

OSTEOGENESIS PROMOTER

[Name of Document]

[Title of Invention]

[Technical Field]

[0001]

The present invention relates to an osteogenesis accelerator and to a preventive and therapeutic agent. It relates more particularly to an osteogenesis accelerator and to a preventive and therapeutic agent having as active ingredient a substance which inhibits binding of semaphorin 4D and plexin B1.

[Background of Invention]

[0002]

As Japan's population ages, there has been an increase in patients suffering from bone fractures, osteoporosis, articular rheumatism, lumbar pain and other bone diseases. The function of tissue in bone tissue is maintained by coordinating osteoblasts which support osteogenesis and osteoclasts which support bone resorption and by maintaining a balance between osteogenesis and bone resorption. The balance of bone metabolism is destroyed by aging, a decline in ovarian function as well as other factors. When osteogenesis decreases or when there is abnormal bone resorption the bone quantity (bone density) decreases and a variety of bone diseases occur. Bone diseases include bone fractures, bone deficiency, osteoporosis, osteomalacia, osteopenia, lumbar pain, Paget's disease of bone, tonic myelitis, articular rheumatism, deformative arthrosis and the like. In particular, when an elderly person is afflicted with a bone disease, it is difficult for that person to function on a level required for daily life and depending on the case, there is a risk that the patient will become bed-ridden. As a result, this is extremely significant in the prevention and treatment of bone diseases in modern society where an increasing number of people are elderly.

[0003]

Activated vitamin D3, biphosphonate, calcitonin, hormone preparations containing estradiol, vitamin K2 preparations and the like are generally used as therapeutic agents for bone diseases. Estradiol derivatives, activated vitamin D3 derivatives, biphosphonate derivatives and the like are being developed as more effective therapeutic agents as they have fewer side effects (see Patent Document 1). However, vitamin D3 has an action which increases the concentration of calcium in the blood and estradiol and biphosphonate have a bone resorption inhibition effect. However, none of these has an effect which directly accelerates osteogenesis through the osteoblasts. A multiple drug therapy is the treatment of choice in this case so that there is a need to develop a novel preventive and therapeutic agent having a different action from that of the conventional preventive and therapeutic agents.

[0004]

Bones are being recreated continuously in stages known as bone remodeling wherein the bone resorption stage and the following osteogenesis stage are repeated. The transition from one stage to another must be controlled precisely by secretion from the bone cell as it contributes to the communication between the osteoclast-osteoblast or by bone reconstruction factors released from the bone matrix. It is well known that TGF- β and IGF-1 released during bone resorption stimulate osteogenesis so that it is known as a coupling factor. Although there is an abundance of *in vitro* data on candidate molecules which are extremely important for the coupling factor, there is still no *in vitro* evidence for this.

[0005]

Axon guidance molecules manifest widely outside the nervous system. Therefore, cell wandering, the immune response, tissue development and angiogenesis and the like are controlled (see Non-Patent Documents 1 and 2). Based on research carried out in recent years, it is suggested that the axon guidance molecules of semaphorin and ephrin and the like contribute to intercellular communication between osteoclasts and osteoblasts (see Non-Patent Documents 3 through 5).

[0006]

It is well known that Semaphorin 4D is secreted from oligodendrocytes and that it induces destruction of the growth cone in the central nervous system. It is also clear that semaphorin 4D is extremely important in maintaining the immune response and the homeostasis of the immune system. One well-known semaphorin 4D receptor is plexin-B1 (see Non-Patent Document 6). Furthermore, Non-Patent Document 7 suggests that osteoclast formation can be accelerated through the osteoblast differentiation inhibition action as well as the osteoblasts. Non-Patent Document 8 discloses that semaphorin 4D is not detected in the osteoblasts, that it is present on the surface of the osteoclasts and the bone quantity has been confirmed to increase as compared to wild-type mice in female sema4D^{-/-} mice where Semaphorin 4D (Sema 4D) is deficient in homo [zygotes].

[Prior Art Documents]

[Patent Documents]

[0007]

Special Table in Publication 2005-509629.

[0008]

[Non-Patent Document 1] B.J. Dickson, Science 298, 1959 (Dec 6, 2002)

[Non-Patent Document 2] A.B. Huber, A.L. Kolodkin, D.D. Ginty, J.F. Cloutier, Annual Review Neuroscience 26, 509 (2003)

[Non-Patent Document 3] N. Takagahara et al., Nat. Cell Biol 8,615 (Jun, 2006)

[Non-Patent Document 4] N. Irie et al., Cell Metab 4, 111 (Aug, 2006)

[Non-Patent Document 5] C. Zhao et al., Cell Metab 4, 111 (Aug, 2006)

[Non-Patent Document 6] I. Oinuma et al., Science, Vol. 305, No. 5685, pp. 862 -865
(August 6, 2004)

[Non-Patent Document 7] Ishida Masanari, Kaneda Toshio, Muto Akihiro, Yoshida Masashi,
Meeting Program Abstracts of the Japan Society of Bone
Metabolism, vol. 25, page 266, published June 2007, [Analysis of
Bioaction in Bone Metabolism in Osteoclast-derived Semaphorin
4D]

[Non-Patent Document 8] Romain Dacquin, Chantal Domenget, Pierre Jurdic, Irma
Machuca-Gayet, ASBMR 2010 Annual Meeting Abstracts,
Presentation Abstracts, Presentation Number MOO152,
“Physiological Control of Bone Resorption by Semaphorin 4D is
Dependent on Ovarian Function”

[Overview of Invention]

[Problems Which the Present Invention is Intended to Solve]

[0009]

It is an object of the present invention to provide an osteogenesis acceleration agent and a preventive and therapeutic agent for bone diseases which involves direct promotion of osteogenesis using osteoblasts.

[Means Used to Solve the Problems]

[0010]

After a great deal of hard work and research under the conditions described in the abovementioned background of technology, the inventors found that (a) when semaphorin 4D which is derived from osteoclasts binds with the plexin B receptor on the osteoblast, it activates the small G protein RhoA which inhibits osteoblast differentiation, decreases the IRS signals (signals which promote the differentiation of the osteoblasts) and inhibit the differentiation of the osteoblasts so that osteogenesis is inhibited; and that (b) the anti-semaphorin 4D antibodies and anti-plexin B1 antibodies directly accelerate osteogenesis using the osteoblasts.

[0011]

This means that the present invention relates to (1) an osteogenesis acceleration agent having as active ingredient a substance which inhibits binding between semaphorin 4D and plexin B1; (2) an osteogenesis acceleration agent as described in (1) above characteristic in that the binding inhibiting substance is an anti-semaphorin 4D antibody; and (3) an osteogenesis acceleration agent as described in (1) above characteristic in that the binding inhibiting substance is an anti-plexin B1 antibody or a protein containing plexin B1 extracellular regions.

[0012]

The present invention also relates to (4) an agent for prevention and treatment of bone diseases having as active ingredient a substance which inhibits binding of semaphorin 4D and plexin B1; (5) an agent for prevention and treatment of bone diseases as described in (4) above characteristic in that the binding inhibition substance is an anti-semaphorin 4D antibody; (6) an agent for prevention and treatment of bone diseases as described in (4) above characteristic in that the binding inhibition substance is an anti-plexin B1 antibody or a protein containing plexin B1 extracellular regions; and (7) an agent for prevention and treatment of bone diseases as described in any of (4) through (6) above characteristic in that the bone diseases are selected from bone fracture, bone deficiency, osteoporosis, osteomalacia, osteopenia, lumbar pain, Paget's disease of bone, tonic myelitis, articular rheumatism and deformative arthrosis.

[0013]

The present invention also relates to (8) a method used to determine a candidate active ingredient for an osteogenesis acceleration agent wherein it is determined whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1, and if the abovementioned substance being studied is the abovementioned substance which inhibits binding, the abovementioned substance being studied is determined to be a candidate active ingredient for an osteogenesis acceleration agent; (9) a method as described in (8) above characteristic in that the method used to determine whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1 consists of the following steps (A) through (D): (A) a step wherein semaphorin 4D and plexin B1 are brought in contact with one another in the presence of the substance being studied, (B) a step wherein the degree of binding between semaphorin 4D and plexin B1 is measured, (C) a step wherein the degree measured in step (B) above is compared with the degree when not in the presence of the substance being studied, and (D) a step wherein the substance being studied is determined to be a substance which inhibits binding between semaphorin 4D and plexin B1 when the degree measured in step (B) above is lower than the degree when not in the presence of the substance being studied; and (10) a method of screening for a candidate active ingredient for an osteogenesis acceleration agent characteristic in that the methods described in (8) or (9) above are used to search substances being studied for a candidate active ingredient for an osteogenesis acceleration agent.

[0014]

The present invention can be used to directly promote osteogenesis using osteoblasts and to prevent and/ or treat bone diseases.

[Brief Explanation of Figures]

[0015]

[Figure 1] A diagram indicating bone quantity in wild mice (WT) measured using microcomputer tomography (μ CT) and semaphorin 4D knockout (Sema 4d^{-/-}) mice.

[Figure 2] A diagram indicating the trabecular width in WT mice measured using μ CT and Sema4d^{-/-} mice.

[Figure 3] A diagram indicating osteogenesis [surface area of osteoblast (left);, surface area of calcified bone (center); and osteogenesis rate (right)] measured using osteomorphogenesis analysis in WT mice and Sema4d^{-/-} mice with calcein double marker every 4 days.

[Figure 4] A diagram indicating the osteoclast surface area and the cell count (at left and in the middle) as well as the parameters (at right) of the bone resorption measured using osteomorphogenesis analysis in WT mice and Sema4d^{-/-} mice.

[Figure 5] A diagram indicating the bone quantity of mice in which adoptive immune cell grafting has been carried out using the bone marrow cells of WT mice and Sema4d^{-/-} mice. The second graph from the left indicates the bone quantity of mice when grafted onto WT mice and the second graph from the right indicates the bone quantity of mice when grafted onto Sema4d^{-/-} mice.

[Figure 6] A diagram indicating the number of bone nodes when Fc-sema4D (recombined Semaphorin 4D fused with the Fc area of the IgG1) are added to the calvarial cells cultured under the osteogenesis conditions.

[Figure 7] A diagram indicating the manifestation of Plexin B type and mRNA of cluster of differentiation 72 (CD72) during osteoblast differentiation.

[Figure 8] A diagram indicating results of pull down analysis using Fc-sema4D. The panel on the right indicates results of sampling analysis prior to pull down and the panel on the left indicates results of sampling analysis after pull down.

[Figure 9] A diagram indicating the bone quantity in WT mice measured using μ CT analysis and in Plexin B1 knockout (Plxnb1^{-/-}) mice.

[Figure 10] A diagram indicating the width of the trabecula in WT mice measured using μ CT mice and Plxnb1^{-/-} mice.

[Figure 11] A diagram indicating the surface area of osteoblasts in WT mice measured using osteomorphogenesis analysis.

[Figure 12] A diagram indicating bone surface area which has become calcified in WT mice measured using osteomorphogenesis analysis and Plxnb1^{-/-} mice.

[Figure 13] A diagram indicating the osteogenesis rate in WT mice measured using osteomorphogenesis analysis and Plxnb1^{-/-} mice.

[Figure 14] A diagram indicating RA mutants (R1661/1662/1968A) of Plexin B1 and Plexin B1 having mutations in the GAP domain as well as the mutant cut for Plexin B1 in which the PDZ bond domain is missing (Δ PDZ).

[Figure 15] A diagram indicating the bone quantity in WT mice (control mice) measured using μ CT analysis and dominant negative RhoA (RhoA DNOB) mice.

[Figure 16] A diagram indicating the trabecula in WT mice measured using μ CT analysis and RhoA DNOB.

[Figure 17] A diagram indicating the formation [osteoblast surface area] in WT mice and RhoA DNOB mice measured using osteomorphogenesis analysis.

[Figure 18] A diagram indicating the formation [surface area of calcified bone] of WT mice and RhoADNOB mice.

[Figure 19] A diagram indicating the formation [osteogenesis rate] in WT mice and RhoA DNOB mice measured using osteomorphogenesis analysis.

[Figure 20] A diagram indicating the surface area of osteoblasts of mice with extracted ovaries (OVX) and OVX mice treated with anti-Sema4D bodies measured using osteomorphogenesis analysis.

[Figure 21] A diagram indicating the bone quantity of OVX mice and OVX mice treated with anti-semaphorin 4D antibodies (anti-Sema4D antibodies) measured using μ CT analysis.

[Figure 22] A diagram indicating the osteogenesis [osteogenesis rate] in OVX mice and in OVX mice treated with anti-Sema4D antibodies measured using osteomorphogenesis analysis.

[Figure 23] A diagram indicating the trabecular intervals in OVX mice and in OVX mice treated with anti-Sema4D antibodies measured using μ CT analysis.

[Figure 24] The upper panel in Figure 24 is a diagram indicating results of studying the calcification formation of mice osteoblasts cultured using osteoclasts of WT mice of Sema4D^{-/-} mice and when cultured in the presence of anti-Sema4D antibodies. The lower panel in Figure 24 is a diagram indicating the results of studying the calcification of mouse osteoblasts when cultured in the presence of Fc-sema4d and / or anti-plexin Ba antibodies.

[Figure 25] A diagram indicating results of studying the calcification formation of human osteoblasts (HOS) cultured in the presence of osteoclasts or the supernatant and / or the anti-Sema4D antibodies and the like.

[Figure 26] A diagram indicating the surface area of osteoblasts of mice with extracted ovaries (OVX) and OVX mice treated with anti-semaphorin 4D antibodies (anti-Sema4D antibodies) 6 weeks after OVX treatment measured using osteomorphogenesis analysis.

[Figure 27] A diagram indicating the bone quantity of OVX mice and OVX mice treated with anti-Sema4D antibodies 6 weeks after OVX treatment measured using μ CT analysis.

[Figure 28] A diagram indicating the osteogenesis [osteogenesis rate] in OVX mice and in OVX mice treated with anti-Sema4D antibodies 6 weeks after OVX treatment measured using osteomorphogenesis analysis.

[Figure 29] A diagram indicating the trabecular intervals in OVX mice and in OVX mice treated with anti-Sema4D antibodies 6 weeks after OVX treatment measured using μ CT analysis.

[Preferred Mode of Working Invention]

[0016]

There are no particular restrictions on the [osteogenesis accelerator] in the present invention and on the [preventive and therapeutic agent for bone diseases] (hereinafter referred to collectively as [agent in the present invention], as long as they have as active ingredient a substance which inhibits binding of semaphorin 4D and plexin B1 (hereinafter, referred to simply as [binding inhibiting substance in the present invention]) as the active ingredient and there are no particular restrictions on the binding inhibitor in the present invention as long as it is a substance which inhibits binding of semaphorin 4D and plexin B1 of vertebrates. However,

protein comprising the extracellular regions of anti-semaphorin 4D antibodies, anti-plexin B1 antibodies, and plexin B1 can be cited as suitable examples. The binding inhibitor in the present invention inhibits binding of semaphorin 4D derived from osteoclasts and plexin B1 receptors on the osteoblasts and by inhibiting suppression of differentiation of osteoblasts through RhoA activation inhibition and through inhibition of lowering of IRS signals so that it is thought to accelerate osteogenesis using osteoblasts. Furthermore, in this Specification, the terms [inhibition] and [suppression] are used interchangeably.

[0017]

The abovementioned anti-semaphorin 4D antibodies and anti-plexin B1 antibodies (hereinafter referred to as [antibodies in this invention]) may be polyclonal antibodies and may be monoclonal and any functional fragment of these. However, monoclonal antibodies are preferred given their high specificity. The abovementioned anti-semaphorin 4D antibodies and anti-plexin B1 antibodies may be produced using a conventional well-known method using semaphorin 4D and plexin B1. The functional fragments which are the antibodies in the present invention indicate fragments of antibodies which bind specifically relative to (a) semaphorin 4D which is an antigen in which the antibodies in the present invention bind specifically and to (b) plexin B. Specific examples are F(ab')₂, Fab', Fab, Fv, disulphide-linked FV, single-chain FV (scFV) and polymers of these (D.J. King, Applications and Engineering of Monoclonal Antibodies, 1998 T.J. International Ltd.). These antibody fragments can be obtained by using a conventional method such as digestion of antibodies molecules using papain, pepsin and other proteases or by using a well-known genetic engineering method.

[0018]

The antibodies in the present invention also include human antibodies. Here, [human antibodies] as these relate to the present invention indicate antibodies which are manifested products of human-derived antibody genes. Human antibodies can be obtained by introducing a human antibody gene locus and administering semaphorin 4D and plexin B1 in transgenic animals which are capable of producing human-derived antibodies. A mouse is an example of said transgenic animal. Mice which are capable of producing human antibodies, for example, are deficient in endogenous mouse immunoglobulin (Ig) heavy chains and mouse κ light chains and mice can be used which simultaneously retain a 14th chromosome fragment (SC20) comprising the human Ig heavy chain genes as well as human Ig κ chain transgenes (KCo5). These mice are produced by breeding strain A mice having a human Ig heavy chain gene locus and strain B mice having a human Ig κ chain transgene. The strain A mice are homozygotes for both endogenous Ig heavy chain and κ light chain destruction and they are a mouse strain (Tomizuka, et al., Proc Natl Acad Sci USA, 200 Vol 97:722) which retain a 14th chromosome fragment (SC20) which is capable of apomorphic transmission. Strain B is also a homozygote for both endogenous mouse Ig heavy chains and κ light chain deficiency and is a mouse strain (Nat Biotechnol., 1996 Vol 14:845) which retains a human Ig κ chain transgene (KCo5).

[0019]

The polyclonal antibodies in the present invention may be produced using the method indicated below. They can be obtained by using semaphorin 4D and plexin B1 and if necessary an immunoactivation agent (Freund's adjuvant and the like) on mice, rabbits, goats, horses and other non-human mammals. The monoclonal antibodies in the present invention are used to produce hybridoma from antibody producing cells from immunosensitized animals and myeloma cells which cannot produce their own antibodies. The hybridoma is cloned and clones are selected which produce monoclonal antibodies indicating specific affinity toward the antigens used for immunization. Said hybridoma can be produced based on the method of Keller and Millstein (Nature, 1975 Vol. 256:495-497). Screening of the hybridoma clone which produces monoclonal antibodies is carried out by culturing the hybridoma in a micro-titer plate. The responsiveness to the immunoantigens in the culturing supernatant in the wells seen to have proliferated can be carried out by measuring using ELISA and other enzyme immunoassay methods, radioimmunoassay, the fluorescent antibody method and other immunological methods.

[0020]

Producing monoclonal antibodies from hybridomas can be carried out by culturing the hybridoma *in vitro* and isolating it from the supernatant. It can also be cultured *in vivo* in the pleural fluid of mice, rats, guinea pigs, hamsters or rabbits and the like and isolated from the pleural fluid. The genes which code monoclonal antibodies from hybridoma and other antibody producing cells are cloned, recombined in a suitable vector and these are introduced in the host (for example, chinese hamster ovary (CHO) cells and other mammalian cell stock, *Escherichia coli*, yeast cells, insect cells, plant cells and the like) and the recombinant antibodies are produced using gene recombination technology (P.J. Delves, Antibody Production Essential Techniques, 1997, Wiley, P. Shepherd and C. Dean, Monoclonal Antibodies, 2000, Oxford University Press, J.W. Goding, Monoclonal Antibodies: Principles and Practice, 1993, Academic Press).

[0021]

Transgenic cows, goats, sheep or pigs wherein the desired antibody genes which are recombined in endogenous genes using transgenic animal production technology are produced and large quantities of antibodies derived from antibody genes are obtained from the milk of these transgenic animals.

[0022]

The antibodies produced can be refined using a well-known method in the field such as a combination of protein A column chromatography, ion exchanged chromatography, hydrophobic

chromatography, the sulfate analytical method, gel filtration, affinity chromatography and the like.

[0023]

The protein comprising the extracellular region of the abovementioned plexin B1 can inhibit binding of semaphorin 4D and plexin B1 by trapping semaphorin 4D. There are no particular restrictions on the protein which comprises the plexin B1 extracellular region, however, a protein which fuses with the constant region of the antibodies (preferably any immunoglobulin Fc fragment) is a suitable example.

[0024]

The protein which comprises the abovementioned semaphorin 4D, plexin B1 and plexin B1 extracellular regions can produce a manifestation vector which comprises that sequence based on the sequence information of these proteins. The manifestation vector is subjected to phenotypic transformation in suitable host cells, the target protein is produced inside the cells and the target protein can be introduced by using an isolating method or other well-known method. For example, the DNA sequence³ (sequence number 1) of human semaphorin 4D and the amino acid sequence (sequence number 2) are disclosed in GenBank Accession Number NM_001142287 and the DNA sequence (sequence number 3) of human plexin B1 and the amino acid sequence (sequence number 4) are disclosed in GenBank Accession Number NM_001130082. Furthermore, the human plexin B1 extracellular region corresponds to amino acid numbers 1 through 1490 of the amino acid sequence of the abovementioned Accession Number NM_001130082.

[0025]

We measured by immunoblotting analysis the binding between semaphorin 4D and plexinB1 both in the presence of and not in the presence of the substance to see whether a certain substance is a substance which inhibits binding of semaphorin 4D and plexinB1 and studied whether the bond between them decreased in the presence of that substance so that it could be easily confirmed.

[0026]

The osteogenesis acceleration effect referred to in the present invention indicates the effect of accelerating osteogenesis and more precisely includes the effect of accelerating osteogenesis by osteoblasts by inhibiting the suppression of differentiation of osteoblasts. Whether or not a certain substance has an osteogenesis acceleration effect can be confirmed by administering the substance to vertebrates having a lower bone quantity than usual (preferably patients with osteoporosis and osteoporosis model vertebrates) and finding whether or not the bone quantity increases.

[0027]

The bone disease prevention and treatment effect in the present invention indicates the effect of preventing and / or treating any of the bone diseases or the effect of improving the symptoms in the present invention. Whether or not a certain substance has a bone disease therapeutic effect can be confirmed by administering the substance to a patient or a vertebrate with a bone disease (preferably a patient with osteoporosis or an osteoporosis model vertebrate) and then studying whether the bone disease is cured or improved.

[0028]

The formulation of the present invention may contain only the binding inhibition substance in the present invention, however, a usually pharmacologically permitted carrier, binding agent, stabilizer, excipient, diluent, pH buffer, disintegrator, solubilizing agent, solubilization adjuvant, isotoner and other adjustment agent compounding components may be added. The formulation of the reducing agent in the present invention may be a powder formulation, granulated formulation, a capsule agent or other solid preparation. It may also be a solution preparation, an emulsion, a suspension or other liquid formulation. These preparations may be used as suitable for the binding inhibition substance in the present invention by treating using the regular method.

[0029]

There are no particular restrictions on the method used to administer the agent in the present invention as long as it has the desired bone disease preventive or therapeutic effect and it may be administered orally or non-orally. The non-oral method used to administer it includes vascular administration, muscular administration, hypodermic administration, transdermal administration, nasal administration, transpulmonary administration. Of these, vascular and intravenous administration can be used to particular advantage. The dose of the preparation in the present invention as well as the number of times administered and the concentration can be adjusted in accordance with the body weight of the subject, the type of bone disease and the bone disease symptoms.

[0030]

The subject for the present invention may be vertebrates such as mammals and animals belonging to the bird family. These may include humans, monkeys, mice, rats, hamsters, guinea pigs, cows, pigs, horses, rabbits, sheep, goats, cats, dogs, chickens, quail and other suitable animals. Of these, humans and domestic animals and fowl are particularly suitable. The binding inhibiting substance contained in the agent of the present invention is suitable from the standpoint of obtaining an outstanding osteogenesis acceleration effect and the bone disease prevention and treatment effect whether the type of vertebrate derived from semaphorin 4D and plexin B1 which bring out the binding inhibition action coincides with the type of vertebrate which is a candidate for administration of the agent in the present invention. Furthermore, a

vertebrate derived from semaphorin 4D and the type of vertebrate derived from plexin B1 may be the same or different.

[0031]

There are no particular restrictions on the type of bone disease which can be treated using the present invention as long as it is a bone disease having a factor which brings about a decline in osteogenesis or a bone disease related to a decline in osteogenesis. However, bone fractures, bone deficiency, osteoporosis, osteomalacia, bone deficiency, lumbar pain, Paget's disease of bone, tonic myelitis, articular rheumatism, dysosteogenesis and deformative arthrosis are all suitable candidates. Of these, osteoporosis, osteomalacia, osteopenia and dysosteogenesis are particularly suitable.

[0032]

The method in the present invention is a method used to determine a candidate active ingredient for an osteogenesis acceleration agent wherein it is determined whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1, and if the abovementioned substance being studied is the abovementioned substance which inhibits binding, the abovementioned substance being studied is determined to be a candidate active ingredient for an osteogenesis acceleration agent. In the abovementioned determination method, the following steps (A) through (D) are a suitable example of the method used to determine whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1:

- (A) a step wherein semaphorin 4D and plexin B1 are brought in contact with one another in the presence of the substance being studied;
- (B) a step wherein the degree of binding between semaphorin 4D and plexin B1 is measured;
- (C) a step wherein the degree measured in step (B) above is compared with the degree when not in the presence of the substance being studied;
- (D) a step wherein the substance being studied is determined to be a substance which inhibits binding between semaphorin 4D and plexin B1 when the degree measured in step (B) above is lower than the degree when not in the presence of the substance being studied.

[0033]

In the abovementioned determination method, the following may be used to measure the degree of binding: for semaphorin 4D, labeled semaphorin 4D extracellular region or fusion protein of semaphorin 4D extracellular region and immunoglobulin Fc region, etc., and for plexin B1, a cell expressing plexin B1 on its surface. The following may also be used to measure the degree of binding: for plexin B1, labeled plexin B1 extracellular region or fusion protein of plexin B1 extracellular region and immunoglobulin Fc region, etc., and for semaphorin 4D, a cell expressing semaphorin 4D on its surface.

[0034]

There are no particular restrictions on the substance being studied in the determination method in the present invention and it may be a substance predetermined to have an activity which inhibits binding of semaphorin 4D and plexin B1 and it may be any substance whose activity is unknown. It is also possible to simultaneously use multiple substances being studied. When simultaneously using multiple substances being studied, it is possible to simultaneously use individual substances being studied each in a separate sample, to simultaneously use multiple substances being studied in a single sample, or to simultaneously use multiple samples individually prepared with multiple substances being studied. When simultaneously using multiple substances being studied in a single sample, it may not be possible in one test to determine which of the substances being studied inhibits binding of semaphorin 4D and plexin B1, but it is possible to determine which of the substances being studied is a binding inhibitor by running the test multiple times and narrowing down the substances being studied in stages. The determination method in the present invention is characterized by searching substances being studied for a candidate active ingredient for an osteogenesis acceleration agent, and the method can be used to screen for a candidate active ingredient for an osteogenesis acceleration agent.

[0035]

Furthermore, other modes of the present invention may involve (a) use of the binding inhibition substance in the present invention in the production of the preventive and therapeutic agent for the abovementioned osteogenesis acceleration agent and bone diseases; (b) a binding inhibition substance in the present invention for use as an osteogenesis acceleration agent and in the prevention and treatment of bone diseases; (c) a method of using the binding inhibition substance for the acceleration of osteogenesis and prevention and treatment of bone diseases; (d) a method of promoting osteogenesis by administering the binding inhibition substance in the present invention; and (e) a method of preventing and treating bone diseases by administering the binding inhibition substance in the present invention.

[0036]

Next we shall describe the present invention in detail referring to practical examples of it, however, it should by no means be construed that the present invention is restricted to these practical examples.

[Practical Examples 1]

[0037]

[Analysis of Mice and Bone Phenotypes]

We produced the *Sema4d*^{-/-}, *Plxnb1*^{-/-}, plexin B2 knockout (*Plxnb2*^{-/-}), CAT-RhoA DN and α 1 (I)-Cre mice in accordance with the method described in the literature (W. Shi *et al.*, *Immunity* 13, 633 (Nov, 2000); R. H. Friedel *et al.*, *J Neurosci* 27, 3921 (Apr 4, 2007); R.H. Friedel *et al.*, *Proc Natl Acad Sci USA* 102, 13188 (Sep 13, 2006); K. Kobayashi *et al.*, *J.*

Neurosci 24, 3480 (Apr 7, 2004); R. Dacquin, M. Starbuck, T. Schinke; G. Karsenty, Dev Dyn 224, 245 (Jun, 2002)]. All of the mice were backcrossed 8 or more times with C57BL/6 mice. All of the mice were maintained in a condition where there was no specific pathogenic fungus. All of the animal experiments were authorized by the Animal Experiment Committee of the Tokyo Medical and Dental University and complied with related guidelines and laws. Analysis of the bone phenotype involved controlling respectively the genetically altered mice and the mice from the same litter and we analyzed at least 8 males and 8 females. Three-dimensional micro CT (μ CT) analysis and histomorphological measurement analysis were carried out in accordance with the methods described in the literature (K. Nishikawa *et al.*, J Clin Invest 120, 3455 (Oct 1, 2010); T. Koga *et al.*, Nature 428, 758 (Apr 15, 2004).

[0038]

[Bone Marrow Chimera Mice]

We produced bone marrow chimera mice by changing in part the method described in the literature (B. Zhao *et al.*, Nat Med 15, 1066 (Sep, 2009). This means that we collected donor bone marrow cells (C57BL/6-Ly5, 2 backgrounds) from wild-type *Sema4d*^{-/-} mice from the same litter. We injected intravenously 2×10^6 cells obtained from each donor in the caudal vein of wild type recipient mice (3 weeks old, C57BL/6-Ly5, 1 background) exposed to lethal radiation or *Sema4d*^{-/-} mice. Eight weeks after bone marrow transplant, a high level donor type chimerism (>95 %) was attained.

[0039]

[GeneChip Analysis]

We carried out GeneChip analysis in accordance with the method described in the literature (K. Nishikawa *et al.*, J Clin Invest 120, 3455 (Oct 1, 2010)). This means that after cDNA synthesis was carried out using reverse transcription using all of the RNA, we synthesized the cRNA which had been subjected to biotinized labeling by transcription *in vitro*. After we fragmented the cRNA, we carried out hybridization using the mouse A430 GeneChip (made by Affymetrix) in accordance with the method described in the literature (T. Koga *et al.*, Nat Med 11, 880 (Aug, 2005); H. Takayanagi *et al.*, Dev Cell 3, 889 (Dec, 2002).

[0040]

[Induction of Differentiation to Osteoblasts *in vitro*]

Induction of differentiation to osteoblasts and osteoclasts *in vitro* was carried out according to the method described in the document (T. Koga *et al.*, Nat Med 11, 880 (Aug, 2005); and H. Takayanagi *et al.*, Dev Cell 3, 889 (Dec, 2002). This means that we carried out differentiation induction by carrying out culturing of calvaria-derived cells in an osteogenesis culture medium (50 μ M of ascorbic acid, 10 nM of dexamethasone, and 10 mM of

β glycerophosphate) and the differentiation induction was confirmed by alkaline phosphatase (ALP) assay (after 7 days) and osteonodosity analysis (21 days later, alizarin red staining). Fc-sema4D, anti-Sema4D antibodies and anti-plexin BA antibodies (anti-Plexin-B1 antibodies) was added every 3 days. The osteoclast supernatant was recovered from each of the culture solutions of the wild type and Sema4d-/- cells after stimulating the intranuclear factor κ B activated receptor ligand (RANKL; made by Peprotech). We cultured the cultured osteoclasts in a collagen code dish (made by IWAKI) and recovered it using trypsin two days after RANKL stimulation. The osteoclast supernatant was used as an osteogenesis culture medium containing the abovementioned reagent and we added it to the cultured osteoclasts (1 x 150 cell/well, 24 well-plate) every 3 days.

[0041]

[Quantitative Real-Time RT-PCR Analysis]

Quantitative real-time RT-PCR was carried out in accordance with the product protocols using a light cycler device (made by Roche) and SYBR Green (made by Toyobo). We used the following primers.

Plxnb1 sense: 5'-tgggtcatgtgcagtagcat-3' (sequence number 5),
 Plxnb1 antisense: 5'-cactgctctccaggttctcc-3' (sequence number 6),
 Plxnb2 sense: 5'-aggggagcctctctacaagc-3' (sequence number 7),
 Plxnb2 antisense : 5'-tcgatcccttcctcctgaac-3' (sequence number 8),
 Plnxb3 sense: 5'-atatgctgagcgtgccttct-3' (sequence number 9),
 Plnxb3 antisense: 5'-tgctgttgagcaaattggag-3' (sequence number 10),
 CD72 sense: 5'-gcctctcctgtcctgtctg-3' (sequence number 11),
 CD72 antisense: 5'-cctcctggaactgctgagac-3' (sequence number 12),
 Alpl sense: 5'-aacccagacacaagcattcc-3' (sequence number 13)
 Alpl antisense: 5'-gccttgagggttttggtca-3' (sequence number 14)
 Bglap sense: 5'-gcgctctgtctctctgacct-3' (sequence number 15),
 Bglap antisense: 5'-acctattgccctcctgctt-3' (sequence number 16),
 Colla1 sense: 5'-gagcggagagtactggatcg-3' (sequence number 17),
 Coll1l1 antisense: 5'-gttcgggctgatgtaccagt-3' (sequence number 18)
 Gapdh sense: 5'-accagaaagactgtggatgg-3' (sequence number 19),

[0042]

[Genetic Introduction of Adenovirus and Retrovirus]

The method of producing the adenovirus vector carrying the configuration active type (CA) of RhoA (Myc-V14Rho) and Rac1 (hRac1 V12) and the dominant negative type (DN) of the RhoA (Myc-N19Rho) and Rac1 (hRac1 V12) and the method of introducing these were carried out in accordance with the method described in the literature (Bito, H. *et al.* A Critical Role for a Rho-Associated Kinase, p160ROCK, in Determining Axon Outgrowth in Mammalian

CNS Neurons, Neuron 26, 431-441 (2000). Producing the retrovirus vector (pMXs-Plexin-B1-EGFP, pMXs-Plexin-B1 RA-EGFP and pMXs-Plexin-B1 DPDS-EGFP where the mutant [Plexin-B1 DPDZ-EGFP]) manifested wherein activation of [Plexin-B1 RA] and RhoA could not be activated was carried out by inserting the cDNA fragment of respectively Plexin-B1, Plexin-B1 RA (I. Oinuma, Y. Ishikawa, H. Katoh, M. Negishi, Science 305, 862 (Aug 6, 2004) and Plexin-B1 DPDZ (V. Perrot, J. Vazquez-Prado, J.S. Gutkind, J Biol Chem 277, 43115 (Nov 8, 2002)) in pMXs-IRES-EGFP. Production of a recombinant retrovirus was carried out in accordance with the method described in the literature (S. Morita, T. Kojima, T. Kitamura, Gene Ther 7, 1063 (Jun, 2000). This means that we carried out a retrovirus packaging by introducing the retrovirus vector produced in the Plat-E cells.

[0043]

[Anti-Sema4D Anti-Body Processing for OVX Induced Decline in Bone Quantity]

Producing osteoporosis model mice induced by OVX (ovaries extracted) was carried out in accordance with the method described in the literature (M. Shinohara *et al.*, J. Bio Chem 282, 17640 (Jun 15, 2007) . This means that we carried out an ovarian extraction operation or pseudo operation on 7 week old female mice. Of these model mice, we carried out tests on more than 6 mice in each group. We validated this using the method indicated below to find out whether or not there was a preventive effect on the decline in bone quantity. This means that we injected intravenously 20 μ g of Sema4D antibodies (made by MBL) or a saline solution from the caudal vein once a week. We sacrificed all of the mice 8 weeks after surgery and carried out μ CT analysis and osteomorphogenesis analysis. We also carried out validation using the following method to study whether or not there was an acceleration effect on the bone quantity which had decreased. This means that after 6 weeks we injected intravenously 20 μ g of anti-Sema4D antibodies (made by MBL) from the caudal vein for 3 weeks every 3 days in OVX mice. We sacrificed all of the mice 9 weeks after the operation and used them for μ CT analysis.

[0044]

[Immunoblotting Analysis, Pull Down Analysis and Immunofluorescent Staining]

We cultured calvarial cells for two days in an osteogenesis culture medium (50 μ M of ascorbic acid, 10 nM of dexamethasone and 10 mM of β -glycerophosphate) and then stimulated these with Fc-Sema4D. We used purified human IgG (Fc part) (made by Beckman Coulter) for native control for pull down analysis (time 0). We collected cells at the point indicated and carried out immunoblotting analysis or pull down analysis using anti-Plexin-B 1 antibodies (clone A-8, made by Santa Cruz); anti-PDZ-RhoGEF antibodies (polyclonal, made by Protein Express) ; anti-LARG antibodies (polyclonal, made by Lifespan Biosciences); anti-phospho-Akt antibodies (Thr308) (polyclonal, made by Cell Signaling); anti-Akt antibodies (polyclonal, made by Cell Signaling); anti-phosphor ERK antibodies (Thr202/Tyr204) (polyclonal, made by Cell Signaling); anti-ERK antibodies (polyclonal, made by Cell Signaling); anti-Met antibodies

(clone 25H2) (made by Cell Signaling); anti-ErbB2 antibodies (clone 29D8) (made by Cell Signaling); anti-Rac1 antibodies (clone 102/Rac1) (made by BD Transduction Laboratories); anti-RhoA antibodies (clone 55/Rho) (made by BD Transduction Laboratories); anti-cadherin-11 antibodies (polyclonal, made by Invitrogen); anti-IRS1 antibodies (clone 53-10C-31) (made by Millipore); and anti-b-actin antibodies (clone AC40) (made by Sigma-Aldrich). The phosphorylation of plexin B1, Met, ErbB2 and IRS1 was detected by anti-phosphotyrosine antibodies (4G10, made by Upstate) after immunoprecipitation using respectively specific antibodies for these. We incubated a cell dissolved product with Fc-sema4D (500 ng) which binded with protein A-agarose beads in order to detect the plexin B1 which binded to semaphorin 4D and carried out immunoblotting analysis using anti-Plexin-B1 antibodies. Detecting activation of the GTPase was carried out in accordance with the description in the literature (M. Shinohara *et al.*, J Bio Chem 282, 17640 (Jun 15, 2007). This means that we treated the calvarial cells with Fc-sema4D and collected them at the point they were displayed. We incubated the cell dissolved product with GST-RBD (using RhoA) or GST-PAK1 (using Rac1) (2 μ g) which binded with glutathione sepharose and we carried out immunoblotting analysis using respectively anti-RhoA antibodies or anti-Rac1 antibodies. We fixed the cells with a 4 % paraform aldehyde for immunofluorescent staining, carried out permeation processing and then stained it using Alex Fluor 488 labeling secondary antibodies and rhodamine conjugate phalloidin (made by Molecular Probes).

[0045]

[Flow Cytometry]

We stained a single cell floating solution using monoclonal antibodies conjugated with 8 types of fluorescent pigments [anti-CD45. 2 antibodies conjugated with PerCP.Cy5.5; anti-CD45.2 antibodies (clone 104) conjugated with FITC; anti-CD11b antibodies (clone M1/70) conjugated with eFLuor450; anti-CD105 antibodies (clone MJ7/18) conjugated with PE; anti-CD10 antibodies (clone 4.29E+02) conjugated with Alex Fluor 647; (anti-CD44 antibodies conjugated with APC-Alexa Fluor 750; anti-Cd 44 antibodies (clone IM7) conjugated with AC-Cy7; and anti-Sca-1 antibodies (clone D7) (made by eBioscience) conjugated with APC in order to analyze the bone marrow derived cells and the calvarial cells. Then, we carried out flow cytometry using FACSCant II using Diva software (made by BD Biosciences).

[0046]

[Cell Proliferation Analysis]

We cultured bone marrow-derived interstitial cells in an osteogenesis condition culture medium and analyzed the cell proliferation rate before osteoblast differentiation (Day 0) or in the osteoblast differentiation process (Day 14) using semaphorin4D stimulation using human IgG Fc partial or using cell proliferation ELISA kit (made by Roche) in the presence of Fc-Semaphorin4D and detected the incorporation of 5-bromo-2' deoxyuridine (BrdU).

[0047]

[Analysis of Colony Formation Unit (CFU)]

We plated the bone marrow cells at 3×10^6 cells per single well on a 24-well plate. Then, we cultured this for 3 days using α -MEM culture medium containing 10 % fetal calf serum and then replaced it with an osteogenesis condition culture medium. The colony formation unit-alkali phosphatase (CFU-ALP) was detected as an ALP positive colony on the 7th day and the CFU-osteoblast (CFU-Ob) was detected as an alizarin red positive colony on day 21. The aggregate number of colonies (CFU-fibroblasts (CFU-F)) was detected by staining with toluidine blue on day 7 and day 21.

[0048]

[Statistical Analysis]

All of the data are indicated as mean $\pm$ SEM (n=15). Statistical analysis was carried out using the Student's test A NOVA and when possible the Bonferroni test (*p < 0.05; ** p<0.01; *** p<0.005; n.s., not significant). Results are indicated in the representative example of 4 or more individual experiments.

[0049]

[Results]

We carried out mRNA genome wide screening in the osteoclasts and the osteoblasts using GeneChip analysis to study whether or not the semaphorin, ephrin, slits and netrin which are axon guidance molecules contribute to the remodeling of the bone. This means that after analyzing 20 kinds of semaphorin, 16 types of ephrin, 6 types of slits and 6 kinds of netrin, an extremely high manifestation of semaphorin 4D was confirmed in the osteoclasts. Meanwhile, this manifestation was not confirmed in the osteoblasts. These results indicate that the semaphorin 4D had a selectively induced manifestation in the osteoclast formation process.

[0050]

We carried out a functional analysis of the skeletal system using Sema4d^{-/-} mice to study the semaphorin 4D function with selectively induced manifestation in the osteoclast formation process. The bone quantity (Figure 1) and the trabecular width (Figure 2) in the Sema4d^{-/-} mice was shown to have increased significantly compared to the wild-type mice (WT mice) by using μ CT and osteomorphogenesis analysis. The surface area of the osteogenesis in Sema4d^{-/-} mice (Figure 3, at left), the surface area of the calcified bone (Figure 3, middle) and the osteogenesis rate (Figure 3, at right) increased conspicuously as compared to the wild type mice. However, there were no changes in the parameters (surface areas of osteoclasts (Figure 4, at left), the number of osteoclasts (Figure 4, center) and the surface area of the corroded bone (Figure 4, at right) indicating the bone resorption of the osteoclasts. We also observed that the *in vitro* osteoclast formation in Sema4d^{-/-} cells was normal. Despite the fact that the semaphorin 4D

manifested specifically in the osteoclasts, these results suggest that the phenotype of high bone density in the *Sema4d*^{-/-} mice increased due to osteogenesis through the osteoblasts.

[0051]

We carried out adoptive immune cell grafting using bone marrow cells which included osteoclast precursor cells to find out whether or not the *Sema4d*^{-/-} mice bone phenotype was based on abnormalities in the bone marrow cell system. As a result, the bone quantity increased when the *Sema4d* deficient bone marrow cells were grafted onto the wild mice as compared to when wild type bone marrow cells were grafted (see the two graphs on the left in Figure 5). Meanwhile, when wild type bone marrow cells were grafted onto *Sema4d*^{-/-} mice, the bone quantity decreased and returned to normal as compared to when the *Sema4d* deficient bone marrow cells were grafted (see the two graphs on the right in Figure 5). Based on these results, it was indicated that the bone phenotype in the *Sema4d*^{-/-} mice was such that abnormalities in the hematopoietic system cells comprising the osteoclasts were the cause. Furthermore, no clear-cut increase in the osteonodes of the *Sema4d*^{-/-} calvarial cells was confirmed in the osteonodosity of the *Sema4d*^{-/-} calvarial cells. These results suggest that the semaphorin 4D which manifests in the osteoclasts functions as a bone remodeling factor inhibiting osteogenesis through the osteoblasts.

[0052]

We produced Fc-sema4D (I. Ishida *et al.*, Int Immunol 15, 1027 (Aug, 2003) to study in detail the inhibition effect of the semaphorin 4D on the osteoblasts. Then, we added the Fc-sema4D to the calvarial cells which had been cultured under osteogenesis conditions. Adding the Fc-sema4D inhibited concentration dependently activation of ALP and manifestation of osteocalcin [Bglap] and collagen I type [Colla1]). These results indicate that the osteonodosity is inhibited as a result of the Sema4D inducing differentiation of the osteoblasts. We also cultured the calvarial cells in the presence of the culturing supernatant of the osteoclasts or in the presence of the osteoblasts to see whether or not the semaphorin 4D derived from the osteoclasts contributed to the regulation of the osteogenesis. In joint culturing with the culture supernatant of the wild-type osteoclasts or with the osteoclasts, there was no effect on the osteonodosity whereas in joint culturing with the culture supernatant of the *Sema4d*^{-/-} osteoclasts or in joint culturing with the osteoclasts, osteonodosity was clearly accelerated. Based on these results, the osteoclasts are indicated as inhibiting osteogenesis through the semaphorin 4D produced as soluble at least partially. It must also be pointed out that one or more factors are contained in the supernatant of the osteoclasts. The osteogenesis acceleration effect brought about by these factors is thought to be brought out to the fullest when semaphorin 4D is not present.

[0053]

We analyzed (a) the manifestation of the plexin B type which is well known as a semaphorin D receptor in non-lymphatic cells and lymph cells and (b) the RNA of CD72 (K.

Suzuki, A. Kumanogoh, H. Kikutani, *Nat Immunol* 9, 17 (Jan, 2008) using quantitative real time RT-PCR in order to specify the semaphorin 4D receptor where the osteoblasts manifested. As a result, the amount of plexin B1 manifested increased conspicuously during osteoblast differentiation (Figure 7). Meanwhile, the amount of plexin B2 manifested was rather low and there was virtually no manifestation plexin B 3 and CD 72 (Figure 7). After carrying out pull down analysis using Fc-sema 4D, it was indicated that the semaphorin 4D interacted with plexin B1 (Figure 8). It was also indicated that Fc-sema 4D induces phosphorylation of plexin B 1. It is well known that when semaphorin 4D binds with plexin B1, a conjugate is formed with tyrosine kinase ErbB2 and ErbB2 phosphorylates itself and plexin B1 is dependent on semaphorin B4. In osteogenesis, after inhibiting ErbB2 kinase activation, the phosphorylation declines dependent on the semaphorin 4D stimulation of plexin B1. Based on these results, it is suggested that the plexin B1 acts as the main receptor for the semaphorin 4D in the osteoblasts. Then, we analyzed the bone phenotype of the *Plxb1*^{-/-} mice. Like the *Sema4d*^{-/-} mice, the bone quantity (Figure 9) and the width of the trabecula (Figure 10) of the *Plxb1*^{-/-} mice increase conspicuously as compared to the wild type mice due to an increase in the osteoblast osteogenesis. The surface area of the osteoblasts in the *Plxb1*^{-/-} mice (Figure 11), the surface area of the calcified bone (Figure 12) and the osteogenesis rate (Figure 13) also increased respectively as compared to the wild-type mice. However, there was no change in the parameters (surface area of the osteoclasts, osteoclast cell count and corrosion of bone surface area) indicating the bone resorption in the osteoclasts. Based on these results, it is suggested that the phenotype of high bone density of the *Plxb1*^{-/-} mice, like the phenotype of the high bone density of the *Sema4d*^{-/-} mice, is caused by an increase in osteogenesis brought about by the osteoblasts. No stimulation effect on the osteonodosity by the osteoblasts confirmed by the wild-type *Sema4d*^{-/-} osteoclasts was confirmed with the *Sema4d*^{-/-} osteoclasts. Therefore, this suggests that plexin B1 recognizes the osteogenesis mainly through semaphorin 4D.

[0054]

How does the semaphorin 4D-plexin B1 transform to the inhibition signals in the osteoblasts? It is well known that the semaphorin-plexin system regulates osteomorphogenesis and cell wandering by regulating the rearrangement of the actin cell bone. It is also well known that when semaphorin 4D is combined with plexin B2, the RhoA activity is stimulated in the presence of tyrosine kinase ErbB2 whereas the semaphorin 4D has a counter action in the presence of other tyrosine kinase Met (J.M. Swiercz, T. Worzfeld, S. Offermanns, *J Bio Chem* 283, 1893 (Jan 25, 2008). Therefore, we studied the possibility of semaphorin 4D activating the RhoA. When we analyzed the amount of ErbB2 and Met manifested in the osteogenesis, it was suggested that ErbB2 manifested in considerably greater quantities than the Met. Although semaphorin 4D stimulation induced phosphorylation of the ErbB2, phosphorylation of Met was not induced. Furthermore, based on immunoblotting analysis and pull down analysis, semaphorin 4D increased the GTP combining activation type for RhoA and it is indicated that this activity is inhibited particularly by the *Plxb1*^{-/-} cells. Meanwhile, the activity of Rac1

which is in another Rho family did not affect the semaphorin 4D stimulation. Coinciding with these results, when we introduced structured activation type RhoA using an adenovirus, the osteonodosity in the calvarial cells was inhibited whereas when dominant negative RhoA was introduced, the osteonodosity was accelerated. Meanwhile, the structured activation type Rac1 and the dominant negative Rac1 did not affect the osteogenesis. Based on these results, it is suggested that the RhoA selectively mediates the inhibition effect for the osteogenesis of semaphorin 4D · plexin B1. Plexin B1 has two RhoGTPase regulation domains, that is, a GTPase activated protein (GAP) domain and a PDZ-binding domain which binds to Rho·GEF (I. Oinuma, Y. Ishikawa, H. Katoh, M. Negishi, *Science* 305, 862 (Aug 6, 2004), J.M. Swiercz, R. Kuner, J. Behrens, S. Offermanns, *Neuron* 35, 51 (July 3, 2002), V. Perrot, J. Vazquez-Prado, J.S. Gutkind, *J Bio Chem* 277, 43115 (Nov 8, 2002), M.H. Driessens, C. Olivo, K. Nagata, M. Inagaki, J.G. Collard, *FEBS Lett* 529, 168 (Oct 9, 2002). Based on pull down analysis and immunoblotting analysis, PDZ-RhoGEF and LARG (leukemia associated RhoGEF) which are known as Rho·GEG were indicated as binding with plexin B1 even in the osteoblasts. Therefore, the abovementioned GAP domain and PDZ-binding domain produced two types of plexin B1 mutants (Plexin B1-Δ PDZ and Plexin B1 · RA) to determine whether the osteoporosis was significantly inhibited (I. Oinuma, Y. Ishikawa, H. Katoh, M. Negishi, *Science* 305, 862 (Aug 6, 2004) (Figure 14). After the two types of plexin B1 mutants and wild type plexin B1 (WT-Plexin · B1) were used respectively to produce Plxnbl^{-/-} calvarial cells with excess manifestation, we stimulated these using Fc-sema 4D and evaluated the osteoblast differentiation inhibition effect. The inhibition effect of the Fc-Sema4d indicated in the manifestation of mRNA of the osteoblast markers (Alpl, Bglap and Colla1) was recovered in the WT-Plexin B1 and PlexinB1-RA excess manifestation, however, it was not recovered in the PlexinB1-Δ PDZ excess manifestation. These results suggest that RhoA mediates the inhibition of osteogenesis specifically by the semaphorin 4D.

[0055]

We crossed CAT-RHoA DN transgenic mice (K. Kobayashi *et al.*, *J. Neurosci* 24, 3480 (Apr 7, 2004) and collagen α 1 (I)-Cre transgenic mice (R. Dacquin, M. Starbuck, T. Schinke, G. Karsenty, *Dev. Dyn* 224, 245 (Jun, 2002)) and produced mice in which dominant negative RhoA was manifested specifically (RhoA DNOB) in the osteoblasts to study the role of RhoA in the osteoblasts *in vivo*. The bone quantity (Figure 15) and the width of the trabecula (Figure 16) of the RhoA DNOB mice increased as compared to the wild type mice when osteogenesis was accelerated by the osteoblasts. In addition, the surface area of the osteoblasts (Figure 17) of RhoA DNOB mice, the surface area of the bone (Figure 18) which had calcified and the osteogenesis rate (Figure 19) increased conspicuously compared to the wild-type mice, however, there were no changes in the parameters which indicated the bone resorption of the osteoclasts (surface area of osteoclasts, number of osteoclasts and surface area of corroded bone). The bone phenotype was the same as the bone phenotype for the Sema4d^{-/-} and Plxbl^{-/-} mice. As the manifestation of the RhoA DNOB increased during osteoblast differentiation, the manifestation

of the osteonodosity and osteoblast marker genes (Alpa, Bglap and Colla1) increased markedly in the calvarial cells originating in the RhoA DNOB mice. The osteonodosity which increased in the RhoA DNOB cells was not inhibited by the Fc-sema4d so that it is suggested that the RhoA mediates the inhibition signals downstream of the semaphorin 4D-plexin B1.

[0056]

In order to study whether or not the Sema4D function inhibition was effective for osteoporosis, we carried out validation using model mice with osteoporosis after menopause treated for OVX (ovaries excised). This means that we administered intravenously the anti-Sema4D antibodies in mice on a weekly basis following OVX treatment and studied whether there was a preventive effect for a decrease in bone quantity. After analyzing the bone tissue, the bone quantity decreased when anti-Sema4d antibodies were not administered whereas when anti-Sema4d antibodies were administered, acceleration in osteogenesis (increase in surface area of osteoblast [Figure 20], bone quantity [Figure 21] and osteogenesis rate [Figure 22] and decrease in trabecular intervals [Figure 23]) was confirmed. Meanwhile, there were no changes in the parameters indicating bone resorption of the osteoclasts (number of osteoclasts and surface area of corroded bone). Based on these results, when the function of semaphorin 4D is inhibited by anti-Sema 4D antibody treatment, a preventive effect on the decrease in bone quantity in osteoporosis is indicated. We also studied whether or not the functional inhibition of semaphorin 4D was effective even for treatment of bone quantity which had already decreased. This means that after OVX treatment, we administered the anti-Sema4d antibodies from the caudal vein for three weeks three times a week 6 weeks after the bone quantity had decreased. The results of analyzing the parameters indicating osteogenesis (surface area of osteoblasts, increase in bone quantity, increase in osteogenesis rate and decrease in trabecular intervals) confirmed acceleration in osteogenesis (increase in surface area of osteoblast [Figure 26], bone quantity [Figure 27] and osteogenesis rate [Figure 28] and decrease in trabecular intervals [Figure 29]). Therefore, it became clear that once the bone quantity had decreased it returned to the same level as when Sema4D antibodies were administered on a weekly basis after the abovementioned OVX treatment. This means there was a return to the same level as when decrease in bone quantity was prevented. Meanwhile, there were no changes in the parameters indicating bone resorption of the osteoclasts (surface area of corroded bone). We also studied the effect of the functional inhibition of semaphorin 4D and plexin B1 on osteonodosity. When mouse osteoblasts and the osteoclasts of WT mice were used, osteonodosity was accelerated as a function of the concentration of anti-Sema 4D antibodies (plates 1 to 3 from the left in the upper panel of Figure 24). Meanwhile, the osteonodosity which was inhibited by Fc-sema4d was accelerated as a function of the concentration of anti-plexin B1 antibodies (lower panel in Figure 24). Based on these results, when the semaphorin 4D-plexin B1 interaction is inhibited by anti-Sema 4d antibody treatment or anti-plexin B1 antibody treatment, not only is the decrease in bone quantity in osteoporosis inhibited but an effect which promotes an increase in bone quantity is indicated as well. We also studied the effect of the functional inhibition of semaphorin 4D in

human osteoblasts on osteonodosity. This means that when we carried out validation using osteoclasts which had differentiated from the human peripheral blood monocyte derived CD14 positive cells (Figure 25, lower row) and the culturing supernatant thereof (Figure 25, upper row) and when we added it to the osteoclasts and the culturing supernatant thereof and added anti-Sema4d antibodies to the osteoblasts, the osteonodosity was accelerated as a function of the concentration of the anti-Sema 4d antibodies (Figure 25). These results indicate that the results in the abovementioned post-menopausal osteoporosis model mice were supported in human cells and that inhibition of the semaphorin 4D-plexin B1 interaction is a new strategy in promoting osteogenesis.

[0057]

We made a detailed analysis of the action of the semaphorin RD osteoblast differentiation inhibition. This means that when we compared the number of hematopoietic cells in the bone marrow (Sca-1+CD105+CD106+CD44+CD45, 2-CD11b) in the Sema4d-/-mice with the wild-type mice by carrying out flow cytometry analysis, there were no differences. We also carried out cell proliferation analysis and although there was somewhat of an increase due to the Sema4d prior to the differentiation of the osteoblasts (Day 0), there were no changes due to the semaphorin 4D stimulation in the osteoblast differentiation process (Day 14). Furthermore, based on CFU analysis, the osteogenesis indicated in the ALP manifestation and the alizarin was inhibited due to the semaphorin 4D stimulation. These results suggest that the semaphorin 4D acts in osteoblast differentiation stages.

[0058]

Insulin receptor substrate (IRS) signals caused by insulin-like growth factors (IGF)-1 are known to accelerate the differentiation of the osteoblasts. Therefore, we studied Akt and ERK phosphorylation relating to the IRS signals with semaphorin 4D stimulation and the phosphorylation level declined. The Tyr phosphorylation which is the IRS activation indicator also declined. These results suggest that semaphorin 4D stimulation decreases the IRS signals. It is also clear that the activated type RhoA lowers the Akt and ERK phosphorylation and conversely Y-27632 and RKI which are RhoA inhibitors promote this phosphorylation. It is also clear that the RhoA inhibitor induces the promotion of activation type phosphorylation in the IRS signals. These results suggest that semaphorin 4D induces a decline in IRS signals by activating the RhoA and inhibits osteoblast differentiation.

[0059]

[Summary]

Based on the abovementioned experiments, we identified osteoclast-derived semaphorin 4D as an extremely important mediator for transmission between osteoclasts-osteoblasts in bone reconstruction. Bone reconstruction is carried out in cycles made up of three stages (beginning of bone resorption due to osteoclasts, transition to new osteogenesis due to osteoblasts and

completion of new bone synthesis (K. Matsuo, N. Irie, Arch Biochem Biophys 473, 201 (May 2008). Semar4d which manifests in osteoclasts functions up to the time osteoclast resorption is completed as an osteogenesis inhibition factor in the initial stage in which differentiation of the osteoblasts is inhibited. Furthermore, activation of RhoA is also indicated as inhibiting osteoblast osteogenesis *in vivo*. EphrinB2/EphB4 indicated as contributing to the transition stage also make use of RhoA to regulate the osteogenesis (C. Zhao *et al.*, Cell Metab 4, 111 (Aug, 2006) which suggests that the Rho family-lower molecular GTPase acts as a coordinator for bone reconstruction single transmission. The PDZ-containing RhoA specific GEFArfgef 12 (LARG) (J.M. Swiercz, R. Kuner, J. Behrens, S. Offermanns, Neuron 35, 51 (Jul 3, 2002); V. Perrot, J. Vazquez-Prado, J.S. Gutkind, J Bio Chem 277, 43115 (Nov 8, 2002)) has been indicated to have high manifestation in osteoblasts in GeneChip analysis. These results back up the importance of the semaphorin 4D-plexin B1-RhoA path. Furthermore, based on analysis using immunofluorescent staining, a down regulation for cadherin 11 has been observed after Fc-ema Rd stimulation and the contribution of the regulated gap binding function is suggested. Intermittent parathyroid hormones (PTH) treatment is the only effective method which has been proved to increase osteogenesis. Anti-Sost antibodies which are currently in development have garnered attention as a novel osteogenesis agent while antibodies targeting factors related to the semaphorin 4D-plexin B1-RhoA path and inhibitors and the like can be expected to be new therapeutic agents for patients with osteopenia.

[Industrial Applicability]

[0060]

The present invention can be used to effect in accelerating osteogenesis and in the prevention and treatment of bone diseases.

[Sequence Table]

Patent Application P11-0052011-108642_0.app

Claim

[Claim 1]

An osteogenesis acceleration agent having as active ingredient a substance which inhibits binding of semaphorin 4D and plexin B1.

[Claim 2]

An osteogenesis acceleration agent as described in Claim 1 characteristic in that the binding inhibition substance is an anti-semaphorin 4D antibody.

[Claim 3]

An osteogenesis acceleration agent as described in Claim 1 characteristic in that the binding inhibition substance is an anti-plexin B1 antibody or a protein containing plexin B1 extracellular regions.

[Claim 4]

An agent for prevention and treatment of bone diseases having as active ingredient a substance which inhibits binding of semaphorin 4D and plexin B1.

[Claim 5]

An agent for prevention and treatment of bone diseases as described in Claim 4 characteristic in that the binding inhibition substance is an anti-semaphorin 4D antibody.

[Claim 6]

An agent for prevention and treatment of bone diseases as described in Claim 4 characteristic in that the binding inhibition substance is an anti-plexin B1 antibody or a protein containing plexin B1 extracellular regions.

[Claim 7]

An agent for prevention and treatment of bone diseases as described in any of Claims 4 through 6 characteristic in that the bone diseases are selected from bone fracture, bone deficiency, osteoporosis, osteomalacia, osteopenia, lumbar pain, Paget's disease of bone, tonic myelitis, articular rheumatism and deformative arthrosis.

[Claim 8]

A method used to determine a candidate active ingredient for an osteogenesis acceleration agent wherein it is determined whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1, and if the abovementioned substance being studied is the abovementioned substance which inhibits binding, the abovementioned substance being studied is determined to be a candidate active ingredient for an osteogenesis acceleration agent.

[Claim 9]

A method as described in Claim 8 characteristic in that the method used to determine whether a substance being studied is a substance which inhibits binding between semaphorin 4D and plexin B1 consists of the following steps (A) through (D):

- (A) a step wherein semaphorin 4D and plexin B1 are brought in contact with one another in the presence of the substance being studied;
- (B) a step wherein the degree of binding between semaphorin 4D and plexin B1 is measured;
- (C) a step wherein the degree measured in step (B) above is compared with the degree when not in the presence of the substance being studied;
- (D) a step wherein the substance being studied is determined to be a substance which inhibits binding between semaphorin 4D and plexin B1 when the degree measured in step (B) above is lower than the degree when not in the presence of the substance being studied.

[Claim 10]

A method of screening for a candidate active ingredient for an osteogenesis acceleration agent characteristic in that the methods described in Claims 8 or 9 are used to search substances being studied for a candidate active ingredient for an osteogenesis acceleration agent.

seqlisting1843073pc01
SEQUENCE LISTING

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<150> 2011-108642

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<223> Inventor: Takayanagi, Hiroshi ;Negishi, Takako

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Glu 1325	Glu	Pro	Cys	His	Val	Asn 1330	Ser	Ser	Gln	Leu	Ile 1335	Thr	Cys	Arg
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Pro 1370	Thr	Pro	Phe	Ser	Tyr	Glu 1375	Ala	Asp	Pro	Thr	Leu 1380	Gln	Pro	Leu
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Val 1400	Phe	Ser	Val	Glu	Gly	Glu 1405	Asn	Leu	Asp	Leu	Ala 1410	Met	Ser	Lys
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Pro 1445	Leu	Pro	Arg	His	His	Ala 1450	Leu	Arg	Glu	Ala	Pro 1455	Asp	Ser	Leu
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Ala 1490	Gln	Val	Gly	Leu	Gly	Val 1495	Gly	Thr	Ser	Leu	Leu 1500	Ala	Leu	Gly
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