Description

Title of Invention

FUSION PROTEIN COMPRISING ANTI-MESOTHELIN ANTIBODY, ANTI-CD3 ANTIBODY OR ANTI-EGFR ANTIBODY, BISPECIFIC OR TRISPECIFIC ANTIBODY COMPRISING SAME, AND USES THEREOF

Technical Field

The present invention relates to a fusion protein including an anti-mesothelin antibody, an anti-CD3 antibody, or an anti-EGFR antibody; and a bispecific antibody specific for mesothelin and CD3, and a trispecific antibody specific for mesothelin, CD3, and EGFR; and uses thereof.

Background Art

In anticancer treatment, monoclonal antibodies as the first cancer immunotherapy have shown excellent effects not only in solid cancers but also in blood cancers; however, they still have limitations. In this regard, techniques are introduced in which the Fc region of an antibody is engineered to increase an antibody-dependent cytotoxicity (ADCC) effect of the antibody, or in which a bispecific or trispecific antibody is developed so that a single antibody can access multiple targets.

A bispecific antibody is an antibody that can bind to two different antibodies at the same time, and can be engineered so that immune cells such as T cells are toxic only to specific target cells such as cancer cells and are not toxic to other normal cells. Such bispecific antibodies can exhibit maximized cancer therapeutic effects with minimized side effects. Thus, several bispecific antibody formats have been developed and their suitability for T cell-mediated immunotherapy has been reported.

However, there is a problem that upon co-expression during a process of producing a bispecific antibody, mispairing of antibody heavy and light chains of different specificities results in a number of non-functional byproducts. Therefore, to solve this problem, there is a need for development of a technique for producing a desired multi-specific antibody construct in clinically sufficient amount and purity.

Disclosure of Invention

Technical Problem

As a result of conducting intensive studies to solve the problems of existing bispecific antibodies, the present inventors have developed a fusion protein, in which heavy and light chains of antibodies are linked through linkers, and thus are included as a single chain, and a bispecific or trispecific antibody comprising the same; and have found that these fusion proteins and antibodies solve the problem of mispairing and have a high killing effect on tumor cells, thereby completing the present invention.

Accordingly, an object of the present invention is to provide a fusion protein including an anti-mesothelin antibody, an anti-CD3 antibody, or an anti-EGFR antibody, and a bispecific or trispecific antibody comprising the same, and a method for treating cancer using the same.

Solution to Problem

To solve the above-mentioned problem, in an aspect of the present invention, there is provided a first fusion protein in the form of a single chain, including an antibody that specifically binds to mesothelin and an antibody that specifically binds to CD3.

In another aspect of the invention, there is provided a second fusion protein in the form of a single chain, including an antibody that specifically binds to mesothelin.

In yet another aspect of the present invention, there is provided a third fusion protein in the form of a single chain, including an antibody that specifically binds to

EGFR.

In still yet another aspect of the present invention, there is provided a bispecific antibody, comprising the first fusion protein and the second fusion protein.

In still yet another aspect of the present invention, there is provided a trispecific antibody, comprising the first fusion protein and the third fusion protein.

In still yet another aspect of the present invention, there is provided a pharmaceutical composition for treating cancer, comprising, as an active ingredient, the fusion protein, the bispecific antibody, or the trispecific antibody.

In still yet another aspect of the present invention, there is provided a use of the fusion protein, the bispecific antibody, or the trispecific antibody.

In still yet another aspect of the present invention, there is provided a method for treating cancer, comprising a step of administering, to an individual, a therapeutically effective amount of the pharmaceutical composition for treating cancer.

Advantageous Effects of Invention

The fusion protein, and the bispecific antibody or the trispecific antibody comprising the same, of the present invention, can be produced in high yield and purity, and can be effectively used for cancer treatment due to their excellent tumor killing and growth inhibitory effects.

Brief Description of Drawings

FIG. 1 illustrates an exemplary schematic diagram of an anti-MSLN/anti-CD3 bispecific antibody (hereinafter referred to as HMI323/HMI323-A15).

FIG. 2 illustrates an exemplary schematic diagram of an anti-EGFR/anti-MSLN/anti-CD3 trispecific antibody (hereinafter referred to as Cetuximab/HMI323-A15).

FIG. 3 illustrates a result obtained by performing size exclusion chromatography on HMI323/HMI323-A15.

FIG. 4 illustrates a result obtained by performing SDS-PAGE on HMI323/HMI323-A15.

FIG. 5 illustrates a result obtained by performing size exclusion chromatography on Cetuximab/HMI323-A15.

FIG. 6 illustrates a result obtained by performing SDS-PAGE on Cetuximab/HMI323-A15.

FIG. 7 illustrates a result obtained by measuring, with fluorescence activated cell sorter (FACS), the binding capacity of HMI323/HMI323-A15 or Cetuximab/HMI323-A15 to H226 cancer cell line.

FIG. 8 illustrates a result obtained by measuring, with FACS, the binding capacity of HMI323/HMI323-A15 or Cetuximab/HMI323-A15 to AsPC-1 cancer cell line.

FIG. 9 illustrates a result obtained by measuring, with FACS, the binding capacity of HMI323/HMI323-A15 or Cetuximab/HMI323-A15 to Jurkat cell line.

FIG. 10 illustrates a result obtained by subjecting a mixture of PBMCs and H226 cancer cell line (at a ratio of PBMC:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 24 hours.

FIG. 11 illustrates a result obtained by subjecting a mixture of PBMCs and AsPC-1 cancer cell line (at a ratio of PBMC:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 24 hours.

FIG. 12 illustrates a result obtained by subjecting a mixture of PBMCs and H226 cancer cell line (at a ratio of PBMC:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death

after 48 hours.

FIG. 13 illustrates a result obtained by subjecting a mixture of PBMCs and AsPC-1 cancer cell line (at a ratio of PBMC:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 48 hours.

FIG. 14 illustrates a result obtained by subjecting a mixture of PBMCs and H226 cancer cell line (at a ratio of PBMC:target cell = 20:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 48 hours.

FIG. 15 illustrates a result obtained by subjecting a mixture of PBMCs and AsPC-1 cancer cell line (at a ratio of PBMC:target cell = 20:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 48 hours.

FIG. 16 illustrates a result obtained by subjecting a mixture of T cells and H226 cancer cell line (at a ratio of T cell:target cell = 5:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 24 hours.

FIG. 17 illustrates a result obtained by subjecting a mixture of T cells and H226 cancer cell line (at a ratio of T cell:target cell = 5:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 48 hours.

FIG. 18 illustrates a result obtained by subjecting a mixture of T cells and H226 cancer cell line (at a ratio of T cell:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death after 24 hours.

FIG. 19 illustrates a result obtained by subjecting a mixture of T cells and H226 cancer cell line (at a ratio of T cell:target cell = 10:1) to treatment with HMI323/HMI323-A15 or Cetuximab/HMI323-A15, and then observing cell death

after 48 hours.

FIG. 20 illustrates a result obtained by identifying the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15 in a NOG mouse model with H226 xenograft.

FIG. 21 illustrates a result by comparing HMI323/HMI323-A15 and Cetuximab/HMI323-A15 with a control, in terms of tumor suppression effect (%), in a NOG mouse model with H226 xenograft, to identify the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15.

FIG. 22 illustrates results by observing the infiltration of immune T cells into tumor tissue in a NOG mouse model with H226 xenograft to identify the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15.

FIG. 23 illustrates a result by observing the tumor size (mm^3) in a NOG mouse model with AsPC-1 xenograft to identify the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15.

FIG. 24 illustrates a result by comparing HMI323/HMI323-A15 and Cetuximab/HMI323-A15 with a control, in terms of tumor suppression effect (%), in a NOG mouse model with AsPC-1 xenograft to identify the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15.

FIG. 25 illustrates results by observing the infiltration of immune T cells into tumor tissue in a NOG mouse model with AsPC-1 xenograft to identify the anticancer effect of HMI323/HMI323-A15 and Cetuximab/HMI323-A15.

FIG. 26 illustrates a result obtained by analyzing the pharmacokinetics (PK) of Cetuximab/HMI323-A15 in mouse serum.

FIG. 27 illustrates a result obtained by analyzing the pharmacokinetics (PK) of HMI323/HMI323-A15 in mouse serum.

Best Mode for Carrying out the Invention

In an aspect of the present invention, there is provided a first fusion protein having Structural Formula 1:

[Structural Formula 1]

N′-A-B-L1-C-D-L2-E-F-L3-G-H-L4-I-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

A is a light chain variable domain of an antibody that specifically binds to mesothelin, the domain including light chain CDR1 of SEQ ID NO: 25, light chain CDR2 of SEQ ID NO: 26, and light chain CDR3 of SEQ ID NO: 27,

B is a light chain constant domain of the antibody,

C is a heavy chain variable domain of the antibody that specifically binds to mesothelin, the domain including heavy chain CDR1 of SEQ ID NO: 28, heavy chain CDR2 of SEQ ID NO: 29, and heavy chain CDR3 of SEQ ID NO: 30,

D is a CH1 domain in heavy chain constant domains of the antibody,

E is a light chain variable domain of an antibody that specifically binds to CD3, the domain including light chain CDR1 of SEQ ID NO: 31, light chain CDR2 of SEQ ID NO: 32, and light chain CDR3 of SEQ ID NO: 33,

F is a light chain constant domain of the antibody,

G is a heavy chain variable domain of the antibody that specifically binds to CD3, the domain including heavy chain CDR1 of SEQ ID NO: 34, heavy chain CDR2 of SEQ ID NO: 35, and heavy chain CDR3 of SEQ ID NO: 36,

H is a CH1 domain in heavy chain constant domains of the antibody,

I is an Fc region or a variant thereof, and

each of L1, L2, L3, and L4 is a peptide linker.

In the present invention, each of L1, L2, L3, and L4 may be a peptide linker

consisting of 1 to 50 amino acids.

In the present invention, each of L1 and L3 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 43.

In the present invention, L2 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 44.

In the present invention, L4 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

In the present invention, L4 may be a hinge region of an antibody.

In the present invention, A may be a light chain variable domain of the antibody that specifically binds to mesothelin, the domain consisting of the amino acid sequence of SEQ ID NO: 7 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 7.

In the present invention, B may be a light chain constant domain of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to mesothelin), the domain consisting of the amino acid sequence of SEQ ID NO: 46 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 46.

In the present invention, C may be a heavy chain variable domain of the antibody that specifically binds to mesothelin, the domain consisting of the amino acid sequence of SEQ ID NO: 9 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 9.

In the present invention, D may be a CH1 domain in heavy chain constant domains of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to mesothelin), the domain consisting of the amino acid sequence of SEQ ID NO: 47 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 47.

In the present invention, E may be a light chain variable domain of an antibody that specifically binds to CD3, the domain consisting of the amino acid sequence of SEQ ID NO: 13 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 13.

In the present invention, F may be a light chain constant domain of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to CD3), the domain consisting of the amino acid sequence of SEQ ID NO: 46 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 46.

In the present invention, G may be a heavy chain variable domain of the antibody that specifically binds to CD3, the domain consisting of the amino acid sequence of SEQ ID NO: 15 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 15.

In the present invention, H may be a CH1 domain in heavy chain constant domains of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to CD3), the domain consisting of the amino acid sequence of SEQ ID NO: 47 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 47.

In the present invention, I may be an Fc region or a variant thereof, of an IgG, IgA, IgM, IgD, or IgE antibody, the region or variant consisting of the amino acid sequence of SEQ ID NO: 48 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 48. In addition, I may be obtained by substitutions at some amino acids of a CH3 domain in heavy chain constant domains of the antibody. That is, the amino acid sequence of SEQ ID NO: 48 may undergo substitutions (Y119C, K130E, and K179W) to form a knob structure in the CH3 domain in the heavy chain constant domains of the antibody; and the amino acid sequence of SEQ ID NO: 48 may

undergo substitutions (Q117R, S124C, D169V, and F175T) to form a hole structure therein.

In the present invention, the first fusion protein may consist of the amino acid sequence of SEQ ID NO: 1 or consist of an amino acid sequence having a homology of at least 90%, at least 95%, and preferably at least 99% to the amino acid sequence of SEQ ID NO: 1.

In an aspect of the present invention, there is provided a second fusion protein having Structural Formula 2:

[Structural Formula 2]

N′-A-B-L1-C-D-L5-J-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

A is a light chain variable domain of an antibody that specifically binds to mesothelin, the domain including light chain CDR1 of SEQ ID NO: 25, light chain CDR2 of SEQ ID NO: 26, and light chain CDR3 of SEQ ID NO: 27,

B is a light chain constant domain of the antibody,

C is a heavy chain variable domain of the antibody that specifically binds to mesothelin, the domain including heavy chain CDR1 of SEQ ID NO: 28, heavy chain CDR2 of SEQ ID NO: 29, and heavy chain CDR3 of SEQ ID NO: 30,

D is a CH1 domain in heavy chain constant domains of the antibody,

J is an Fc region or a fragment thereof, and

each of L1 and L5 is a peptide linker.

In the present invention, each of L1 and L5 may be a peptide linker consisting of 1 to 50 amino acids.

In the present invention, L1 may be a peptide linker consisting of the amino

acid sequence of SEQ ID NO: 43.

In the present invention, L5 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

In the present invention, L5 may be a hinge region of an antibody.

In the present invention, the second fusion protein may consist of the amino acid sequence of SEQ ID NO: 3 or consist of an amino acid sequence having a homology of at least 90%, at least 95%, and preferably at least 99% to the amino acid sequence of SEQ ID NO: 3.

J may be an Fc region or a variant thereof, of an IgG, IgA, IgM, IgD, or IgE antibody, the region or variant consisting of the amino acid sequence of SEQ ID NO: 48 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 48. In addition, J may be obtained by substitutions at some amino acids of a CH3 domain in heavy chain constant domains of the antibody. That is, the amino acid sequence of SEQ ID NO: 48 may undergo substitutions (Y119C, K130E, and K179W) to form a knob structure in the CH3 domain in the heavy chain constant domains of the antibody; and the amino acid sequence of SEQ ID NO: 48 may undergo substitutions (Q117R, S124C, D169V, and F175T) to form a hole structure therein.

In an aspect of the present invention, there is provided a third fusion protein having Structural Formula 3:

[Structural Formula 3]

N′-K-M-L6-N-O-L7-P-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

K is a light chain variable domain of an antibody that specifically binds to EGFR, the domain including light chain CDR1 of SEQ ID NO: 37, light chain CDR2 of SEQ ID NO: 38, and light chain CDR3 of SEQ ID NO: 39,

M is a light chain constant domain of the antibody,

N is a heavy chain variable domain of the antibody that specifically binds to EGFR, the domain including heavy chain CDR1 of SEQ ID NO: 40, heavy chain CDR2 of SEQ ID NO: 41, and heavy chain CDR3 of SEQ ID NO: 42,

O is a CH1 domain in heavy chain constant domains of the antibody,

P is an Fc region or a fragment thereof, and

each of L6 and L7 is a peptide linker.

In the present invention, each of L6 and L7 may be a peptide linker consisting of 1 to 50 amino acids.

In the present invention, L6 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 43.

In the present invention, L7 may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

In the present invention, L7 may be a hinge region of an antibody.

In the present invention, the third fusion protein may consist of the amino acid sequence of SEQ ID NO: 5.

In the present invention, K is a light chain variable domain of the antibody that specifically binds to EGFR, the domain consisting of the amino acid sequence of SEQ ID NO: 17 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 17.

In the present invention, M may be a light chain constant domain of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to EGFR), the domain consisting of the amino acid sequence of SEQ ID NO: 46 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 46.

In the present invention, N is a heavy chain variable domain of the antibody

that specifically binds to EGFR, the domain consisting of the amino acid sequence of SEQ ID NO: 19 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 19.

In the present invention, O may be a CH1 domain in heavy chain constant domains of an IgG, IgA, IgM, IgD, or IgE antibody (preferably an antibody that specifically binds to EGFR), the domain consisting of the amino acid sequence of SEQ ID NO: 47 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 47.

J may be an Fc region or a variant thereof, of an IgG, IgA, IgM, IgD, or IgE antibody, the region or variant consisting of the amino acid sequence of SEQ ID NO: 48 or consisting of an amino acid sequence that has a homology of at least 95% and preferably at least 99% to the amino acid sequence of SEQ ID NO: 48. In addition, I may be obtained by substitutions at some amino acids of a CH3 domain in heavy chain constant domains of the IgG, IgA, IgM, IgD, or IgE antibody. That is, the amino acid sequence of SEQ ID NO: 48 may undergo substitutions (Y119C, K130E, and K179W) to form a knob structure in the CH3 domain in the heavy chain constant domains of the antibody; and the amino acid sequence of SEQ ID NO: 48 may undergo substitutions (Q117R, S124C, D169V, and F175T) to form a hole structure therein.

In the present invention, the third fusion protein may consist of the amino acid sequence of SEQ ID NO: 5 or consist of an amino acid sequence having a homology of at least 90%, at least 95%, and preferably at least 99% to the amino acid sequence of SEQ ID NO: 5.

As used herein, the term "mesothelin (MSLN)" is meant to include wild-type MSLN, and variants, isotypes, and paralogs thereof, which are present in animals, preferably humans and monkeys. In addition, the term "human MSLN" refers to human-derived MSLN. Mesothelin is a 40-kDa cell surface glycoprotein that is present on normal mesothelial cells and is overexpressed in several human tumors, including mesothelioma, ovarian adenocarcinoma, and pancreatic adenocarcinoma.

Mesothelin has been shown to exert megakaryocyte colony-forming activity in the presence of interleukin-3. Mesothelin is a tumor differentiation antigen that is present at low levels in normal adult tissues, such as mesothelium, and is aberrantly overexpressed in a wide variety of human tumors, including mesothelioma, ovarian cancer, pancreatic cancer, squamous cell carcinoma of the cervix, head and neck, vulva, lung, and esophagus, lung adenocarcinoma, endometrial carcinoma, biphasic synovial sarcoma, desmoplastic small round cell tumor, and gastric adenocarcinoma.

As used herein, the term "CD3" is meant to include wild-type CD3, and variants, isotypes, and paralogs thereof, which are present in animals, preferably humans and monkeys. Also, the term "human CD3" refers to human-derived CD3. CD3, which is an antigen molecule present on the surface of T cells, is associated with T cell antigen receptor, and thus acts as a signaling system, thereby activating T cells.

As used herein, the term "EGFR" refers to a membrane protein that is the epidermal growth factor receptor, and is also called ErbB-1 or HER1. In addition, the term "human EGFR" refers to human-derived EGFR.

As used herein, the term "antibody" refers to an immunoglobulin (Ig) molecule that is immunologically reactive with a particular antigen, that is, a protein molecule acting as a receptor that specifically recognizes an antigen. The antibody is used as a concept encompassing a whole antibody and an antibody fragment.

As used herein, the term "heavy chain" is meant to include both a full-length heavy chain and a fragment thereof, in which the full-length heavy chain includes a variable domain (VH) sufficient to confer specificity for an antigen and three constant domains (CH1, CH2, and CH3).

As used herein, the term "light chain" is meant to include both a full-length light chain and a fragment thereof, in which the full-length light chain includes a variable domain (VL) sufficient to confer specificity for an antigen and a constant domain (CL).

As used herein, the term "CDR" refers to a complementarity determining

region that is part of the heavy chain variable domain (VH) and light chain variable domain (VL) of an antibody.

As used herein, the term "Fc" refers to a C-terminal region of an immunoglobulin, this region being one of the functional structural units which consists of only CH2 and CH3 among heavy chain constant domains. Fc has no capacity to bind an antigen. However, Fc exhibits complement-binding activity and the like, and has a constant amino acid sequence.

As used herein, the term "hinge region" refers to a peptide present between CH1 and CH2 of a heavy chain. The hinge region has a highly flexible structure in which one or more cysteine residues are present so that two heavy chains are linked to each other by disulfide bond(s).

In the present invention, the hinge region may include 1 to 3 cysteine residues, for example, 1, 2, or 3 cysteine residues.

In addition, in the present invention, there is provided a polynucleotide encoding the above-described fusion protein.

In the present invention, the DNA encoding the first fusion protein may consist of the nucleotide sequence of SEQ ID NO: 2 or consist of a nucleotide sequence having a homology of at least 80%, preferably at least 90%, more preferably at least 95%, and most preferably at least 99% to the nucleotide sequence of SEQ ID NO: 2.

In the present invention, the DNA encoding the second fusion protein may consist of the nucleotide sequence of SEQ ID NO: 4 or consist of a nucleotide sequence having a homology of at least 80%, preferably at least 90%, more preferably at least 95%, and most preferably at least 99% to the nucleotide sequence of SEQ ID NO: 4.

In the present invention, the DNA encoding the third fusion protein may consist of the nucleotide sequence of SEQ ID NO: 6 or consist of a nucleotide sequence having a homology of at least 80%, preferably at least 90%, more preferably at least 95%, and most preferably at least 99% to the nucleotide sequence of SEQ ID

NO: 6.

In addition, in the present invention, there is provided an expression vector, comprising the above-described fusion protein-encoding polynucleotide.

In addition, in the present invention, there is provided a host cell transfected by introduction of the expression vector.

In addition, in the present invention, there is provided a method for producing the fusion protein, comprising a step of culturing the host cell.

In another aspect of the present invention, there is provided a bispecific antibody, comprising the first fusion protein and the second fusion protein.

As used herein, the term "bispecific antibody" refers to a single antibody engineered to recognize two different antigens.

The bispecific antibody of the present invention contains an anti-MSLN antibody and an anti-CD3 antibody at the same time, and thus can specifically bind to MSLN-expressing cancer cells and T cells so that inhibited proliferation or death of the MSLN-expressing cancer cells is caused through induction of immune cell activation.

In the present invention, to provide a single antibody, that is, a bispecific antibody, through binding of the first fusion protein to the second fusion protein, each fusion protein may undergo substitutions at some amino acid residues in the heavy chain constant domain. That is, the Fc regions of the first fusion protein and the second fusion protein may be linked to each other via a disulfide bond or a knob-into-hole structure (with the knob-into-hole structure being preferred) to form a bispecific antibody.

As used herein, the term "knob-into-hole structure" refers to a structure obtained by inducing mutations in the respective CH3 domains of two different Ig heavy chains so that a knob structure is induced in one Ig heavy chain CH3 domain and a hole structure is induced in the other Ig heavy chain CH3 domain, and allowing the two domains to form a heterodimer.

Typically, in amino acid residues that form the knob structure, a hydrophobic amino acid residue having a large side chain is substituted with a hydrophobic amino acid residue having a small side chain; and in amino acid residues that form the hole structure, a hydrophobic amino acid residue having a small side chain is substituted with a hydrophobic amino acid residue having a large side chain. However, the present invention is not limited thereto. In this way, in a case where the respective antibodies are induced to form a knob structure and a hole structure, a heterodimer may be formed more easily than a homodimer.

In the present invention, the bispecific antibody may include a knob modification in I of the first fusion protein and a hole modification in J of the second fusion protein. Alternatively, the bispecific antibody may include a hole modification in I of the first fusion protein and a knob modification in J of the second fusion protein.

In the present invention, the bispecific antibody may be in the form of 2+1 IgGs and may consist of two single chains obtained by linking the heavy and light chains, which constitute the anti-MSLN or anti-CD3 Fab fragment, to each other with a linker so that the respective fragments are expressed as single chains, and linking the anti-MSLN fragment and the anti-CD3 Fab fragment, which are single chains, to each other with a peptide linker.

In another aspect of the present invention, there is provided a trispecific antibody, comprising the first fusion protein and the third fusion protein.

As used herein, the term "trispecific antibody" refers to a single antibody engineered to recognize three different antigens.

The trispecific antibody of the present invention contains anti-EGFR antibody, an anti-MSLN antibody, and an anti-CD3 antibody at the same time, and thus can specifically bind to EGFR- or MSLN-expressing cancer cells and T cells so that inhibited proliferation or death of the EGFR- or MSLN-expressing cancer cells is caused through induction of immune cell activation.

In the present invention, to provide a single antibody, that is, a trispecific antibody, through binding of the first fusion protein to the third fusion protein, each fusion protein may undergo substitutions at some amino acid residues in the heavy chain constant domain. That is, the Fc regions of the first fusion protein and the third fusion protein may be linked to each other via a disulfide bond or a knob-into-hole structure (with the knob-into-hole structure being preferred) to form a trispecific antibody.

In the present invention, the trispecific antibody may include a knob modification in I of the first fusion protein and a hole modification in P of the third fusion protein. Alternatively, the trispecific antibody may include a hole modification in I of the first fusion protein and a knob modification in P of the third fusion protein.

In the present invention, the trispecific antibody may be in the form of 2+1 IgGs and may consist of two single chains obtained by linking the heavy and light chains, which constitute the anti-EGFR Fab fragment, to each other with a linker so that the fragment is expressed as a single chain, linking the heavy and light chains, which constitute the anti-MSLN or anti-CD3 Fab fragment, to each other with a linker so that the respective fragments are expressed as single chains, and linking the anti-MSLN fragment and the anti-CD3 Fab fragment, which are single chains, to each other with a peptide linker.

In yet another aspect of the present invention, there are provided a pharmaceutical composition for treating cancer, comprising, as an active ingredient, the first fusion protein, the second fusion protein, the third fusion protein, the bispecific antibody, or the trispecific antibody; and a method for treating cancer in an individual, comprising a step of administering, to the individual, a therapeutically effective amount of the pharmaceutical composition.

The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier. As the pharmaceutically acceptable carrier, a binder, a glidant, a disintegrant, an excipient, a solubilizer, a dispersant, a stabilizer, a suspending agent, a

pigment, a flavor, and the like may be used for oral administration; a buffer, a preserving agent, a pain-relieving agent, a solubilizer, an isotonic agent, a stabilizer, and the like may be used in admixture for injections; and a base, an excipient, a lubricant, a preserving agent, and the like may be used for topical administration.

Preparations of the pharmaceutical composition may be prepared in various ways by being mixed with the pharmaceutically acceptable carrier as described above. For example, for oral administration, the pharmaceutical composition may be formulated in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, or the like. For injections, the pharmaceutical composition may be formulated in the form of unit dosage ampoules or multiple dosage forms.

The pharmaceutical composition may be administered in a pharmaceutically effective amount to treat cancer cells or metastasis thereof or to inhibit cancer growth. The effective amount may vary depending on various factors such as type of cancer, the patient's age, weight, nature and severity of symptoms, type of current therapy, number of treatments, dosage form, and route of administration, and may be easily determined by experts in the corresponding field.

The pharmaceutical composition may be administered together or sequentially with the above-mentioned pharmacological or physiological components, and may also be administered in combination with additional conventional therapeutic agents, in which case the pharmaceutical composition may be administered sequentially or simultaneously with the conventional therapeutic agents. Such administration may be single or multiple administration. Taking all of the above factors into consideration, it is important to administer an amount that is a minimum amount and allows the maximum effect to be obtained without adverse effects, and such an amount may be easily determined by those skilled in the art.

In the present invention, the cancer may include, without limitation, any carcinoma known in the art.

In still yet another aspect of the present invention, there is provided a use of the first fusion protein, the second fusion protein, the third fusion protein, the

bispecific antibody, or the trispecific antibody, for the manufacture of a medicament for treating cancer in an individual in need thereof.

Mode for the Invention

Hereinafter, preferred examples are provided to help understand the present invention. However, the following examples are merely given for easier understanding of the present invention, and the scope of the present invention is not limited thereto.

Example 1. Production of anti-MSLN/anti-CD3 bispecific antibody and anti-EGFR/anti-MSLN/anti-CD3 trispecific antibody

Example 1.1. Production of MSLN-, CD3-, or EGFR-specific antibody

To select MSLN-specific antibodies, mice were immunized with recombinant human MSLN, and then B cells were extracted therefrom to clone various antibody genes. Using phage display, these genes were used to prepare an antibody library, and cloning of antibodies binding to the recombinant human MSLN was performed. Finally, antibody humanization was performed to obtain HMI323 clone consisting of the amino acid sequence of SEQ ID NO: 1, this amino acid sequence being encoded by a gene consisting of the nucleotide sequence of SEQ ID NO: 4.

To select antibodies specific for human and monkey CD3, the mouse SP34 antibody was humanized, and the antibodies, which bind to CD3 with various affinity, were selected. Among these, Clone A07 consisting of the amino acid sequence of SEQ ID NO: 21, Clone A15 consisting of the amino acid sequence of SEQ ID NO: 11, and Clone E15 consisting of the amino acid sequence of SEQ ID NO: 23 were obtained, and these amino acid sequences are encoded by genes consisting of the nucleotide sequences of SEQ ID NOs: 22, 12, and 24, respectively.

For the EGFR-specific antibody, Cetuximab, which is a therapeutic agent for colorectal cancer, was used. The antibody consists of the amino acid sequence of SEQ ID NO: 5, and this amino acid sequence is encoded by a gene consisting of the

nucleotide sequence of SEQ ID NO: 6.

Example 1.2. Transfection of vector for antibody expression

Example 1.2.1. Construction of vector for antibody expression

As illustrated in FIG. 1, vectors were constructed as follows in such a manner that co-expression of anti-MSLN antibody (HMI323) and an antibody obtained by linking anti-CD3 Fab (A15) to anti-MSLN Fab (HMI323) was performed so that a bispecific antibody (HMI323/HMI323-A15) having a knob-into-hole structure is expressed.

Using In-fusion HD (Takara, Mountain View, CA, USA), DNA (SEQ ID NO: 4) for anti-MSLN (HMI323) antibody sequence or DNA (SEQ ID NO: 2) for the antibody sequence obtained by linking anti-CD3 Fab (A15) to anti-MSLN Fab (HMI323) was inserted into each pCI vector (Promega, Madison, WI, USA), and then E. coli competent cells were subjected to heat shock transformation therewith at 42°C for 45 seconds. The transformants were plated on a carbenicillin-containing LB plate and cultured for 14 hours in an incubator at 37°C. After culture, the transformant colonies cultured on the plate were inoculated into 2 ml of carbenicillin-containing LB medium and cultured at 37°C for 16 hours in a shaking incubator. Then, plasmids were extracted from the cultured transformants, and sequencing thereof showed that the genes for antibody expression were cloned into the vectors.

As illustrated in FIG. 2, vectors were constructed as follows in such a manner that co-expression of anti- EGFR antibody (Cetuximab) and an antibody obtained by linking anti-CD3 Fab (A15) to anti-MSLN Fab (HMI323) was performed so that a trispecific antibody (Cetuximab/HMI323-A15) having a knob-into-hole structure is expressed.

Using In-fusion HD (Takara, Mountain View, CA, USA), DNA (SEQ ID NO: 6) for anti-EGFR (Cetuximab) antibody sequence or DNA (SEQ ID NO: 2) for the antibody sequence obtained by linking anti-CD3 Fab (A15) to anti-MSLN Fab (HMI323) was inserted into each pCI vector (Promega, Madison, WI, USA), and then

E. coli competent cells were subjected to heat shock transformation therewith at 42°C for 45 seconds. The transformants were plated on a carbenicillin-containing LB plate and cultured for 14 hours in an incubator at 37°C. After culture, the transformant colonies cultured on the plate were inoculated into 2 ml of carbenicillin-containing LB medium and cultured at 37°C for 16 hours in a shaking incubator. Then, plasmids were extracted from the cultured transformants, and sequencing thereof showed that the genes for antibody expression were cloned into the vectors.

Example 1.2.2. Transfection of vector for antibody expression into host cells

24 hours before, Expi293F™ cells (Thermo Fisher Scientific) at a density of 2.0×10^6 cells/ml were passaged with Expi293™ expression medium (Thermo Fisher Scientific) at 125 ± 10 rpm in a shaking incubator at 37°C and 8% CO_2. At the time of transfection, the number of cells and cell viability were measured to identify whether cell viability of 95% or higher is exhibited. The cells were dispensed at 5×10^8 cells in a 500 mL culture flask, and then Expi293™ expression medium was added to adjust the final volume to 170 mL (based on 200 mL).

Using Opti-MEM I™ medium (Thermo Fisher Scientific), 200 μg of antibody-expressing vector was mixed therewith to a total of 1,500 μl, and culture was performed for 5 minutes at room temperature. Using Opti-MEM I™ medium, 540 μl of transfection reagent (ExpiFectamine™ 293 Reagent, Thermo Fisher Scientific) was mixed therewith to a total of 1,500 μl, and culture was performed for 5 minutes at room temperature. The Opti-MEM I™ media, containing the vector and the transfection reagent, respectively, were mixed gently and allowed to react at room temperature for 20 minutes. Then, the resultant was placed in a flask containing Expi293F™ cells.

Culture was performed for 16 to 20 hours at 125 ± 10 rpm in a shaking incubator at 37°C and 8% CO_2. Then, 1 ml of transfection enhancer I (ExpiFectamine™ 293 Enhancer I, Thermo Fisher Scientific) and 10 ml of transfection enhancer II (ExpiFectamine™ 293 Enhancer II, Thermo Fisher Scientific)

were added thereto, and culture was performed for 5 days to obtain candidate antibodies.

Example 2. Purification of anti-MSLN/anti-CD3 bispecific antibody and anti-EGFR/anti-MSLN/anti-CD3 trispecific antibody

Example 2.1. Purification using affinity chromatography

As an experiment to separate only antibodies using specific interaction between Protein A and the antibody, the culture solution was centrifuged at 4000 rpm for 30 minutes and filtered with a 0.22 μm bottle top filter to prepare a supernatant from which cell debris was removed. Then, a column packed with 5 ml of Mabselect PrismA resin (GE Healthcare) was connected with ÄKTA PrimePlus (GE Healthcare) and used. Subsequently, washing with binding buffer (Pierce) was performed, and then the supernatant was loaded at a rate of 5 ml/min. After all of the prepared supernatant was loaded, washing with binding buffer (Pierce) was performed at the same rate to remove non-specific binding.

After washing, while flowing IgG elution buffer (Pierce), a section, in which the UV 280nm value rose, was collected portionwise by 5 ml in a tube containing 5 ml of binding buffer (Pierce), and the eluate was immediately neutralized. The neutralized eluate was subjected to buffer exchange with PBS using a Zeba spin desalting column (Thermo Fisher Scientific).

Example 2.2. Purification using size exclusion chromatography (SEC)

As an experiment to separate, from among the separated antibodies, only a target antibody of 200 kDa using a purification method for separation by size, a Superdex 200 SEC column (GE Healthcare) was connected with ÄKTA pure 150L equipment (GE Healthcare) and used. A sample loop was filled with the antibody sample, which was subjected to buffer exchange with PBS, and connected with the ÄKTA pure 150L equipment (GE Healthcare). Then, the process was conducted under a flow rate condition of 1 ml/min, and the elution sample was collected portionwise by 1 ml depending on its peak pattern. The portionwise-collected eluate

was subjected to SDS-PAGE (NuPAGE®, Novex 4-12% Bis-Tris Gel, Invitrogen), and then checked by staining with Coomassie Blue. Only the highly pure target antibody of 200 kDa was pooled.

By doing so, the results as shown in Table 1 were obtained.

[Table 1]

Construct	Yield (mg/L)	Aggregation after SEC (%)	Monomer (%)
HMI323/HMI323-A15	3.3	30	100
Cetuximab/HMI323-A15	3.2	30	100

Example 3. Measurement of affinity of antibodies to MSLN, CD3, and EGFR

Using the Octet system, affinity of the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) to human MSLN, human CD3, and human EGFR, respectively, was measured.

Specifically, recombinant human MSLN, CD3, and EGFR were prepared at 20 μg/ml in 1X kinetic buffer, and plated in 96-well plates at 200 μl/well. The plated MSLN, CD3, and EGFR were immobilized on an Aminopropylsilane (APS) sensor (Cat # 18-5045, Fortebio). The bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) were prepared at 100, 50, 25, 12.5, and 6.25 nM in 10X kinetic buffer, and plated at a concentration of 200 μl/well. The interaction between each antibody and each of the recombinant human MSLN (Table 2), human CD3 (Table 3), and human EGFR (Table 4), which were immobilized on the sensor, was analyzed to calculate the antigen-antibody affinity. The results are as shown in Tables 2 to 4, respectively.

[Table 2]

Antibody	ka (1/M·s)	kd (1/s)	KD (nM)
HMI323/HMI323-A15	9.94×10^{4}	7.74×10^{-4}	7.79
Cetuximab/HMI323-A15	6.20×10^{4}	1.36×10^{-3}	22.6

[Table 3]

Antibody	ka (1/M·s)	kd (1/s)	KD (nM)
HMI323/HMI323-A15	4.61×10^{5}	2.42×10^{-2}	52.6
Cetuximab/HMI323-A15	4.63×10^{5}	6.25×10^{-3}	13.5

[Table 4]

Antibody	ka (1/M·s)	kd (1/s)	KD (nM)
Cetuximab/HMI323-A15	2.08×10^{6}	2.54×10^{-3}	1.22

From the measurement results as shown in Tables 2 to 4, it was found that the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) exhibited excellent affinity to human MSLN, human CD3, and human EGFR.

Example 4. Analysis of affinity of antibodies to MSLN-, EGFR-, and CD3-expressing cells

It was identified, using flow cytometry, whether the bispecific antibody (HMI323/HMI323-A15) or the trispecific antibody (Cetuximab/HMI323-A15) exhibits affinity to specific cell lines.

Specifically, tubes were prepared, each tube containing 100 μl of FACS buffer (2% FBS/ sheath buffer) to which each of three types of cell line, that is, H226 cancer cell line (ATCC®, CRL-5826™) overexpressing MSLN and EGFR, AsPC-1 cancer cell line (ATCC®, CRL-1682™) overexpressing MSLN and EGFR, and Jurkat E6-1 T cell line (ATCC®, TIB-152™) expressing CD3, was added at a concentration of 0.5×10^{6} cells, and then each tube was treated with 1 μg of primary antibody (HMI323/HMI323-A15 or Cetuximab/HMI323-A15). Then, light was blocked for one and a half hours and incubation was performed at 4°C. Subsequently, 200 μl of FACS buffer was added thereto. Centrifugation was performed at 2,000 rpm for 3 minutes at 4°C, and then the supernatant was removed.

Next, each tube was treated with 0.2 μg of fluorochrome-labeled secondary antibody (PE-labeled anti-human IgG), which is capable of specifically binding to the primary antibody, in 100 μl of FACS buffer. Then, light was blocked for 30 minutes and incubation was performed at 4°C. Subsequently, 200 μl of FACS buffer was

added thereto, and centrifugation was performed at 2,000 rpm for 3 minutes at 4°C; and then the supernatant was removed to obtain a sample.

200 μl of BD Cytofix™ was added to the sample so that the cells were suspended, and analysis was performed by BD LSR Fortessa™. EC_{50} values for binding affinity to specific cells are as shown in Table 5 below.

[Table 5]

Cell line	HMI323/HMI323-A15 (nM)	Cetuximab/HMI323-A15 (nM)
H226	6.2	1.2
AsPC-1	4.7	0.48
Jurkat	58	113

As a result of the analysis, as illustrated in FIGS. 7 to 9, it was found that both the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) bound to the three types of cell line. The negative control antibody used as an irrelevant control did not bind to any of the three types of cell line.

Example 5. Evaluation of cell killing efficacy of antibodies against tumor cell lines in presence of PBMCs

Example 5.1. Construction of target cell lines

Two types of MSLN+ tumor cells (H226, AsPC-1) were transduced with IncuCyte® NucLight Red Lentivirus Reagent (EF-1 Alpha Promoter, Puromycin selection) and treated with puromycin, so that the target cell lines, which are NucLight Red-transduced tumor cell lines, were finally constructed.

Example 5.2. Preparation of target cell lines

The cells were harvested with 1× trypsin-EDTA solution, and centrifuged at 1,200 rpm for 5 minutes at 4°C. Subsequently, the supernatant was removed and resuspension was performed in cRPMI (RPMI, A10491-01 + 10% FBS + 55 μM β-ME). Then, the number of cells was quantified. The respective cell line suspensions were prepared, added to 96-well plates at 10,000 cells/well, and the plates

were incubated in a CO_2 incubator at 37°C for one day to prepare the target cell lines.

Example 5.3. Preparation of peripheral blood mononuclear cells

Cryopreserved peripheral blood mononuclear cells (PBMCs) were rapidly thawed in a water bath at 37°C, and then transferred to a 50 ml conical tube. Thawing medium (RPMI, 11875-093 + 10% FBS + 55 μM β-ME) was added thereto dropwise, and mixing was performed with shaking. Then, the supernatant was removed by performing centrifugation at 1,200 rpm for 10 minutes at 4°C, and resuspension was performed in cRPMI. Then, the number of cells was quantified.

Example 5.4. Plating of peripheral blood mononuclear cells and antibodies

The target cells were plated in wells. 24 hours later, each of the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) was diluted with cRPMI, and then diluted 1/5 starting from 20 nM (at a concentration range of 0.26 pM to 20 nM). Then, PBMCs were prepared at a ratio of PBMC:target cell = 20:1 or 10:1, suspended in cRPMI, and then added to the wells.

Example 5.5. Real-time cell measurement and analysis using IncuCyteS3

Bright field and red fluorescence were measured at 24 hours and 48 hours at 10× magnification using IncuCyteS3 while performing incubation in a CO_2 incubator at 37°C for 2 days. IC_{50} values for PBMC-mediated cell killing by antibodies against MSLN-expressing tumor cells are as shown in Table 6 below, and maximum effects (%) of PBMC-mediated cell killing by antibodies against MSLN-expressing tumor cells are as shown in Table 7 below.

[Table 6]

PBMC: target cell	Hours	Cancer cell line	IC_{50} value (pM)	
			HMI323/HMI323-A15	Cetuximab/HMI323-A15
10:1	24hr	H226	84.38	14.93
		AsPC-1	405.8	3.845
10:1	48hr	H226	50.31	8.913

		AsPC-1	143.6	1.351
20:1	48hr	H226	15.32	3.918
		AsPC-1	30.36	1.374

[Table 7]

PBMC: target cell	Hours	Cancer cell line	Maximum killing effect (%)	
			HMI323/HMI323-A15	Cetuximab/HMI323-A15
10:1	24hr	H226	56.57	49.67
		AsPC-1	38.3	43.8
10:1	48hr	H226	90.66	92
		AsPC-1	74.62	80.99
20:1	48hr	H226	93.46	94.54
		AsPC-1	84.24	81.29

As a result of the evaluation, as illustrated in FIGS. 10 to 15, it was identified that cell-killing effects of the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) against two types of MSLN- and EGFR-expressing cancer cell line (H226, AsPC-1) were exhibited in a dose-dependent manner at a condition of PBMC:target cell = 20:1 or 10:1. In addition, the bispecific antibody (irrelevant/CD3) obtained by linking irrelevant Ab to CD3 antibody did not induce cell death.

Example 6. Evaluation of cell killing efficacy of antibodies against MSLN+ tumor cell lines in presence of T cells

Example 6.1. Construction of target cell lines

Two types of MSLN+ tumor cells (H226, AsPC-1) were transduced with IncuCyte® NucLight Red Lentivirus Reagent (EF-1 Alpha Promoter, Puromycin selection) and treated with puromycin, so that the target cell lines, which are NucLight Red-transduced tumor cell lines, were finally constructed.

Example 6.2. Preparation of target cell lines

The cells were harvested with 1× trypsin-EDTA solution, and centrifuged at 1,200 rpm for 5 minutes at 4°C. Subsequently, the supernatant was removed and

resuspension was performed in cRPMI (RPMI, A10491-01 + 10% FBS + 55 μM β-ME). Then, the number of cells was quantified. The respective cell line suspensions were prepared, added to 96-well plates at 10,000 cells/well, and the plates were incubated in a CO_2 incubator at 37°C for one day to prepare the target cell lines.

Example 6.3. Preparation of proliferated T cells

Cryopreserved peripheral blood mononuclear cells (PBMCs) were rapidly thawed in a water bath at 37°C, and then transferred to a 50 ml conical tube. Thawing medium (RPMI, 11875-093 + 10% FBS + 55 μM β-ME) was added thereto dropwise, and mixing was performed with shaking. Then, the supernatant was removed by performing centrifugation at 1,200 rpm for 10 minutes at 4°C, and suspended in MACS medium (PBS + 0.5% FBS + 2 mM EDTA). CD3 microbeads were added thereto depending on the number of cells, and staining was performed at 4°C for 15 minutes. Only CD3 T cells were isolated using an LS column. The isolated CD3 T cells were suspended in X-VIVO. Then, αCD3/28 Dynabeads®, IL-2 (200 U/ml, Proleukin®), and human plasma (5%) were added thereto, and incubation was performed in an incubator at 37°C. The number of cells was counted 4 days and 7 days after initiation of the incubation, and X-VIVO, IL-2, and human plasma were additionally added so that the cell concentration reached 1×10^6 cells/ml.

Example 6.4. Plating of T cells and antibodies

The target cells were plated in wells. 24 hours later, each of the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) was diluted with cRPMI, and then diluted 1/5 starting from 20 nM (at a concentration range of 0.26 pM to 20 nM). Then, the proliferated T cells were finally prepared at a ratio of T cell:target cell = 10: 1 or 5: 1, suspended in cRPMI, and then added to the wells.

Example 6.5. Real-time cell measurement and analysis using IncuCyteS3

Bright field and red fluorescence were measured at 24 hours and 48 hours at 10× magnification using IncuCyteS3 (Essenbio) while performing incubation in a CO_2

incubator at 37°C for 2 days. IC_{50} values for T cell-mediated cell killing by antibodies against MSLN-expressing tumor cells are as shown in Table 8 below, and maximum effects (%) of T cell-mediated cell killing by antibodies against MSLN-expressing tumor cells are as shown in Table 9 below.

[Table 8]

T cell: target cell	Hours	IC_{50} value	
		HMI323/HMI323-A15	Cetuximab/HMI323-A15
5:1	24 hr	8.57	0.86
	48 hr	9.31	0.91
10:1	24 hr	5.57	0.50
	48 hr	5.78	0.51

[Table 9]

T cell: target cell	Hours	Maximum killing effect (%)	
		HMI323/HMI323-A15	Cetuximab/HMI323-A15
5:1	24 hr	96.33	95.28
	48 hr	99.40	99.82
10:1	24 hr	97.71	99.07
	48 hr	99.52	99.95

As a result of the evaluation, as illustrated in FIGS. 16 to 19, it was identified that cell-killing effects of the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15) against two types of MSLN- and EGFR-expressing cancer cell line (H226, AsPC-1) were exhibited in a dose-dependent manner at a condition of T cell:target cell = 10:1 or 5:1. In addition, the bispecific antibody (irrelevant/CD3) obtained by linking irrelevant Ab to CD3 antibody did not induce cell death.

Example 7. Analysis of tumor growth inhibition efficacy of antibodies in mice

To check whether the bispecific antibody (HMI323/HMI323-A15) and the trispecific antibody (Cetuximab/HMI323-A15), which were under development as T-cell engager antibodies, have tumor growth inhibition efficacy, these antibodies were

administered to a tumor-implanted mouse model.

Example 7.1. Establishment of cell line xenograft model

Mice, which were brought into an animal room, had an acclimatization period for about a week before start of the experiment. H226 tumor cell line (1×10^7/200 μL PBS) or AsPC-1 tumor cell line (5×10^6/200 μL PBS) was injected subcutaneously into the axillary fossa of the mice. On day 5 after injection of the tumor cells, purified and activated human T cells were intraperitoneally injected thereinto at a ratio of T cell:tumor cell = 2:1. On day 7 after injection of the tumor cells, the mice were divided into 4 groups (each group containing 5 mice), and then statistical analysis was performed. As a result, it was identified that there was no significant difference in tumor size between the groups.

Example 7.2. Antibody administration

Starting from day 2 after injection of T cells, PBS or the antibody (HMI323/HMI323-A15 or Cetuximab/HMI323-A15) was intraperitoneally administered at a concentration of 3 mg/kg twice a week (4 times in total).

Example 7.3. Measurement of tumor size

From the day of grouping to the end point, the tumor size (tumor volume (mm^3) = short axis × long axis × height × 0.5) was measured twice a week. The lengths of the short axis, the long axis, and the height were measured by the same test person using a ruler.

Example 7.4. Calculation of tumor growth rate (RTV %), relative tumor growth rate (T/C %), and tumor growth inhibition rate (inhibitory effect)

The tumor growth rate (relative tumor volume (RTV) %) was calculated using a V1/V0 method. V1 means a volume at a time point when the experimental effect is measured, and V0 means a volume at a time point when the experiment starts. The relative tumor growth rate (T/C%) of each group is a value obtained by dividing the average tumor growth rate of each group by the average tumor growth rate of the PBS

group (in which the average tumor growth rate is obtained at a time point when the measurement is performed), and then multiplying the resulting value by 100; and the tumor growth inhibition rate (inhibitory effect) is calculated as 100 minus the relative tumor growth rate. For statistical analysis, one-way ANOVA or two-way ANOVA was used depending on the type of experiment, and a case where the p value is lower than 0.05 was determined to be statistically significant.

Example 7.5. Immunohistochemistry

One week after subjecting the tumor-grown mice to treatment with the antibody (HMI323/HMI323-A15 or Cetuximab/HMI323-A15), tumor samples were removed therefrom and fixed with formalin. Thereafter, the samples were subjected to paraffin infiltration and embedding, and then a microtome was used to obtain paraffin sections. The paraffin sections were deparaffinated, hydrated, and washed; and then stained with human CD3 (anti-CD3, Abcam) or human CD8 (anti-CD8, Abcam). The tissue sections were counterstained with Mayer's hematoxylin (Dako) and observed under an Olympus BX51 microscope.

Example 7.6. End of experiment and results

The mice were euthanized by cervical dislocation or CO_2. As a result, for a mouse model xenografted with the mesothelin-overexpressing lung adenocarcinoma cell line H226, in a case where the Cetuximab/HMI323-A15 or HMI323/HMI323-A15 antibody was intraperitoneally administered thereto 4 times in total at a concentration of 3 mg/kg, all of the antibody-administered groups showed excellent tumor growth inhibition efficacy as compared with the PBS-administered group (FIGS. 20 and 21).

Immunohistochemical staining showed that infiltration of immune T cells (stained in dark color) into the tumor tissue was higher in the antibody (Cetuximab/HMI323-A15 or HMI323/HMI323-A15)-administered group than in the PBS group (FIG. 22). As shown in Table 10 below, a 100% tumor growth inhibition effect was exhibited from day 20 after tumor xenograft for Cetuximab/HMI323-A15, which is an antibody that binds to mesothelin, EGFR, and CD3, and from day 25 after

tumor xenograft for HMI323/HMI323-A15, which is an antibody that binds to mesothelin and CD3.

[Table 10]

H226 group	Inhibition rate (median)						
	Day 7	Day 10	Day 13	Day17	Day 20	Day 25	Day 28
HMI323/HMI323-A15	0%	-53%	-35%	74%	80%	100%	100%
Cetuximab/HMI323-A15	0%	-119%	-40%	86%	100%	100%	100%

In addition, for a mouse model xenografted with AsPC-1, which is a pancreatic cancer cell line with a relatively low expression level of mesothelin relative to H226, in a case where the Cetuximab/HMI323-A15 or HMI323/HMI323-A15 antibody was intraperitoneally administered thereto 4 times in total at a concentration of 3 mg/kg, all of the antibody-administered groups similarly showed excellent tumor growth inhibition efficacy as compared with the PBS-administered group (FIGS. 23 and 24).

Immunohistochemical staining also showed that infiltration of immune T cells (stained in dark color) into the tumor tissue was higher in the antibody (Cetuximab/HMI323-A15 or HMI323/HMI323-A15)-administered group than in the PBS group (FIG. 25). As shown in Table 11 below, a 100% tumor growth inhibition effect was exhibited from day 20 after tumor xenograft for Cetuximab/HMI323-A15, which is an antibody that binds to mesothelin, EGFR, and CD3, and from day 28 after tumor xenograft for HMI323/HMI323-A15, which is an antibody that binds to mesothelin and CD3.

[Table 11]

AsPC-1 groups	Inhibition rate (median)							
	Day 7	Day 10	Day 13	Day 17	Day 20	Day 25	Day 28	Day 32
HMI323/HMI323-A15	0%	23%	58%	94%	97%	97%	100%	100%
Cetuximab/HMI323-A15	0%	26%	57%	95%	100%	100%	100%	100%

Example 8. PK analysis of antibodies in mice

To obtain pharmacokinetics (PK) data of Cetuximab/HMI323-A15 or HMI323/HMI323-A15, which were under development as T-cell engager antibodies, PK parameters of the antibodies in mouse serum were analyzed.

Example 8.1. Obtaining of test solution (mouse serum)

The Cetuximab/HMI323-A15 or HMI323/HMI323-A15 antibody was injected into the tail vein of the mice at a concentration of 3 mg/kg. Blood collection from the retro-orbital plexus was performed at 5 minutes, 1 hour, 4 hours, 8 hours, 24 hours, 48 hours, 72 hours, 120 hours, 168 hours, 240 hours, 336 hours, 504 hours, and 672 hours after antibody injection. 100 μL of blood was collected from 3 or more animals in each group. The blood was coagulated for about 20 to 30 minutes at room temperature, and then centrifuged at 10,000 rpm for 10 minutes to separate the serum.

Example 8.2. ELISA assay

Anti-human Fab (50 ng/100 μL/well) was added to a plate and reaction was allowed to proceed at 4°C overnight. Then, the remaining solution was completely removed. 1% BSA/PBS solution was added thereto at 200 μL/well and reaction was allowed to proceed at room temperature for 1 hour. Then, the remaining solution was completely removed. The standard solution (standard antibody) was adjusted to 1 μg/mL using 1% serum/1% BSA/PBS solution, and then prepared by being diluted therewith by 1/2. The test solution was prepared by being diluted 100-fold with 1% BSA/PBS solution.

Each 100 μL of the prepared standard solution and test solution was dispensed into two wells for each concentration, and reaction was allowed to proceed at room temperature for one hour. After the reaction, washing was performed 3 times in total by dispensing 300 μL of PBST (0.05% Tween 20) solution per well. Anti-human Fc-HRP was diluted 5000-fold with 1% BSA/PBST solution, and then 100 μL thereof was dispensed into each well. Reaction was allowed to proceed at room temperature for 1 hour. After the reaction, washing was performed 3 times in total by dispensing 300 μL of PBST (0.05% Tween 20) solution per well. A TMB peroxidase substrate solution was brought to room temperature before the experiment, and 100 μL thereof

was dispensed into each well. Reaction was allowed to proceed at room temperature for 30 minutes. 100 μL of TMB stop solution was dispensed into each well, gentle shaking was performed for better mixing, and then absorbance was measured at a wavelength of 450 nm.

Cetuximab/HMI323-A15, which is an antibody that binds to mesothelin, EGFR, and CD3, or HMI323/HMI323-A15, which is an antibody that binds to mesothelin and CD3, was injected at a dose of 3 mg/kg into the tail vein of nude mice. Then, the blood was collected at 5 minutes, 1 hour, 4 hours, 8 hours, 24 hours, 48 hours, 72 hours, 96 hours, 168 hours, 240 hours, 336 hours, 504 hours, and 672 hours after injection, and the antibody concentration in mouse serum was quantified by ELISA technique. The results are illustrated in FIGS. 26 and 27.

PK parameters were analyzed based on the ELISA results. As a result, it was identified that the Cetuximab/HMI323-A15 antibody had a half-life of about 207.94 hours, and the HMI323/HMI323-A15 antibody had a half-life of about 202.58 hours (Tables 12 and 13). These half-lives fall within a half-life range for common bispecific antibodies in an IgG form. In addition, the values obtained by extrapolation of the area under the curve (AUC) were 20% or lower, and thus reliability of the calculated PK parameters was ensured.

The PK parameter results for Cetuximab/HMI323-A15 are as shown in Table 12 below, and the PK parameter results for HMI323/HMI323-A15 are as shown in Table 13.

[Table 12]

Parameter (units)	Parameter value
Material	Cetuximab/HMI323-A15
Dose (mg/kg)	3
$T_{1/2}$ (h)	207.94 ± 23.91
C_{max} (μg/mL)	41.40 ± 6.87
AUC_{0-t} (μg.h/mL)	4512.59 ± 601.50
AUC_{0-inf} (μg.h/mL)	5071.93 ± 891.25

AUC_%Extrap_obs (%)	10.48	±	5.43
CL (mL/hr/kg)	0.61	±	0.11
MRT (hr)	223.19	±	11.76
Vss (mL/kg)	180.08	±	13.15

[Table 13]

Parameter (units)	Parameter value		
Material	HMI323/HMI323-A15		
Dose (mg/kg)	3		
$T_{1/2}$ (h)	202.58	±	84.68
C_{max} (µg/mL)	45.46	±	5.52
AUC_{0-t} (µg.h/mL)	4568.32	±	821.75
AUC_{0-inf} (µg.h/mL)	5270.89	±	1719.18
AUC_%Extrap_obs (%)	10.65	±	11.09
CL (mL/hr/kg)	0.61	±	0.17
MRT (hr)	210.51	±	30.99
Vss (mL/kg)	166.09	±	19.28

Claims

[Claim 1]

A first fusion protein having Structural Formula 1:

[Structural Formula 1]

N′-A-B-L1-C-D-L2-E-F-L3-G-H-L4-I-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

A is a light chain variable domain of an antibody that specifically binds to mesothelin, the domain including light chain CDR1 of SEQ ID NO: 25, light chain CDR2 of SEQ ID NO: 26, and light chain CDR3 of SEQ ID NO: 27,

B is a light chain constant domain of the antibody,

C is a heavy chain variable domain of the antibody that specifically binds to mesothelin, the domain including heavy chain CDR1 of SEQ ID NO: 28, heavy chain CDR2 of SEQ ID NO: 29, and heavy chain CDR3 of SEQ ID NO: 30,

D is a CH1 domain in heavy chain constant domains of the antibody,

E is a light chain variable domain of an antibody that specifically binds to CD3, the domain including light chain CDR1 of SEQ ID NO: 31, light chain CDR2 of SEQ ID NO: 32, and light chain CDR3 of SEQ ID NO: 33,

F is a light chain constant domain of the antibody,

G is a heavy chain variable domain of the antibody that specifically binds to CD3, the domain including heavy chain CDR1 of SEQ ID NO: 34, heavy chain CDR2 of SEQ ID NO: 35, and heavy chain CDR3 of SEQ ID NO: 36,

H is a CH1 domain in heavy chain constant domains of the antibody,

I is an Fc region or a variant thereof, and

each of L1, L2, L3, and L4 is a peptide linker.

[Claim 2]

The first fusion protein of claim 1, wherein each of L1, L2, L3, and L4 is a peptide linker consisting of 1 to 50 amino acids.

[Claim 3]

The first fusion protein of claim 1, wherein each of L1 and L3 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 43.

[Claim 4]

The first fusion protein of claim 1, wherein L2 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 44.

[Claim 5]

The first fusion protein of claim 1, wherein L4 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

[Claim 6]

The first fusion protein of claim 1, wherein the first fusion protein consists of the amino acid sequence of SEQ ID NO: 1.

[Claim 7]

A second fusion protein having Structural Formula 2:

[Structural Formula 2]

N′-A-B-L1-C-D-L5-J-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

A is a light chain variable domain of an antibody that specifically binds to mesothelin, the domain including light chain CDR1 of SEQ ID NO: 25, light chain

CDR2 of SEQ ID NO: 26, and light chain CDR3 of SEQ ID NO: 27,

B is a light chain constant domain of the antibody,

C is a heavy chain variable domain of the antibody that specifically binds to mesothelin, the domain including heavy chain CDR1 of SEQ ID NO: 28, heavy chain CDR2 of SEQ ID NO: 29, and heavy chain CDR3 of SEQ ID NO: 30,

D is a CH1 domain in heavy chain constant domains of the antibody,

J is an Fc region or a fragment thereof, and

each of L1 and L5 is a peptide linker.

[Claim 8]

The second fusion protein of claim 7, wherein each of L1 and L5 is a peptide linker consisting of 1 to 50 amino acids.

[Claim 9]

The second fusion protein of claim 7, wherein L1 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 43.

[Claim 10]

The second fusion protein of claim 7, wherein L5 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

[Claim 11]

The second fusion protein of claim 7, wherein the second fusion protein consists of the amino acid sequence of SEQ ID NO: 3.

[Claim 12]

A third fusion protein having Structural Formula 3:

[Structural Formula 3]

N′-K-M-L6-N-O-L7-P-C′

wherein N' is the N-terminus of the fusion protein,

C' is the C-terminus of the fusion protein,

K is a light chain variable domain of an antibody that specifically binds to EGFR, the domain including light chain CDR1 of SEQ ID NO: 37, light chain CDR2 of SEQ ID NO: 38, and light chain CDR3 of SEQ ID NO: 39,

M is a light chain constant domain of the antibody,

N is a heavy chain variable domain of the antibody that specifically binds to EGFR, the domain including heavy chain CDR1 of SEQ ID NO: 40, heavy chain CDR2 of SEQ ID NO: 41, and heavy chain CDR3 of SEQ ID NO: 42,

O is a CH1 domain in heavy chain constant domains of the antibody,

P is an Fc region or a fragment thereof, and

each of L6 and L7 is a peptide linker.

[Claim 13]

The third fusion protein of claim 12, wherein each of L6 and L7 is a peptide linker consisting of 1 to 50 amino acids.

[Claim 14]

The third fusion protein of claim 12, wherein L6 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 43.

[Claim 15]

The third fusion protein of claim 12, wherein L7 is a peptide linker consisting of the amino acid sequence of SEQ ID NO: 45.

[Claim 16]

The third fusion protein of claim 12, wherein the third fusion protein consists of the amino acid sequence of SEQ ID NO: 5.

[Claim 17]

A bispecific antibody, comprising:

the first fusion protein of claim 1; and

the second fusion protein of claim 7.

[Claim 18]

The bispecific antibody of claim 17, wherein a knob modification is included in I of the first fusion protein and a hole modification is included in J of the second fusion protein; or a hole modification is included in I of the first fusion protein and a knob modification is included in J of the second fusion protein.

[Claim 19]

A trispecific antibody, comprising:

the first fusion protein of claim 1; and

the third fusion protein of claim 12.

[Claim 20]

The trispecific antibody of claim 19, wherein a knob modification is included in I of the first fusion protein and a hole modification is included in P of the third fusion protein; or a hole modification is included in I of the first fusion protein and a knob modification is included in P of the third fusion protein.

[Claim 21]

A pharmaceutical composition for treating cancer, comprising as an active ingredient:

the first fusion protein of claim 1;

the second fusion protein of claim 7;

the third fusion protein of claim 12,

the bispecific antibody of claim 17; or

the trispecific antibody of claim 19.

[Claim 22]

A use of the following protein or antibody for the manufacture of a medicament for treating cancer in an individual in need thereof:

the first fusion protein of claim 1;

the second fusion protein of claim 7;

the third fusion protein of claim 12,

the bispecific antibody of claim 17; or

the trispecific antibody of claim 19.

[Claim 23]

A method for treating cancer in an individual, comprising:

a step of administering, to the individual, a therapeutically effective amount of the pharmaceutical composition of claim 21.