by SEQ ID NO: 77 in the Sequence Listing. The sequence comprising amino acid residues 1 to 121 of the amino acid sequence of SEQ ID NO: 77 and the sequence comprising amino acid residues 122 to 451 thereof correspond to the heavy chain variable region and the heavy chain constant region, respectively. The amino acid sequence of SEQ ID NO: 77 is also shown in Fig. 39.

[0259]

c) Humanization of #32A1 light chain

c)-i) h#32A1-T1L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T1L-type light chain".

[0260]

The amino acid sequence of the h#32A1-T1L-type light chain is represented by SEQ ID NO: 61 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 61, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain

constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 61 is represented by SEQ ID NO: 60 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 60, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 60 and the amino acid sequence of SEQ ID NO: 61 are also shown in Fig. 32.

[0261]

c)-ii) h#32A1-T2L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T2L-type light chain".

[0262]

The amino acid sequence of the h#32A1-T2L-type light chain is represented by SEQ ID NO: 63 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 63, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 63 is represented by SEQ ID NO: 62 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 62, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 62 and the amino acid sequence of SEQ ID NO: 63 are also shown in Fig. 33.

[0263]

c)-iii) h#32A1-T3L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid),

106 (isoleucine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with aspartic acid, serine, glutamic acid, serine, serine, leucine, glutamic acid, valine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T3L-type light chain".

[0264]

The amino acid sequence of the h#32A1-T3L-type light chain is represented by SEQ ID NO: 65 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 65, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 65 is represented by SEQ ID NO: 64 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 64, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 64 and the amino acid sequence of SEQ ID NO: 65 are also shown in Fig.

34.

[0265]

c)-iv) h#32A1-T4L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 36 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, serine, serine, leucine, glutamic acid, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T4L-type light chain".

[0266]

The amino acid sequence of the h#32A1-T4L-type light chain is represented by SEQ ID NO: 67 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 67, the sequence comprising amino acid residues 21 to 132 thereof, and the sequence comprising amino acid residues 133 to 237 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 67 is represented by SEQ ID NO: 66 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 66, the sequence comprising nucleotides 61 to 396 thereof, and the sequence comprising nucleotides 397 to 711 thereof encode the signal sequence, the light chain variable

region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 66 and the amino acid sequence of SEQ ID NO: 67 are also shown in Fig. 35.

[0267]

c)-v) h#32A1-T5L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 68 (arginine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 110 (phenylalanine), 122 (alanine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with aspartic acid, serine, glutamic acid, asparagine, proline, lysine, serine, serine, leucine, glutamic acid, valine, tyrosine, glycine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T5L-type light chain".

[0268]

The amino acid sequence of the h#32A1-T5L-type light chain is represented by SEQ ID NO: 69 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 69, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising

amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 69 is represented by SEQ ID NO: 68 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 68, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 68 and the amino acid sequence of SEQ ID NO: 69 are also shown in Fig. 36.

[0269]

c)-vi) h#32A1-T6L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with aspartic acid, serine, glutamic acid, proline, serine, serine, leucine, glutamic acid, valine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T6L-type light

chain".

[0270]

The amino acid sequence of the h#32A1-T6L-type light chain is represented by SEQ ID NO: 71 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 71, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 71 is represented by SEQ ID NO: 70 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 70, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 70 and the amino acid sequence of SEQ ID NO: 71 are also shown in Fig. 37.

[0271]

c)-vii) h#32A1-T7L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36

(glutamine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T7L-type light chain".

[0272]

The amino acid sequence of the h#32A1-T7L-type light chain is represented by SEQ ID NO: 78 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 78 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 78 is also shown in Fig. 40.

[0273]

c)-viii) h#32A1-T8L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 81 (isoleucine), 99

(asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T8L-type light chain".

[0274]

The amino acid sequence of the h#32A1-T8L-type light chain is represented by SEQ ID NO: 79 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 79 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 79 is also shown in Fig. 40.

[0275]

c)-ix) h#32A1-T9L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine),

104 (aspartic acid), 106 (isoleucine), 108 (threonine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T9L-type light chain".

[0276]

The amino acid sequence of the h#32A1-T9L-type light chain is represented by SEQ ID NO: 80 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 80 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 80 is also shown in Fig. 40.

[0277]

c)-x) h#32A1-T10L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110

(phenylalanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T10L-type light chain".

[0278]

The amino acid sequence of the h#32A1-T10L-type light chain is represented by SEQ ID NO: 81 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 81 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 81 is also shown in Fig. 40.

[0279]

c)-xi) h#32A1-T11L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and

132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T11L-type light chain".

[0280]

The amino acid sequence of the h#32A1-T11L-type light chain is represented by SEQ ID NO: 82 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 82 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 82 is also shown in Fig. 40.

[0281]

c)-xii) h#32A1-T12L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the

Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T12L-type light chain".

[0282]

The amino acid sequence of the h#32A1-T12L-type light chain is represented by SEQ ID NO: 83 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 83 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 83 is also shown in Fig. 41.

[0283]

c)-xiii) h#32A1-T13L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine,

glutamic acid, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T13L-type light chain".

[0284]

The amino acid sequence of the h#32A1-T13L-type light chain is represented by SEQ ID NO: 84 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 84 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 84 is also shown in Fig. 41.

[0285]

c)-xiv) h#32A1-T14L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, valine, serine, serine,

leucine, glutamic acid, valine, valine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T14L-type light chain".

[0286]

The amino acid sequence of the h#32A1-T14L-type light chain is represented by SEQ ID NO: 85 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 85 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 85 is also shown in Fig. 41.

[0287]

c)-xv) h#32A1-T15L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, valine,

isoleucine, lysine, and threonine, respectively, was named "h#32A1-T15L-type light chain".

[0288]

The amino acid sequence of the h#32A1-T15L-type light chain is represented by SEQ ID NO: 86 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 86 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 86 is also shown in Fig. 41.

[0289]

c)-xvi) h#32A1-T16L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, valine, isoleucine, lysine, and threonine, respectively, was named

"h#32A1-T16L-type light chain".

[0290]

The amino acid sequence of the h#32A1-T16L-type light chain is represented by SEQ ID NO: 87 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 87 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 87 is also shown in Fig. 41.

[0291]

c)-xvii) h#32A1-T17L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 122 (alanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, glycine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T17L-type light chain".

[0292]

The amino acid sequence of the h#32A1-T17L-type light chain is represented by SEQ ID NO: 88 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 88 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 88 is also shown in Fig. 42.

[0293]

c)-xviii) h#32A1-T18L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, tyrosine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T18L-type light chain".

[0294]

The amino acid sequence of the h#32A1-T18L-type light

chain is represented by SEQ ID NO: 89 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 89 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 89 is also shown in Fig. 42.

[0295]

c)-xix) h#32A1-T19L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 66 (glutamine), 68 (arginine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, proline, lysine, valine, serine, serine, leucine, glutamic acid, valine, valine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T19L-type light chain".

[0296]

The amino acid sequence of the h#32A1-T19L-type light chain is represented by SEQ ID NO: 90 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the

amino acid sequence of SEQ ID NO: 90 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 90 is also shown in Fig. 42.

[0297]

c)-xx) h#32A1-T20L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), 29 (alanine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 36 (glutamine), 41 (serine), 66 (glutamine), 81 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, aspartic acid, serine, glutamic acid, asparagine, proline, valine, serine, serine, leucine, glutamic acid, valine, valine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T20L-type light chain".

[0298]

The amino acid sequence of the h#32A1-T20L-type light chain is represented by SEQ ID NO: 91 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 91 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light

chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 91 is also shown in Fig. 42.

[0299]

c)-xxi) h#32A1-T21L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 21 (aspartic acid), 24 (leucine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, methionine, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, tyrosine, glycine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T21L-type light chain".

[0300]

The amino acid sequence of the h#32A1-T21L-type light chain is represented by SEQ ID NO: 92 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the

amino acid sequence of SEQ ID NO: 92 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 92 is also shown in Fig. 42.

[0301]

c)-xxii) h#32A1-T22L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 21 (aspartic acid), 24 (leucine), between 29 and 30 (i.e inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, methionine, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T22L-type light chain".

[0302]

The amino acid sequence of the h#32A1-T22L-type light chain is represented by SEQ ID NO: 93 in the Sequence Listing.

The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 93 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 93 is also shown in Fig. 43.

[0303]

c)-xxiii) h#32A1-T23L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 24 (leucine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with methionine, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T23L-type light chain".

[0304]

The amino acid sequence of the h#32A1-T23L-type light chain is represented by SEQ ID NO: 94 in the Sequence Listing.

The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 94 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 94 is also shown in Fig. 43.

[0305]

c)-xxiv) h#32A1-T24L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers 21 (aspartic acid), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, tyrosine, glycine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T24L-type light chain".

[0306]

The amino acid sequence of the h#32A1-T24L-type light

chain is represented by SEQ ID NO: 95 in the Sequence Listing. The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 95 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 95 is also shown in Fig. 43.

[0307]

c)-xxv) h#32A1-T25L-type light chain:

A humanized #32A1 light chain designed by replacing amino acid numbers between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 110 (phenylalanine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of the #32A1 light chain represented by SEQ ID NO: 30 in the Sequence Listing with threonine, serine, leucine, proline, glutamic acid, leucine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, tyrosine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-T25L-type light chain".

[0308]

The amino acid sequence of the h#32A1-T25L-type light chain is represented by SEQ ID NO: 96 in the Sequence Listing.

The sequence comprising amino acid residues 1 to 113 of the amino acid sequence of SEQ ID NO: 96 and the sequence comprising amino acid residues 114 to 218 thereof correspond to the light chain variable region and the light chain constant region, respectively. The amino acid sequence of SEQ ID NO: 96 is also shown in Fig. 43.

Example 23

[0309]

Production of gene of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

a) Construction of h#32A1-T1L, h#32A1-T2L, h#32A1-T3L, h#32A1-T4L, h#32A1-T5L, and h#32A1-T6L-type light chain expression vectors

DNAs each containing a gene encoding a h#32A1-T1L, h#32A1-T2L, h#32A1-T3L, h#32A1-T4L, h#32A1-T5L, or h#32A1-T6L-type light chain variable region fused to a secretory signal represented by: amino acid numbers 1 to 133 of SEQ ID NO: 61 in the Sequence Listing; amino acid numbers 1 to 133 of SEQ ID NO: 63; amino acid numbers 1 to 133 of SEQ ID NO: 65; amino acid numbers 1 to 132 of SEQ ID NO: 67; amino acid numbers 1 to 133 of SEQ ID NO: 69; or amino acid numbers 1 to 133 of SEQ ID NO: 71 were synthesized (Medical & Biological Laboratories Co., Ltd., Artificial Gene Synthesis Service). Then, each of the DNA fragments obtained by cleaving the

synthesized DNAs with the restriction enzymes NheI and BsiWI was inserted into a universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NheI and BsiWI, whereby h#32A1-T1L, h#32A1-T2L, h#32A1-T3L, h#32A1-T4L, h#32A1-T5L, and h#32A1-T6L-type light chain expression vectors were constructed. The thus obtained expression vectors were named "pEF6KCL/h#32A1-T1L", "pEF6KCL/h#32A1-T2L", "pEF6KCL/h#32A1-T3L", "pEF6KCL/h#32A1-T4L", "pEF6KCL/h#32A1-T5L", and "pEF6KCL/h#32A1-T6L", respectively.

[0310]

b) Construction of h#32A1-T1H, h#32A1-T2H, h#32A1-T3H, h#32A1-T5H, and h#32A1-T6H heavy chain expression vectors

DNAs each containing a gene encoding a h#32A1-T1H, h#32A1-T2H, h#32A1-T3H, h#32A1-T5H, or h#32A1-T6H-type heavy chain variable region fused to a secretory signal represented by amino acid numbers 1 to 140 of SEQ ID NO: 51, 53, 55, 57, or 59 in the Sequence Listing were synthesized (Medical & Biological Laboratories Co., Ltd., Artificial Gene Synthesis Service). Then, each of the DNA fragments obtained by cleaving the synthesized DNAs with the restriction enzyme BspI was inserted into a universal humanized antibody heavy chain expression vector (pEF1/FCCU-1) at the site cleaved with the restriction enzyme BspI, whereby h#32A1-T1H, h#32A1-T2H,

h#32A1-T3H, h#32A1-T5H, and h#32A1-T6H heavy chain expression vectors were constructed. The thus obtained expression vectors were named "pEF1/FCCU/h#32A1-T1H", "pEF1/FCCU/h#32A1-T2H", "pEF1/FCCU/h#32A1-T3H", "pEF1/FCCU/h#32A1-T5H", and "pEF1/FCCU/h#32A1-T6H", respectively.

Example 24

[0311]

Preparation of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

a) Production of humanized antibody

1.5×10^8 cells of 293 FreeStyle cells in logarithmic growth phase were seeded in 100 ml of fresh FreeStyle 293 Expression Medium (Invitrogen, Inc.) and subjected to shaking culture (125 rpm) at 37°C in an 8% CO₂ incubator. 1 mg of polyethyleneimine (manufactured by Polyscience, Inc. #24765) was dissolved in 4 ml of Opti-Pro SFM medium (manufactured by Invitrogen, Inc.), and the resulting solution was left at room temperature for 5 minutes. A heavy chain expression plasmid (0.05 mg) and a light chain expression plasmid (0.15 mg) prepared using PureLink HiPure Plasmid Kit (Invitrogen, Inc.) were suspended in 4 ml of Opti-Pro SFM medium (Invitrogen, Inc.). Then, 4 ml of the resulting expression plasmids/Opti-Pro SFM mixed liquid was added to 4 ml of the

polyethyleneimine/Opti-Pro SFM mixed liquid which had been left at room temperature for 5 minutes, and the resulting mixture was left at room temperature for an additional 5 minutes. Subsequently, 8 ml of the polyethyleneimine/the expression plasmids/Opti-Pro SFM mixed liquid was added to the 293 FreeStyle cell suspension, and the shaking culture was continued. After the cells were cultured for 7 days at 37°C in 8% CO₂, the culture supernatant was collected.

[0312]

b) Purification of humanized antibody

The culture supernatant obtained in the above a) was purified by Protein A affinity chromatography. 100 ml of the culture supernatant was put in a 500-ml stoppered flask, and 1 ml of a suspension (50% slurry) of MabSelect SuRe (manufactured by GE Healthcare Bio-science Co., Ltd.) equilibrated with PBS was added thereto. The resulting mixture was stirred overnight at 100 rpm in an incubator at 10°C. Then, the 293F culture supernatant/MabSelect SuRe suspension was applied to Zeba Spin Column empty 5 mL (PIERCE, Inc.). After all resin was poured into the column, the column was washed with 10 ml of 1 M NaCl. Subsequently, 1 ml of 1 M arginine solution (pH 4.0) was applied to the column, and a fraction containing the antibody was collected. The fraction was added to a centrifugal filter device (Amicon Ultra-4, fractional molecular weight: 50 K, Millipore Co.,

Ltd.), and liquid replacement with a citrate buffer and condensation were performed. The final volume was made up to 200 μ l, which was used as a purified sample.

[0313]

A humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T1L and pEF1/FCCU/h#32A1-T1H was named "h#32A1-T1"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T2L and pEF1/FCCU/h#32A1-T2H was named "h#32A1-T2"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T3L and pEF1/FCCU/h#32A1-T3H was named "h#32A1-T3"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T4L and pEF1/FCCU/h#32A1-T4H was named "h#32A1-T4"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T5L and pEF1/FCCU/h#32A1-T5H was named "h#32A1-T5"; and a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T6L and pEF1/FCCU/h#32A1-T6H was named "h#32A1-T6".

Example 25

[0314]

Evaluation of binding property of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 to mouse Siglec-15 protein

The binding property of the humanized antibodies of the

rat anti-mouse Siglec-15 #32A1 T1 to T6 to mouse Siglec-15-Fc and human Siglec-15-Fc was evaluated by the following method. The mouse Siglec-15-Fc purified in Example 4 or the human Siglec-15-Fc purified in Example 12 was diluted to 1 $\mu\text{g}/\text{ml}$ with PBS, and the diluted solution was dispensed at 100 $\mu\text{l}/\text{well}$ onto an immunoplate (manufactured by Nunc, Inc., #437111), and the plate was left to stand overnight at 4°C, whereby each protein was adsorbed to the plate. On the next day, each well was washed 5 times with a PBS-T solution (PBS, 0.05% (v/v) Tween 20), and a solution obtained by diluting skim milk (manufactured by Morinaga Milk Industry Co., Ltd.) to 5% with PBS was dispensed at 350 $\mu\text{l}/\text{well}$, and the plate was left stand at room temperature for 2 hours. The liquid in each well was removed, and each well was washed 5 times with the PBS-T solution. Thereafter, each of the purified humanized antibodies of the rat anti-mouse Siglec-15 #32A1 T1 to T6 prepared in Example 24 or the human chimeric antibody prepared in Example 19 was diluted to a final concentration of from 1 to 0.004 $\mu\text{g}/\text{ml}$ (4-fold dilution series) with a PBS solution containing 0.5% skim milk, the diluted solution was dispensed at 100 $\mu\text{l}/\text{well}$, and the plate was left to stand at room temperature for 2 hours. At this time, the concentration of each antibody was determined by performing measurement at 280 nm using a spectrophotometer DU-7400 (manufactured by Beckman Coulter, Inc.) and performing calculations based on a molar molecular extinction coefficient

of each antibody. After each well had been washed 5 times with the PBS-T solution, alkaline phosphatase-conjugated AffiniPure goat anti-human IgG (manufactured by Jackson ImmunoResearch, Inc., #109-055-097) diluted to 2500-fold with a TBS-T solution (TBS, 0.05% (v/v) Tween 20) was added at 100 μ l/well, and the plate was left to stand at room temperature for 1 hour. The liquid in each well was removed, and each well was washed 5 times with the TBS-T solution. Thereafter, a fluorescent substrate solution (manufactured by Roche Co., Ltd., #11681982001) was added at 100 μ l/well, and a fluorescence reaction was allowed to proceed. After 10 minutes from the addition of the fluorescent substrate solution in the case of the plate having the mouse Siglec-15-Fc adsorbed thereto, and after 15 minutes in the case of the plate having the human Siglec-15-Fc adsorbed thereto, a fluorescence intensity was measured using Spectra Max M5 (manufactured by Molecular Devices, Inc.). As a result, it was confirmed that all of the examined six humanized antibodies of the rat anti-mouse Siglec-15 bind to the mouse Siglec-15 protein (Fig. 18) and the human Siglec-15 protein (Fig. 19) in an antibody concentration-dependent manner.

Example 26

[0315]

Effect of addition of chimeric antibody of rat anti-mouse

Siglec-15 monoclonal antibody #32A1 on osteoclast differentiation of mouse bone marrow nonadherent cells (stimulation with TNF α)

By using the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 6 and the chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 19, an effect on osteoclast differentiation of mouse bone marrow nonadherent cells by stimulation with TNF α was examined. Mouse bone marrow nonadherent cells prepared by the method of Example 8 were prepared at 1.5×10^5 cells/ml in α -MEM medium containing 10% fetal bovine serum (FBS), 10 ng/ml of M-CSF, and 2 ng/ml of TGF- β (manufactured by R&D Systems, Inc.), the resulting cell preparation was seeded in each well of a 96-well plate in an amount of 200 μ l and the cells were cultured for 2 days in a CO₂ incubator. The old culture solution in the 96-well plate was removed, and 100 μ l of MEM- α medium was added to each well, the 100 μ l of MEM- α medium containing 10% FBS to which recombinant human TNF α (manufactured by R&D Systems, Inc.) and M-CSF had been added to give final concentrations of 30 ng/ml and 10 ng/ml, respectively. To the cell culture solution, the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 6 or the chimeric antibody of the rat anti-mouse Siglec-15 monoclonal antibody #32A1 prepared in Example 19 was added at a concentration of from 0.125 to 4 μ g/ml, and the cells

were cultured for an additional 3 days in a CO₂ incubator. At the same time, a well in which the cells were cultured by adding recombinant human OCIF/OPG prepared by the method described in the description of Patent Application Publication No. WO 96/26217 at a concentration of 100 ng/ml was also prepared. After completion of the culturing, TRAP staining was performed using a leukocyte acid phosphatase kit (manufactured by Sigma Co., Ltd.) according to the protocol accompanying the kit, and the formation of TRAP-positive multinucleated osteoclasts was observed. As a result, the formation of TRAP-positive giant multinucleated osteoclasts was inhibited by the addition of the anti-mouse Siglec-15 polyclonal antibody (Fig. 20). Although small mononuclear osteoclasts were formed even in the case where the rat anti-mouse Siglec-15 monoclonal antibody #32A1 or the chimeric antibody thereof was added, the formation of large multinucleated osteoclasts induced by TNF α was almost completely inhibited. On the other hand, even when a sufficient amount of OCIF/OPG, which is an RANKL blocker, was added, the formation of large multinucleated osteoclasts was inhibited only a little. Further, in the same manner, the activity of tartrate-resistant acid phosphatase (TRAP) of the formed osteoclasts was measured by the procedure described in Example 9. After stopping the enzymatic reaction, an absorbance of each well at 405 nm was measured, and the obtained measurement was used as an index of TRAP activity. The results

are shown in Fig. 21. A significant inhibition of TRAP activity was observed in the case of the #32A1 antibody and the chimeric antibody of #32A1 within the range of from 125 ng/ml to 4 µg/ml. From these results, it was shown that the rat anti-mouse Siglec-15 monoclonal antibody and the chimeric antibody thereof strongly inhibit the process of cell fusion in osteoclast differentiation and maturation induced by TNFα.

Example 27

[0316]

Evaluation of biological activity of rat anti-mouse Siglec-15 monoclonal antibodies #8A1 and #32A1 based on test for rat osteoclast formation

By using the rat anti-mouse Siglec-15 monoclonal antibodies #8A1 and #32A1 prepared in Example 6, an effect on osteoclast differentiation of rat bone marrow nonadherent cells was examined. Rat bone marrow nonadherent cells were prepared by modifying the method of Example 8. That is, the femur was excised from both legs of a female F344 rat at the age of 12 weeks and soft tissues were removed. Both ends of the femur were cut off, and MEM-α medium was injected using a syringe with a 23-gauge injection needle to push out bone marrow cells, which were collected in a centrifugal tube. Centrifugation was performed at room temperature for 5 minutes at 100 g, and the supernatant was removed. To the resulting

cell pellet, 1 ml of a hemolytic buffer (Red Blood Cell Lysing Buffer, manufactured by Sigma Co., Ltd.) was added to suspend it, and the resulting suspension was left at room temperature for 5 minutes. 20 ml of D-PBS was added thereto, the suspension was centrifuged at room temperature for 5 minutes at 100 g, and the supernatant was removed. To the resulting cell pellet, 10 ml of MEM- α medium (manufactured by Invitrogen, Inc.) containing 5 ng/ml of M-CSF (manufactured by R&D Systems, Inc.) and 10% fetal bovine serum (FBS) was added to suspend it. Then, the resulting suspension was passed through a cell strainer (40 μ m Nylon, manufactured by BD Falcon) to remove aggregates. The resulting cells were transferred to a 75 cm² T-flask (for the attachment of adherent cells) and cultured overnight in a CO₂ incubator. After the overnight culture, the cells which did not adhere to the T-flask were recovered and used as rat bone marrow nonadherent cells.

[0317]

The rat bone marrow nonadherent cells prepared by this method were cultured and a test for rat osteoclast formation was performed according to the method of Example 9. That is, the thus prepared rat bone marrow nonadherent cells were prepared at 1.5×10^5 cells/ml in α -MEM medium containing 10% FBS and 10 ng/ml of M-CSF (manufactured by R&D Systems, Inc.), and the resulting cell preparation was seeded in each well of a 96-well plate in an amount of 200 μ l and the cells were cultured

for 2 days in a CO₂ incubator. The old culture solution in the 96-well plate was removed, and 100 μ l of MEM- α medium was added to each well, the 100 μ l of MEM- α medium containing 10% FBS to which human RANKL (RANKL, manufactured by Peprotech, Inc.) and M-CSF had been added to give final concentrations of 20 ng/ml and 10 ng/ml, respectively. To the cell culture solution, the rat anti-mouse Siglec-15 monoclonal antibody #8A1 or #32A1 produced in Example 6 was added at a concentration of from 31 to 1000 ng/ml, and the cells were cultured for an additional 3 days in a CO₂ incubator. After completion of the culturing, the activity of tartrate-resistant acid phosphatase (TRAP) of the formed osteoclasts was measured by the method described in Example 9. After stopping the enzymatic reaction, an absorbance of each well at 405 nm was measured, and the obtained measurement was used as an index of TRAP activity. The results are shown in Fig. 22. In the case of the #8A1 antibody, a dose-dependent inhibition of TRAP activity was observed at a concentration of 63 ng/ml or higher, and in the case of the #32A1 antibody, a dose-dependent inhibition of TRAP activity was observed at a concentration of 31 ng/ml or higher. From these results, it was shown that the rat anti-mouse Siglec-15 monoclonal antibodies #8A1 and #32A1 strongly inhibit the rat osteoclast formation in the same manner as the mouse osteoclast formation, and the in vitro activity of inhibiting rat osteoclast formation of the #32A1 antibody is higher than that

of #8A1 antibody.

Example 28

[0318]

Designing of humanized antibody of rat anti-mouse
Siglec-15 monoclonal antibody #32A1 (2)

a) Designing of humanized version of #32A1

a)-i) Designing of amino acid sequence of humanized #32A1

A humanized #32A1 antibody was constructed according to a method generally known as CDR grafting (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). As for the definitions of CDR regions, the Kabat definitions (SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST VOL. I, FIFTH EDITION (1991)) were used for all except for CDRH2. As for CDRH2, the sequence thereof was made shorter by five residues at the C terminus than the Kabat definition using a definition of its own. In the heavy chain sequence containing the thus defined CDRH2, the CDR sequence derived from a rat is made shorter and more of a human framework sequence is incorporated, and therefore, when it is administered to humans, it is much less likely to be recognized as a heterologous antigen. An acceptor antibody was selected based on the amino acid homology within the framework region. The sequence of the framework region of #32A1 was compared with the sequences of all human frameworks in the Kabat Database (Nuc. Acid Res. 29, 205-206 (2001)) involving antibody amino

acid sequences. As a result, for the L-chain, GF4/1.1'CL was selected as an acceptor based on a sequence homology of 71% in the framework region. As for the H-chain, M37G037'CL was selected as an acceptor based on a sequence homology of 73% in the framework region. In respect of the L-chain, the amino acid residues in the framework region of GF4/1.1'CL, and in respect of the H-chain, the amino acid residues in the framework region of M37G037'CL were aligned with the amino acid residues of #32A1, and the positions where different amino acids were used were identified. The positions of these residues were analyzed using the three-dimensional model of #32A1 constructed in a)-i) of Example 22. Then, donor residues to be grafted on the acceptor were selected according to the criteria provided by Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). By transferring some selected donor residues to the acceptor antibody, humanized #32A1 sequences were constructed as described in the following Examples.

[0319]

a)-ii) Humanization of #32A1 heavy chain

a)-ii)-i) h#32A1-H1-1-type heavy chain:

A humanized 32A1 heavy chain designed by replacing amino acid numbers 23 (isoleucine), 24 (leucine), 26 (threonine), 32 (lysine), 43 (threonine), 61 (glutamic acid), 83 (glutamic acid), 85 (leucine), 86 (glutamic acid), 89 (valine), 95 (aspartic acid), 96 (serine), 97 (glutamic acid), 98 (serine),

100 (valine), 104 (valine), 105 (serine), 114 (isoleucine), 118 (threonine), 134 (valine), 135 (methionine), and 140 (leucine) of 32A1 represented by SEQ ID NO: 28 in the Sequence Listing with leucine, valine, serine, glutamine, alanine, glycine, alanine, valine, lysine, phenylalanine, asparagine, alanine, lysine, asparagine, leucine, methionine, asparagine, valine, alanine, threonine, leucine, and serine, respectively, was named "h#32A1-H1-1-type heavy chain".

[0320]

The amino acid sequence of the h#32A1-H1-1-type heavy chain is represented by SEQ ID NO: 99 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 99, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 466 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 99 is represented by SEQ ID NO: 98 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 98, the sequence comprising nucleotides 58 to 420 thereof, and the sequence comprising nucleotides 421 to 1398 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 98 and

the amino acid sequence of SEQ ID NO: 99 are also shown in Fig. 54.

[0321]

a)-iii) Humanization of #32A1 light chain

a)-iii)-i) h#32A1-L2-15-type light chain:

A humanized 32A1 light chain designed by replacing amino acid numbers 21 (aspartic acid), 23 (valine), 24 (leucine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 110 (phenylalanine), 122 (alanine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and 132 (alanine) of 32A1 represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, leucine, methionine, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, tyrosine, glycine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-L2-15-type light chain".

[0322]

The amino acid sequence of the h#32A1-L2-15-type light chain is represented by SEQ ID NO: 103 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino

acid sequence of SEQ ID NO: 103, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 103 is represented by SEQ ID NO: 102 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 102, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 102 and the amino acid sequence of SEQ ID NO: 103 are also shown in Fig. 56.

[0323]

a)-iii)-ii) h#32A1-L2-16-type light chain:

A humanized 32A1 light chain designed by replacing amino acid numbers 21 (aspartic acid), 23 (valine), 24 (leucine), between 29 and 30 (i.e. inserting a residue missing from the rat framework), 31 (alanine), 32 (valine), 34 (leucine), 36 (glutamine), 40 (isoleucine), 66 (glutamine), 99 (asparagine), 100 (proline), 101 (valine), 102 (glutamine), 103 (alanine), 104 (aspartic acid), 106 (isoleucine), 108 (threonine), 123 (glycine), 127 (leucine), 129 (leucine), 130 (arginine), and

132 (alanine) of 32A1 represented by SEQ ID NO: 30 in the Sequence Listing with glutamic acid, leucine, methionine, threonine, serine, leucine, proline, glutamic acid, leucine, alanine, serine, serine, leucine, glutamic acid, proline, glutamic acid, phenylalanine, leucine, glutamine, valine, isoleucine, lysine, and threonine, respectively, was named "h#32A1-L2-16-type light chain".

[0324]

The amino acid sequence of the h#32A1-L2-16-type light chain is represented by SEQ ID NO: 105 in the Sequence Listing. The sequence comprising amino acid residues 1 to 20 of the amino acid sequence of SEQ ID NO: 105, the sequence comprising amino acid residues 21 to 133 thereof, and the sequence comprising amino acid residues 134 to 238 thereof correspond to the signal sequence, the light chain variable region, and the light chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 105 is represented by SEQ ID NO: 104 in the Sequence Listing. The sequence comprising nucleotides 1 to 60 of the nucleotide sequence of SEQ ID NO: 104, the sequence comprising nucleotides 61 to 399 thereof, and the sequence comprising nucleotides 400 to 714 thereof encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 104 and the amino acid sequence of SEQ ID NO: 105 are also shown

in Fig. 57.

[0325]

b) Production of gene of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

b)-i) Construction of universal human heavy chain expression vector pEG2

A DNA fragment containing a DNA sequence encoding amino acids of the signal sequence and the constant region of human IgG2 represented by SEQ ID NO: 106 was synthesized (Medical & Biological Laboratories Co., Ltd., Artificial Gene Synthesis Service), and digested with the restriction enzymes NheI and PmeI, whereby a DNA fragment of about 1100 bp was obtained. This DNA fragment was ligated to a DNA fragment of about 6200 bp obtained by digesting pEF1/FCCU-1 with NheI and PmeI and removing a DNA fragment of about 1100 bp, whereby a universal humanized antibody (hIgG2 type) heavy chain expression vector pEG2 was constructed.

[0326]

b)-ii) Construction of h#32A1-H1-1 heavy chain expression vector

A DNA fragment obtained by synthesizing a DNA containing a gene encoding the h#32A1-H1-1 heavy chain variable region fused to a secretory signal represented by amino acid numbers 1 to 140 of SEQ ID NO: 99 in the Sequence Listing (Invitrogen), and cleaving the DNA with the restriction enzyme BspI was

inserted into the universal humanized antibody (hIgG2 type) heavy chain expression vector (pEG2) at the site cleaved with the restriction enzyme BspI, whereby a h#32A1-H1-1 heavy chain expression vector was constructed. The thus obtained expression vector was named "pEG2/h#32A1-H1-1".

[0327]

b)-iii) Construction of h#32A1-H5 heavy chain expression vector

A humanized 32A1 heavy chain in which the signal sequence and the constant region of h#32A1-T5H-type heavy chain designed in b)-iv) of Example 22 were changed to those of IgG2 type was named "h#32A1-H5-type heavy chain".

[0328]

The amino acid sequence of the h#32A1-H5-type heavy chain is represented by SEQ ID NO: 101 in the Sequence Listing. The sequence comprising amino acid residues 1 to 19 of the amino acid sequence of SEQ ID NO: 101, the sequence comprising amino acid residues 20 to 140 thereof, and the sequence comprising amino acid residues 141 to 466 thereof correspond to the signal sequence, the heavy chain variable region, and the heavy chain constant region, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 101 is represented by SEQ ID NO: 100 in the Sequence Listing. The sequence comprising nucleotides 1 to 57 of the nucleotide sequence of SEQ ID NO: 100, the sequence comprising nucleotides

58 to 420 thereof, and the sequence comprising nucleotides 421 to 1398 thereof encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 100 and the amino acid sequence of SEQ ID NO: 101 are also shown in Fig. 55.

[0329]

A DNA fragment obtained by cleaving the pEF1/FCCU/h#32A1-T5H produced in Example 23 with the restriction enzyme BlnI was inserted into the universal humanized antibody (hIgG2 type) heavy chain expression vector (pEG2) at the site cleaved with the restriction enzyme BlnI, whereby a h#32A1-H5 heavy chain expression vector was constructed. The thus obtained expression vector was named "pEG2/h#32A1-H5".

[0330]

b)-iv) Construction of h#32A1-L2-15 and h#32A1-L2-16-type light chain expression vectors

DNAs each containing a gene encoding a h#32A1-L2-15 or h#32A1-L2-16-type light chain variable region fused to a secretory signal represented by amino acid numbers 1 to 133 of SEQ ID NO: 103 in the Sequence Listing or amino acid numbers 1 to 133 of SEQ ID NO: 105 were synthesized (Invitrogen), and h#32A1-L2-15 and h#32A1-L2-16-type light chain expression vectors were constructed in the same manner as in Example 23.

The thus obtained expression vectors were named "pEF6KCL/h#32A1-L2-15" and "pEF6KCL/h#32A1-L2-16", respectively.

[0331]

c) Preparation of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1

c)-i) Production of humanized antibody

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen) in logarithmic growth phase were seeded in 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen) and subjected to shaking culture at 37°C in an 8% CO₂ incubator at 90 rpm for 1 hour. 3.6 mg of polyethyleneimine (Polyscience, #24765) was dissolved in 20 ml of Opti-Pro SFM medium (Invitrogen). Subsequently, an H-chain expression plasmid (0.4 mg) and an L-chain expression plasmid (0.8 mg) prepared using PureLink HiPure Plasmid Kit (Invitrogen, Inc.) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the resulting expression plasmids/Opti-Pro SFM mixed liquid was added to 20 ml of the polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells, and shaking culture was performed for 7 days at 37°C in an 8% CO₂ incubator at 90 rpm. The resulting culture supernatant was filtered through a Disposable Capsule Filter (Advantec #CCS-045-E1H).

[0332]

A humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T5L and pEG2/h#32A1-H5 was named "h#32A1-H5/L5"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-T5L and pEG2/h#32A1-H1-1 was named "h#32A1-H1-1/L5"; a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-L2-15 and pEG2/h#32A1-H1-1 was named "h#32A1-H1-1/L2-15"; and a humanized antibody of #32A1 obtained by a combination of pEF6KCL/h#32A1-L2-16 and pEG2/h#32A1-H1-1 was named "h#32A1-H1-1/L2-16".

[0333]

c)-ii) Purification of humanized antibody

The culture supernatant obtained in the above c)-i) was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bioscience Co., Ltd., 2 x 1 ml HiTrap columns in series) equilibrated with PBS. After all culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with 2 M arginine hydrochloride solution (pH 4.0), and a fraction

containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bioscience Co., Ltd., 2 x 5 ml HiTrap desalting columns in series) and the liquid was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to the replacement with the buffer was applied to a ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1 hydroxyapatite column (2 ml volume)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bioscience Co., Ltd., 2 x 5 ml HiTrap desalting columns in series) and the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), the concentration of IgG was adjusted to 1.0 mg/ml or more, and the thus obtained solution was used as a purified sample. The volumes of the respective purified samples were as follows: h#32A1-H1-1/L5: 2.2 ml, h#32A1-H5/L5: 1.8 ml, h#32A1-H1-1/L2-16: 6.0 ml, and h#32A1-H1-1/L2-15: 2.2 ml.

Example 29

[0334]

Evaluation of biological activity of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 based on test for mouse osteoclast formation

An inhibitory effect on mouse osteoclast formation exhibited by the humanized antibodies obtained in Examples 24 and 28 was evaluated by partially modifying the method described in Example 9. Mouse bone marrow nonadherent cells prepared by the method of Example 8 were prepared at 1.5×10^5 cells/ml in α -MEM medium containing 10% FBS and 10 ng/ml of M-CSF (manufactured by R&D Systems, Inc.), the resulting cell preparation was seeded in each well of a 96-well plate in an amount of 200 μ l and the cells were cultured for 2 days in a CO₂ incubator. The old culture solution in the 96-well plate was removed, and 100 μ l of MEM- α medium was added to each well, the 100 μ l of MEM- α medium containing 10% FBS to which human RANKL (RANKL, manufactured by Peprotech, Inc.) and M-CSF had been added to give final concentrations of 20 ng/ml and 10 ng/ml, respectively. To the cell culture solution, the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) prepared in Example 6 or each of the 6 types of humanized #32A1 antibodies (h#32A1-T1, h#32A1-T2, h#32A1-T3, h#32A1-T4, h#32A1-T5, and h#32A1-T6) prepared in Example 24 was added at a concentration of from 3 to 100 ng/ml, and the cells were

cultured for an additional 3 days in a CO₂ incubator. After completion of the culturing, the activity of tartrate-resistant acid phosphatase (TRAP) of the formed osteoclasts was measured by the following procedure. The culture solution in each well of the 96-well plate was removed by suction, and 50 µl of 50 mM sodium citrate buffer (pH 6.1) containing 1% Triton X-100 was added to each well. Then, the plate was shaken for 5 minutes on a plate shaker to lyse the cells. To each well, 50 µl of a substrate solution (50 mM sodium citrate buffer (pH 6.1) containing 5 mg/ml p-nitrophenyl phosphate and 0.46% sodium tartrate) was added, and the plate was incubated at room temperature for 10 minutes. After the incubation, 50 µl of a 1 N sodium hydroxide solution was added to each well of the 96-well plate to stop the enzymatic reaction. After stopping the enzymatic reaction, an absorbance of each well at 405 nm was measured, and the obtained measurement was used as an index of TRAP activity. The results are shown in Figs. 44 and 45. Further, for the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) prepared in Example 6, the human chimeric antibody of the #32A1 antibody prepared in Example 19, and 4 types of the humanized #32A1 antibodies (h#32A1-H1-1/L5, h#32A1-H5/L5, h#32A1-H1-1/L2-16, and h#32A1-H1-1/L2-15) prepared in Example 28, the biological activity was evaluated by the same method as in this test, and the results are shown in Figs. 46 and 47. In all cases of the

10 types of humanized #32A1 antibodies (h#32A1-T1, h#32A1-T2, h#32A1-T3, h#32A1-T4, h#32A1-T5, h#32A1-T6, h#32A1-H1-1/L5, h#32A1-H5/L5, h#32A1-H1-1/L2-16, and h#32A1-H1-1/L2-15) for which the activity was evaluated, a strong activity of inhibiting osteoclast formation substantially comparable to that of the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) or the human chimeric antibody of the #32A1 antibody was observed.

Example 30

[0335]

Effect of addition of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 on bone resorption activity of normal human osteoclast precursor cells (Evaluation using collagen-coated plate)

Normal human osteoclast precursor cells (Normal Human Natural Osteoclast Precursor Cells, purchased from Sanko Junyaku Co., Ltd., Cat. No. 2T-110) were seeded in a 96-well OsteoLyse cell culture plate (purchased from Sanko Junyaku Co., Ltd., Cat. No. PA-1500) at 1×10^4 cells/well according to the protocol accompanying the cells. As the medium, a minimal essential medium for osteoclast precursor cells (OPBM, purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-8201) supplemented with an OPGM supplement set (purchased from Sanko Junyaku Co., Ltd., Cat. No. PT-9501) containing fetal bovine

serum (final concentration: 10%), human RANKL (final concentration: 63.8 ng/ml), human M-CSF (final concentration: 33 ng/ml), and the like was used. To the resulting culture supernatant, each of the rat anti-mouse Siglec-15 monoclonal antibody (#32A1 antibody) prepared in Example 6, the human chimeric antibody of the #32A1 antibody prepared in Example 19, and the 10 types of humanized #32A1 antibodies (h#32A1-T1, h#32A1-T2, h#32A1-T3, h#32A1-T4, h#32A1-T5, h#32A1-T6, h#32A1-H1-1/L5, h#32A1-H5/L5, h#32A1-H1-1/L2-16, and h#32A1-H1-1/L2-15) prepared in Examples 24 and 28 was added at a final concentration of 4, 20, 100, or 500 ng/ml, and the cells were cultured for 5 days in a CO₂ incubator. A 10 μ l aliquot of the culture supernatant was collected, 200 μ l of Fluorophore Releasing Reagent included in the OsteoLyse Assay Kit was added thereto, and by using a fluorescence plate reader (ARVO MX, manufactured by Perkin Elmer Inc.), time-resolved fluorometry (time-resolved fluorescence fluorometer) (Excitation: 340 nm, Emission: 615 nm) suitable for fluorescence measurement using europium was performed, whereby the amount of free fluorescent collagen fragments released in the culture supernatant was determined (Figs. 48 and 49). As a result, the amount of fluorescent collagen fragments increased by the addition of RANKL was reduced by the addition of the rat #32A1 antibody or the human chimeric #32A1 antibody in a concentration-dependent manner. In the

same manner, also by all of the examined 10 types of humanized #32A1 antibodies, a concentration-dependent inhibition was observed. From this result, it was revealed that the bone resorption activity of human osteoclasts is inhibited by the humanized #32A1 antibodies specifically binding to the Siglec-15 protein.

Example 31

[0336]

Biological evaluation of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 using ovariectomized rats

An inhibitory effect on a decrease in bone mineral density in ovariectomized rats exhibited by the humanized antibodies obtained in Examples 24 and 28 can be evaluated by the method described in Example 15.

Example 32

[0337]

Evaluation of biological activity of humanized antibody of rat anti-mouse Siglec-15 monoclonal antibody #32A1 based on test for mouse osteoclast formation by stimulation with TNF α

An inhibitory effect on mouse osteoclast formation by stimulation with TNF α exhibited by the humanized antibodies obtained in Examples 24 and 28 can be evaluated by the method

described in Example 26.

Example 33

[0338]

Determination of thermal stability of humanized antibody using differential scanning calorimetry (DSC)

For h#32A1-H5/L5, h#32A1-H1-1/L5, h#32A1-H1-1/L2-15, and h#32A1-H1-1/L2-16, thermal stability was determined. Each of these samples was prepared at a concentration of 0.5 mg/ml (in a CBS solution), and a 400 μ l aliquot thereof was used as a sample solution for the DSC measurement. The conditions for the DSC measurement were set as follows. That is, the initial temperature was set to 20°C, the final temperature was set to 100°C, the temperature increase rate was set to 60°C/hour, and the filter time was set to 10 seconds. As a reference solution, CBS was used. As an instrument for the DSC measurement, VP-Capillary DSC platform manufactured by MicroCal Inc. USA was used. The baseline (a scanning curve obtained by filling the reference solution also in a sample cell) was subtracted from a scanning curve obtained for each sample solution, whereby baseline correction was performed. Fig. 50 shows a thermogram for h#32A1-H5/L5, Fig. 51 shows a thermogram for h#32A1-H1-1/L5, Fig. 52 shows a thermogram for h#32A1-H1-1/L2-15, and Fig. 53 shows a thermogram for h#32A1-H1-1/L2-16. When a peak top value in each thermogram

was taken as a thermal denaturation midpoint temperature (T_m) in the Fab region, the T_m value of h#32A1-H5/L5 was 81.2°C, the T_m value of h#32A1-H1-1/L5 was 84.1°C, the T_m value of h#32A1-H1-1/L2-15 was 80.0°C,, and the T_m value of h#32A1-H1-1/L2-16 (IgG2) was 77.9°C.

Industrial Applicability

[0339]

The chimeric or humanized anti-Siglec-15 antibody of the invention has an ability to inhibit osteoclast differentiation or bone resorption activity, and a pharmaceutical composition containing the anti-Siglec-15 antibody can be a therapeutic or preventive agent for a disease of abnormal bone metabolism.

Accession Number

[0340]

FERM BP-10999

CLAIMS

[Claim 1]

An isolated antibody which binds to a polypeptide comprising amino acid residues 39 to 165 of an amino acid sequence represented by SEQ ID NO: 2 and inhibits osteoclast formation and/or osteoclastic bone resorption, or a functional fragment of the antibody, wherein the antibody contains a heavy chain variable region sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 41 and a light chain variable region sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 43.

[Claim 2]

The functional fragment of the isolated antibody according claim 1 which is selected from the group consisting of Fab, F(ab')₂, Fab' and Fv.

[Claim 3]

The isolated antibody according to claim 1, wherein the antibody is an scFv.

[Claim 4]

The isolated antibody or a functional fragment of the isolated antibody according to any one of claims 1 to 3, wherein the antibody is a chimeric antibody.

[Claim 5]

The isolated antibody or a functional fragment of the isolated antibody according to claim 4, wherein the antibody or functional fragment thereof comprises a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 41 and a light chain sequence comprising amino acid residues 21 to 237 of an amino acid sequence represented by SEQ ID NO: 43.

[Claim 6]

The isolated antibody or a functional fragment of the isolated antibody according to any one of claims 1, 2 or 4, wherein the antibody is humanized.

[Claim 7]

The isolated antibody according to claim 5 or claim 6, wherein the heavy chain has a constant region of a human immunoglobulin G2 heavy chain and the light chain has a constant region of a human immunoglobulin κ light chain.

[Claim 8]

An isolated antibody which inhibits osteoclast formation and/or osteoclastic bone resorption or a functional fragment of the antibody, wherein the antibody contains:

(a) a heavy chain variable region selected from the group consisting of the following amino acid sequences:

a1) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 51;

a2) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 53;

a3) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 55;

a4) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 57;

a5) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 59;

a6) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by

SEQ ID NO: 99;

a7) an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 101;

a8) an amino acid sequence having a homology of at least 95% with any one of the amino acid sequences selected from a1) to a7);

a9) an amino acid sequence having a homology of at least 99% with any one of the amino acid sequences selected from a1) to a7); and

a10) an amino acid sequence including a substitution, deletion, or addition of one amino acid residue in any one of the amino acid sequences selected from a1) to a7); and

(b) a light chain variable region selected from the group consisting of the following amino acid sequences:

b1) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 61;

b2) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 63;

b3) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 65;

b4) an amino acid sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 67;

b5) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69;

b6) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 71;

b7) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 103;

b8) an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 105;

b9) an amino acid sequence having a homology of at least 95% with any one of the amino acid sequences selected from b1) to b8);

b10) an amino acid sequence having a homology of at least 99% with any one of the amino acid sequences selected from b1) to b8); and

b11) an amino acid sequence including a substitution, deletion, or addition of one amino acid residue in any one of the amino acid sequences selected from b1) to b8).

[Claim 9]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 51 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 61.

[Claim 10]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 53

and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 63.

[Claim 11]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 65.

[Claim 12]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 132 of an amino acid sequence represented by SEQ ID NO: 67.

[Claim 13]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 57

and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 14]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 59 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 71.

[Claim 15]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 16]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 101

and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 17]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 103.

[Claim 18]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 140 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 133 of an amino acid sequence represented by SEQ ID NO: 105.

[Claim 19]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 51 and a light chain sequence comprising amino acid residues 21 to 238 of

an amino acid sequence represented by SEQ ID NO: 61.

[Claim 20]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 53 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 63.

[Claim 21]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 65.

[Claim 22]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 55 and a light chain sequence comprising amino acid residues 21 to 237 of an amino acid sequence represented by SEQ ID NO: 67.

[Claim 23]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 57 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 24]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 470 of an amino acid sequence represented by SEQ ID NO: 59 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 71.

[Claim 25]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 26]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 101 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 69.

[Claim 27]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 103.

[Claim 28]

The isolated antibody or a functional fragment of the isolated antibody according to claim 8, wherein the antibody or functional fragment thereof contains a heavy chain sequence comprising amino acid residues 20 to 466 of an amino acid sequence represented by SEQ ID NO: 99 and a light chain sequence comprising amino acid residues 21 to 238 of an amino acid sequence represented by SEQ ID NO: 105.

[Claim 29]

A pharmaceutical composition, wherein said pharmaceutical composition comprises at least one of the antibodies or functional fragments of the antibodies according to claims 1 to 28.

[Claim 30]

The pharmaceutical composition according to claim 29, wherein said pharmaceutical composition is a therapeutic and/or preventive agent for abnormal bone metabolism.

[Claim 31]

A pharmaceutical composition for treating and/or preventing abnormal bone metabolism, which comprises at least one of the antibodies or functional fragments of the antibodies according to claims 1 to 28 and at least one member selected from the group consisting of bisphosphonates, active vitamin D₃, calcitonin and derivatives thereof, hormones, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents, soluble TNF receptors, anti-TNF- α antibodies or functional fragments of the antibodies, anti-PTHrP (parathyroid hormone-related protein) antibodies or functional fragments of the antibodies, IL-1 receptor antagonists, anti-IL-6 receptor antibodies or functional fragments of the antibodies, anti-RANKL antibodies or functional fragments of the antibodies,

and OCIF (osteoclastogenesis inhibitory factor).

[Claim 32]

The pharmaceutical composition according to claim 30 or 31, wherein the abnormal bone metabolism is selected from the group consisting of osteoporosis, bone destruction accompanying rheumatoid arthritis, cancerous hypercalcemia, bone destruction accompanying multiple myeloma or cancer metastasis to bone, giant cell tumor, osteopenia, tooth loss due to periodontitis, osteolysis around a prosthetic joint, bone destruction in chronic osteomyelitis, Paget's disease of bone, renal osteodystrophy, and osteogenesis imperfecta.

[Claim 33]

The pharmaceutical composition according to claim 32, wherein the abnormal bone metabolism is osteoporosis, bone destruction accompanying rheumatoid arthritis, or bone destruction accompanying cancer metastasis to bone.

[Claim 34]

The pharmaceutical composition according to claim 33, wherein the abnormal bone metabolism is osteoporosis.

[Claim 35]

The pharmaceutical composition according to claim 34, wherein the osteoporosis is postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent, or osteoporosis accompanying rheumatoid arthritis.

[Claim 36]

Use of an antibody according to any one of claims 1 to 28 or a functional fragment thereof for the manufacture of a medicament for treating and/or preventing abnormal bone metabolism.

[Claim 37]

Use of an antibody according to any one of claims 1 to 28 or a functional fragment thereof for the manufacture of a

medicament for treating and/or preventing abnormal bone metabolism, wherein said medicament is for simultaneous or successive administration with at least one member selected from the group consisting of bisphosphonates, active vitamin D₃, calcitonin and derivatives thereof, hormones, SERMs (selective estrogen receptor modulators), ipriflavone, vitamin K₂ (menatetrenone), calcium preparations, PTH (parathyroid hormone), nonsteroidal anti-inflammatory agents, soluble TNF receptors, anti-TNF- α antibodies or functional fragments of the antibodies, anti-PTHrP (parathyroid hormone-related protein) antibodies or functional fragments of the antibodies, IL-1 receptor antagonists, anti-IL-6 receptor antibodies or functional fragments of the antibodies, anti-RANKL antibodies or functional fragments of the antibodies, and OCIF (osteoclastogenesis inhibitory factor).

[Claim 38]

The use according to claim 36 or 37, wherein the abnormal bone metabolism is osteoporosis, bone destruction accompanying rheumatoid arthritis, or bone destruction accompanying cancer metastasis to bone.

[Claim 39]

The use according to claim 38, wherein the abnormal bone metabolism is osteoporosis.

[Claim 40]

The use according to claim 39, wherein the osteoporosis is postmenopausal osteoporosis, senile osteoporosis, secondary osteoporosis due to the use of a therapeutic agent, or osteoporosis accompanying rheumatoid arthritis.

[Claim 41]

The pharmaceutical composition according to claims 31 to 35, or the use according to claim 37, wherein the

hormones are estradiol.

[Claim 42]

The pharmaceutical composition according to claim 35 or the use according to claim 40 wherein the therapeutic agent is a steroid or an immunosuppressant.

[Claim 43]

An isolated polynucleotide encoding the antibody according to any one of claims 1, 5, and 8 to 28.

[Claim 44]

The isolated polynucleotide according to claim 43, wherein said polynucleotide contains a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 40 and a nucleotide sequence comprising nucleotides 61 to 396 of a nucleotide sequence represented by SEQ ID NO: 42.

[Claim 45]

The isolated polynucleotide according to claim 44, wherein said polynucleotide contains a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 40 and a nucleotide sequence comprising nucleotides 61 to 711 of a nucleotide sequence represented by SEQ ID NO: 42.

[Claim 46]

The isolated polynucleotide according to claim 43, wherein said polynucleotide contains:

(a) a polynucleotide selected from the group consisting of the following nucleotide sequences:

a1) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 50;

a2) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 52;

a3) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54;

a4) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 56;

a5) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 58;

a6) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98;

a7) a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 100;

a8) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from a1) to a7) under stringent conditions; and

a9) a nucleotide sequence including a substitution, deletion, or addition of one nucleotide in any one of the nucleotide sequences selected from a1) to a7); and

(b) a polynucleotide selected from the group consisting of the following nucleotide sequences:

b1) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 60;

b2) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 62;

b3) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 64;

b4) a nucleotide sequence comprising nucleotides 61 to 396 of a nucleotide sequence represented by SEQ ID NO: 66;

b5) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68;

b6) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 70;

b7) a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 102;

b8) a nucleotide sequence comprising nucleotides 61 to

399 of a nucleotide sequence represented by SEQ ID NO: 104;

b9) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from b1) to b8) under stringent conditions; and

b10) a nucleotide sequence including a substitution, deletion, or addition of one nucleotide in any one of the nucleotide sequences selected from b1) to b8).

[Claim 47]

The polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 50, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 60.

[Claim 48]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 52, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 62.

[Claim 49]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 64.

[Claim 50]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 396 of a nucleotide sequence represented by SEQ ID NO: 66.

[Claim 51]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 56, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 52]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 58, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 70.

[Claim 53]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 54]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 100, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 55]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 102.

[Claim 56]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 420 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 399 of a nucleotide sequence represented by SEQ ID NO: 104.

[Claim 57]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 50, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 60.

[Claim 58]

The isolated polynucleotide according to claim 46,

wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 52, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 62.

[Claim 59]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 64.

[Claim 60]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 54, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 711 of a nucleotide sequence represented by SEQ ID NO: 66.

[Claim 61]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 56, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 62]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide

comprising a nucleotide sequence comprising nucleotides 58 to 1410 of a nucleotide sequence represented by SEQ ID NO: 58, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 70.

[Claim 63]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 64]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 100, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 68.

[Claim 65]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 102.

[Claim 66]

The isolated polynucleotide according to claim 46, wherein said polynucleotide contains a polynucleotide comprising a nucleotide sequence comprising nucleotides 58

to 1398 of a nucleotide sequence represented by SEQ ID NO: 98, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 714 of a nucleotide sequence represented by SEQ ID NO: 104.

[Claim 67]

A vector, comprising any one of the polynucleotides according to claims 43 to 66.

[Claim 68]

A transformed host cell, comprising any one of the polynucleotides according to claims 43 to 66.

[Claim 69]

A transformed host cell, comprising the vector according to claim 67.

[Claim 70]

A method of producing the antibody according to any one of claims 1, 5, and 8 to 28, comprising culturing the host cell according to claim 68 or 69, and purifying an antibody from the resulting culture product.