Description

Title of Invention

BISPECIFIC ANTIBODY SPECIFICALLY BINDING TO TIE2 AND VEGF, AND USE THEREOF

Technical Field

The present invention relates to a bispecific antibody comprising a novel anti-Tie2 antibody or a binding fragment thereof and a VEGF binding site, and a pharmaceutical use thereof.

Background Art

Tie2 (tyrosine kinase receptor 2) protein is a receptor-type tyrosine kinase (TK) specifically expressed in vascular endothelial cells. It is activated by phosphorylation by its specific ligand, angiopoietin-1 (Ang-1) protein. Tie2 protein is known to play a crucial role in angiogenesis and in the structural and functional normalization and stabilization of blood vessels in various physiological and pathological conditions (Korean Patent No. 10-2195957). Therefore, Tie2 protein is attracting attention as a therapeutic agent for ischemic diseases. In addition, the activation of Tie2 protein is known to inhibit vascular leakage, vascular inflammation, and vascular abnormalization induced by vascular endothelial growth factor (VEGF). Recently, methods have been proposed to suppress abnormal angiogenesis and to induce vascular normalization and stabilization by inhibiting VEGF activation and activating Tie2 protein via Ang-1 protein as therapeutic strategies for diabetic retinopathy, sepsis, and tumors.

However, Ang-1 tetramer, the physiologically activated form of the Ang-1 protein, is very difficult to produce as a recombinant protein and has a short half-life *in vivo*, thereby making its use as a therapeutic agent difficult. In order to address the problems associated with Ang-1, research is underway on various Tie2-specific activators that could replace the Ang-1 protein. However, issues such as low productivity and concerns about inducing immune responses limit their use as therapeutics. Furthermore, VEGF inhibitors are known to exhibit various side effects.

Therefore, there is a need for research and development of methods for treating various vascular abnormality-related disease, such as angiogenesis, endothelial signaling, inflammation, and/or vascular leakage regulation, through more effective induction of Tie2 protein activity and inhibition of VEGF activity.

Detailed Description of Invention

Technical Problem

Accordingly, the present inventors have conducted research to develop a therapeutic agent for vascular abnormality-related disease. As a result, the present inventors have found that a novel anti-Tie2 antibody and a bispecific antibody comprising the antibody and a VEGF binding site improved a vascular abnormality-related disease in a mouse model of vascular disease. Based on the above, the present inventors have completed the present invention.

Solution to Problem

In order to achieve the above object, in one aspect of the present invention, there is provided an antibody or fragment thereof that specifically binds to Tie2, comprising (a) a heavy chain variable region (VH) comprising i) a heavy chain complementarity determining region 1 (HCDR1) represented by the amino acid sequence of SEQ ID NO: 84, ii) a heavy chain complementarity determining region 2 (HCDR2) represented by the amino acid sequence of SEQ ID NO: 85, and iii) a heavy chain complementarity determining region 3 (HCDR3) represented by any one amino acid sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 15, and SEQ ID NO: 86; and (b) a light chain variable region (VL) comprising iv) a light chain complementarity determining region 1 (LCDR1) represented by the amino acid sequence of SEQ ID NO: 87, v) a light chain complementarity determining region 2 (LCDR2) represented by the amino acid sequence of SEQ ID NO: 88, and vi) a light chain complementarity determining region 3 (LCDR3) represented by the amino acid sequence of SEQ ID NO: 89. In the amino acid sequence of $X_1X_2X_3FX_4X_5X_6X_7$ of SEQ ID NO: 84, X_1 is G or S, X_2 is F or D, X_3 is T or S, X_4 is N, T or A, X_5 is S or G, X_6 is Y, N, or Q, and X_7 is G, W, or A; in the amino acid sequence of $X_8X_9X_{10}DX_{11}X_{12}X_{13}X_{14}$ of SEQ ID NO: 85, X_8 is T or I, X_9 is S or Y, X_{10} is N, P, or W, X_{11} is G, D, or S, X_{12} is S or G, X_{13} is T, D, or G, and X_{14} is T or I; in the amino acid sequence of ARKVVRGY$X_{15}X_{16}$HDAFDI of SEQ ID NO: 86, X_{15} is S, H, or L, and X_{16} is Y or P; in the amino acid sequence of $QX_{17}X_{18}X_{19}SX_{20}$ of SEQ ID NO: 87, X_{17} is S, G, or D, X_{18} is V or I, X_{19} is S or D, and X_{20} is Y or N; in the amino acid sequence $X_{21}X_{22}S$ of SEQ ID NO: 88, X_{21} is G or A, and X_{22} is A or S; in the amino acid sequence of $QQX_{23}X_{24}X_{25}X_{26}PX_{27}T$ of SEQ ID NO: 89, X_{23} is Y, A, or G, X_{24} is G, N, or Y, X_{25} is T, S, or D, X_{26} is T or F, and X_{27} is Y, L, or W.

In another aspect of the present invention, there is provided a polynucleotide encoding the antibody or fragment thereof, an expression vector comprising the polynucleotide, and a cell transformed with the vector.

In another aspect of the present invention, there is provided a method for producing an antibody or fragment thereof, comprising culturing the transformed cell, and obtaining the antibody or fragment thereof from the culture solution.

In another aspect of the present invention, there is provided a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof; and a VEGF-specific binding site.

In another aspect of the present invention, there is provided a polynucleotide encoding the bispecific antibody, a vector comprising the polynucleotide, and a cell transformed with the vector.

In another aspect of the present invention, there is provided a method for producing a bispecific antibody, comprising culturing the transformed cell, and obtaining the bispecific antibody from the culture solution.

In another aspect of the present invention, there is provided a pharmaceutical composition for preventing or treating a vascular abnormality-related disease, comprising the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof, and a VEGF-specific binding site as an active ingredient.

In another aspect of the present invention, there is provided a use of the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof, and a VEGF-specific binding site for the prevention or treatment of a vascular abnormality-related disease.

In another aspect of the present invention, there is provided a method for preventing or treating a vascular abnormality-related disease, comprising administering to a subject the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof, and a VEGF-specific binding site.

Effects of Invention

The anti-Tie2 antibody according to the present invention induced phosphorylation by specifically binding to the Tie2 protein. At this time, the antigen specificity and affinity of the anti-Tie2 antibody were excellent compared to existing ligands (Ang-1, Ang-2). In addition, the bispecific antibody comprising the anti-Tie2 antibody and the VEGF binding site inhibited VEGF while activating the Tie2 protein. In particular, the bispecific antibody effectively inhibited side effects that can be caused by an inhibitor of VEGF. In addition, the anti-Tie2 antibody or bispecific antibody exhibited a therapeutic effect on diseases in a mouse model of vascular abnormality-related diseases. Thus, the anti-Tie2 antibody and the bispecific antibody comprising the anti-Tie2 antibody and the VEGF-specific binding site according to the present invention can be used as a therapeutic agent for vascular abnormality-related diseases.

Brief Description of Drawings

Figures 1a to 1c are diagrams showing the results obtained by confirming the level of phosphorylation of Tie2 and its downstream signaling factors using an anti-Tie2 antibody (MT-100) in HEK293 cells, 3T3-J2 cells, or HUVEC (human umbilical vein endothelial cells), which are cells expressing human or mouse Tie2, through western blotting.

Figures 2a to 2d are diagrams showing the results obtained by confirming the level of phosphorylation of Tie2 and its downstream signaling factors after treatment with an anti-Tie2 antibody (MT-101, MT-110, or MT-111) in HUVECs, through western blotting.

Figure 3 is a diagram showing the results obtained by confirming the level of phosphorylation of Tie2 and its downstream signaling factors after treatment with an IgG1 type antibody (MT-101 (IgG1)), an antibody having the constant region of isotype IgG4 (MT-101 (IgG4)), and an antibody having the L234A/L235A amino acid substitution mutation in the IgG1 type CH2 region (MT-101 (IgG1-LALA)) of the anti-Tie2 antibody (MT-101) in HUVECs, through western blotting.

Figure 4 is a diagram showing the results obtained by screening for phosphorylation induction of 49 human tyrosine receptor proteins (receptor tyrosine kinases (RTKs)) following treatment with an anti-Tie2 antibody (MT-100) in HUVECs.

Figures 5a to 5d are graphs showing the results obtained by confirming the species cross-binding (cross-reactivity) of an anti-Tie2 antibody (MT-100, MT-101 (IgG1), MT-101 (IgG1-LALA), or MT-101 (IgG4)).

Figure 6 is a diagram showing the results obtained by confirming the vascular network stabilization inducing effect of an anti-Tie2 antibody (MT-100).

Figures 7a and 7b are graphs showing the results obtained by confirming apoptosis in HUVECs cultured under serum-free and hypoxic conditions after treatment with an anti-Tie2 antibody (MT-101 (IgG1), MT-101 (IgG1-LALA), or MT-101 (IgG4)).

Figures 8a and 8b are graphs and diagrams showing the results obtained by confirming vascular permeability (vascular leakage phenomenon) in HUVECs after treatment with VEGF (vascular endothelial growth factor) and an anti-Tie2 antibody (MT-100 or MT-101). Figure 8c is a diagram showing the results obtained by confirming the expression of ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), which are inflammatory response mediators, in HUVECs after treatment with VEGF and an anti-Tie2 antibody (MT-100 or MT-101), through western blotting.

Figures 9a and 9b are graphs showing the results obtained by confirming the effects of an anti-Tie2 antibody (MT-101) on the mutual binding of Tie2 protein and angiopoietin-2 (Ang-2) protein, using biolayer interferometry (BLI).

Figure 10 is a diagram showing the results obtained by confirming the level of phosphorylation of Tie2 and its downstream signaling factors after pretreatment or co-treatment with an anti-Tie2 antibody (MT-101) of Ang-2 protein in HUVEC, through western blotting.

Figures 11a to 11c are diagrams showing the results obtained by confirming the efficacy of inhibiting acute kidney injury after administration of an anti-Tie2 antibody (MT-101 (MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA)) in an acute kidney injury (AKI) mouse model induced by ischemia and reperfusion (I/R). Figure 11a is a diagram

showing the experimental schedule, and Figure 11b is a diagram showing the results of serum creatinine analysis. Figure 11c is a diagram showing the results of blood urea nitrogen (BUN) analysis.

Figures 12a and 12b are diagrams showing the results obtained by confirming renal tubular injury (tubular interstitial injury) after administration of an anti-Tie2 antibody (MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA)) in an I/R-induced AKI mouse model, through H&E (hematoxylin & eosin) staining. Figure 12a is a diagram showing the staining results, and Figure 12b is a graph showing the results in numerical form.

Figure 13a is a diagram showing the results obtained by confirming the phosphorylation of Tie2 protein (P-Tie2) in renal blood vessels after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in an I/R-induced AKI mouse model, through immunostaining. Figure 13b shows the results of immunostaining for vascular endothelial cell marker protein (CD31) and vascular endothelial pericyte marker protein (PDGFRβ).

Figure 14a is a diagram showing the results obtained by confirming the expression of an inflammatory response mediator (VCAM-1) in renal blood vessels (CD31) after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in an I/R-induced AKI mouse model through immunostaining. Figure 14b is a diagram showing the results obtained by confirming the degree of adhesion or influx of neutrophil cells through immunostaining of Gr-1 protein. Figure 14c is a graph showing the results obtained by confirming the expression of cytokines (CCL2, IL-6, or IL-1β) that induce an immune response in kidney tissue through polymerase chain reaction (real-time PCR) analysis.

Figures 15a to 15d are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) after intravenous administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) to mice in an acute kidney injury mouse model (LPS-induced AKI) caused by lipopolysaccharide (LPS)-induced sepsis. Figure 15a is a diagram showing the experimental schedule. Figures 15b and 15c are graphs showing the results obtained by measuring serum creatinine and blood urea nitrogen (BUN). Figure 15d is a diagram showing the results obtained by confirming the degree of kidney injury and renal vascular (CD31) injury through immunostaining for KIM-1 (kidney injury molecule-1) protein and CD31 protein, respectively.

Figures 16a to 16c are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in a mouse model of chronic renal failure (chronic kidney disease, CKD) caused by ischemia and reperfusion. Figure 16a is a diagram showing the experimental schedule. Figure 16b is a diagram showing the results obtained by confirming renal tubular injury in kidney tissue through H&E staining. Figure 16c is a diagram showing the results obtained by confirming renal fibrosis through Masson's trichrome staining, Sirius red staining, and immunostaining for fibrosis markers (collagen IV, α-SMA).

Figures 17a to 17d are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in an adenine-induced CKD mouse model. Figure 17a is a diagram showing the experimental schedule. Figure 17b is a diagram showing the results of serum creatinine measurement, and Figure 17c is a diagram showing the results obtained by confirming the degree of kidney injury through gene expression of KIM-1 and NGAL (neutrophil gelatinase-associated lipocalin). Figures 17d and 17e are diagrams and graphs showing the results of H&E and PAS (Periodic acid-Schiff) staining.

Figures 18a to 18d are diagrams showing the results obtained by confirming renal fibrosis in mice after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) at different concentrations (2 mg/kg, 5 mg/kg, 10 mg/kg) in an adenine-induced CKD mouse model. Figures 18a to 18c are diagrams showing the results of Sirius red staining and immunostaining for fibrosis markers (collagen IV or α-SMA), respectively. Figure 18d is a diagram showing the results obtained by confirming the expression of Col1a1, Col3a1, ACTA2, or TGF-β gene through polymerase chain reaction.

Figures 19a and 19c are diagrams showing the results obtained by confirming injury to renal tubules (lotus tetragonolobus lectin, LTL) and vascular endothelial cells after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) at different concentrations (2 mg/kg, 5 mg/kg, 10 mg/kg) in an adenine-induced CKD mouse model. Figure 19a is a diagram showing the results of immunostaining, and Figure 19b is a diagram showing the results obtained by confirming the gene expression of immune response inducers (MCP-1, TNF-α, or IL-1β) and immune cell marker proteins (CD4, CD11c, F4/80, or CD206) through polymerase chain reaction.

Figures 20a to 20c are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in a unilateral ureteral obstruction (UUO)-induced CKD mouse model. Figure 20a is a diagram showing the experimental schedule. Figures 20b to 20e are diagrams showing the results obtained by confirming the degree of renal tubular injury (tubular interstitial injury) and fibrosis through H&E staining, Masson's trichrome staining, and immunostaining for fibrosis marker proteins (collagen IV or α-SMA).

Figures 21a and 21b are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) after administration of an anti-Tie2 antibody (MT-101 (IgG1-LALA)) in a lipopolysaccharide (LPS)-induced sepsis mouse model (LPS-induced sepsis). Figure 21a is a diagram showing the experimental schedule. Figure 21b is a graph showing the results obtained by confirming the percent survival (%) of mice.

Figures 22a to 22d are diagrams showing the results obtained by confirming the efficacy of anti-Tie2 antibodies (MT-100 and MT-101) after administration of anti-Tie2 antibodies (MT-100 and MT-101) at different concentrations (1 mg/kg, 5 mg/kg) in a hindlimb

ischemic mouse model. Figure 22a is a diagram showing the results obtained by observing bloodstream flow and tissue necrosis. Figure 22b is a diagram showing the results obtained by analyzing bloodstream flow (LDPI ratio), and Figure 22c is a diagram showing the results obtained by analyzing tissue necrosis. Figure 22d is is a diagram showing the results obtained by analyzing angiogenesis (α-SMA^+ $CD31^+$ vessels).

Figures 23a to 23c are diagrams showing the results obtained by measuring the intracavernosal pressure (ICP; indicating the degree of erectile power) of the corpus cavernosum penis according to penile nerve electrical stimulation after administration of an anti-Tie2 antibody (MT-100 or MT-101) at different concentrations (1 μg, 10 μg) in a diabetic erectile dysfunction mouse model. Figure 23a is a diagram showing the results obtained by measuring the intracavernosal pressure, and Figure 23b is a diagram showing the results obtained by analyzing the maximum intracavernosal pressure, and Figure 23c is a diagram showing the results obtained by analyzing the total intracavernosal pressure.

Figures 24a to 24c are diagrams showing the results obtained by confirming angiogenesis through immunostaining for vascular endothelial cell marker protein (CD31) and vascular endothelial pericyte marker protein (NG2) in corpus cavernosum penis tissue after administration of anti-Tie2 antibodies (MT-100 and MT-101) in a diabetic erectile dysfunction mouse model. Figure 24a is a diagram showing the results of immunostaining, and Figure 24b is a diagram showing the results obtained by analyzing the expression area of vascular endothelial cell marker protein (CD31), and Figure 24c is a diagram showing the results obtained by analyzing the expression area of vascular endothelial pericyte marker protein (NG2).

Figures 25a and 25b are graphs showing the results obtained by confirming the degree of capillary-like tube formation after treatment with an anti-Tie2 antibody (MT-100 or MT-101) in HUVECs treated with high glucose (HG), and the results obtained by analyzing the vascular network formation value.

Figures 26a to 26c are diagrams showing the results obtained by confirming the efficacy of an anti-Tie2 antibody (MT-100 or MT-101) or control drug (EYLEA) after intravitreal administration of an anti-Tie2 antibody (MT-100 or MT-101) or control drug (EYLEA) in a laser-induced choroidal neovascularization (CNV) mouse model. Figure 26a is a diagram showing the experimental schedule. Figure 26b is a diagram showing the results obtained by observing choroidal neovascularization, and Figure 26c is a diagram showing the results obtained by measuring the choroidal vascular leakage value relative to choroidal neovascularization.

Figures 27a to 27d are diagrams showing the results obtained by analyzing endomucin expression, CNV mass, area, and volume after intravitreal administration of an anti-Tie2 antibodies (MT-100 or MT-101) in a laser-induced choroidal neovascularization (CNV) mouse model.

Figures 28a to 28c are diagrams showing the results obtained by confirming the avascular area and neovascular tuft area in the retina after intravitreal administration of an anti-Tie2 (MT-101) antibody or a control drug (EYLEA) in an oxygen-induced retinopathy (OIR) mouse model. Figure 28a is a diagram showing the experimental schedule. Figures 28b and 28c are diagrams showing the results obtained by observing the avascular area and neovascular tuft area in the retina, respectively.

Figures 29a and 29b are diagrams showing the results obtained by confirming the degree of ischemia-induced retinal vascular endothelial cell proliferation and leakage after intravitreal administration of an anti-Tie2 antibody (MT-101) in an oxygen-induced retinopathy (OIR) mouse model.

Figure 30 is a diagram showing the structure of a bispecific antibody (MT-103-1, MT-103-2, MT-103-3, MT-103-4, and MT-103-5) comprising an anti-Tie2 antibody (MT-101) and a vascular endothelial growth factor (VEGF) binding site, which is one specific example of the present invention.

Figures 31a to 31e are diagrams showing showing the results obtained by confirming the phosphorylation of VEGFR2 and its downstream signaling factors after treatment with VEGF and a bispecific antibody (MT-103-1, MT-103-2, MT-103-3, MT-103-4, or MT-103-5) or a control drug (EYLEA, Avastin, or Beovu) in HUVECs, through western blotting.

Figures 32a and 32b are diagrams showing showing the results obtained by confirming the phosphorylation of Tie2 and its downstream signaling factors after treatment with an anti-Tie2 antibody (MT-101) or a bispecific antibody (MT-103-1 or MT-103-4) in HUVECs, through western blotting.

Figure 33a is a graph showing the results obtained by confirming apoptosis after treatment with an anti-Tie2 antibody (MT-101) or a bispecific antibody (MT-103-1 or MT-103-4) at different concentrations (1 nM, 10 nM) in HUVECs. Figures 33b and 33c are diagrams and graphs showing the results obtained by confirming the increase in vascular endothelial cell permeability induced by VEGF through immunostaining of the adherens junction protein (VE-cadherin) after treatment with VEGF and a bispecific antibody (MT-103-1 or MT-103-4) at different concentrations (0.125 nM, 0.5 nM, 2 nM, 8 nM, 32 nM) in HREC cells (human retinal endothelial cells). Figure 33d is a graph showing the increase in vascular endothelial cell permeability induced by VEGF and Ang-2, as measured by the degree of permeation of fluorescently labeled dextran, after treatment with VEGF, Ang-2, and a bispecific antibody (MT-103-1 or MT-103-4) at different concentrations (0.05 nM, 0.5 nM, and 5 nM) in HREC cells.

Figure 34 is a diagram showing the results obtained by confirming the binding of Ang-2 and Tie2 following the binding of a bispecific antibody (MT-103-1) and VEGF using biolayer interferometry.

Figure 35a is a diagram showing experimental conditions for confirming activation of VEGFR and Tie2 downstream signaling pathways after treatment with Ang-1, Ang-2, and a bispecific antibody (MT-103-1 or MT-103-4) in HUVECs. Figure 5b is a diagram showing the results obtained by confirming the phosphorylation of VEGFR2, Tie2, and their downstream signaling factors under each condition through western blotting.

Figure 36a is a diagram showing the results obtained by confirming the phosphorylation of VEGFR2 and Tie2 downstream signaling factors after treatment with VEGF, a bispecific antibody (MT-103-1 or MT-103-4), or a control drug (Vabysmo) in HUVECs, through western blotting. Figure 36b is a diagram showing the results obtained by comparing the degree of apoptosis after treating HUVEC with a bispecific antibody (MT-103-1 or MT-103-4) or a control drug (Vabysmo, EYLEA, or Beovu) and culturing under serum-free and hypoxic conditions.

Figures 37a to 37c are diagrams showing the results obtained by confirming the neovascularization inhibitory efficacy after intravitreal administration of an anti-Tie2 antibody (MT-101) or a bispecific antibody (MT-103-1 or MT-103-4) in a laser-induced choroidal neovascularization (CNV) mouse model. Figure 37a is a diagram showing the experimental schedule. Figure 37b is a diagram showing the results obtained by confirming the choroidal vascular leakage, and Figure 37c is a diagram showing the results obtained by analyzing the CNV vascular area.

Figures 38a to 38c are diagrams showing the results obtained by confirming the efficacy after intravitreal administration of a bispecific antibody (MT-103-1 or MT-103-4) or a control drug (EYLEA) in a laser-induced choroidal neovascularization (CNV) mouse model. Figure 38a is a diagram showing the experimental schedule. Figure 38b is a diagram showing the results obtained by confirming the choroidal vascular leakage, and Figure 38c is a diagram showing the results obtained by analyzing the CNV vascular area.

Figures 39a to 39c are diagrams showing the results obtained by confirming the efficacy after intravitreal administration of a bispecific antibody (MT-103-1 or MT-103-4) or a control drug (EYELA) in an oxygen-induced retinopathy mouse model (OIR). Figure 39a is a diagram showing the experimental schedule. Figures 39b and 39c are diagrams showing the results obtained by observing the avascular area and neovascular tuft area in the retina of a mouse, respectively.

Best Mode for Carrying out the Invention

Definition of Terms

As used herein, the term "antibody" refers to an immunoglobulin molecule and a multimer thereof having a structure in which one light chain is linked by a disulfide bond to each of two heavy chains by a disulfide bond. The light chain (LC) comprises two regions: one variable region (variable region of light chain, VL) and one constant region (constant region of

light chain, CL). In addition, the heavy chain (HC) comprises four regions: one variable region (variable region of heavy chain, VH) and three constant regions (constant region of heavy chain, CH; CH1, CH2, and CH3). The constant regions of the light and heavy chains are responsible for biological properties such as binding between light and/or heavy chains, secretion, complement binding, and binding to Fc receptors (FcRs), while the variable regions of the light and heavy chains determine antigen recognition and binding specificity. The antibody may act as an agonist or an antagonist.

As used herein, the term "antigen binding fragment" refers to a portion of an intact antibody, particularly any polypeptide or glycoprotein comprising the antigen binding site or variable region of an intact antibody. The antigen binding fragments as described above may be produced by recombinant DNA techniques or by enzymatic or chemical degradation of intact antibodies, and examples of such antigen binding fragments include, but are not limited to, Fv, Fab, F(ab')2, Fab', dsFv, (dsFv)2, scFv, sc(Fv)2, diabodies, and the like, as well as bispecific and multispecific antibodies formed therefrom.

As used herein, the term "single-chain Fv" or "scFv" refers to a protein in which the VH and VL domains of an antibody are linked by a linker consisting of a peptide chain of approximately 15 amino acids. The sequence may be either light chain variable region (VL)-linker-heavy chain variable region (VH) or heavy chain variable region (VH)-linker-light chain variable region (VL), and possesses antigen specificity identical to or similar to that of the original antibody.

As used herein, the term "heavy chain" encompasses both full-length heavy chains and fragments thereof, including the variable region (VH) and three constant regions, CH1, CH2, and CH3. The heavy chain constant regions (CH) exhibit different amino acid compositions and sequences, and thus possesses different types of antigenicity. Therefore, immunoglobulins may be classified into five categories and referred to as immunoglobulin isotypes: IgM, IgD, IgG, IgA, and IgE. The corresponding heavy chains are the μ chain, δ chain, γ chain, α chain, and ε chain, respectively. In addition, immunoglobulins of the same type may be classified into different subtypes based on the amino acid composition of the hinge region and the number and location of heavy chain disulfide bonds. For example, IgG may be classified into IgG1, IgG2, IgG3, and IgG4.

As used herein, the term "light chain" refers to two types, λ and κ, consisting of approximately 211 to 217 amino acids. Each human antibody has only one such chain. The light chain consists of a continuous variable region (VL) and a constant region (CL). The light chain may include both full-length light chains and fragments thereof. Any CL and CH1 regions of these antibody classes may be used in the present disclosure.

The "variable region" refers to the region of an antibody that binds to an antigen. The variable region comprises three hypervariable regions, called complementarity determining regions (CDRs), and four framework regions (FRs).

As used herein, the term "complementarity determining region (CDR)" refers to the variable region of an antibody that confers binding specificity to an antigen. The above CDRs primarily play a role in binding to epitopes of antigens. The heavy and light chains each comprise three complementarity determining regions. The CDRs of each chain are typically designated CDR1, CDR2, and CDR3, sequentially starting from the N-terminus, and are identified by the chain on which the particular CDR is located. The FRs of each chain are typically designated FR1, FR2, FR3, or FR4, sequentially starting from the N-terminus, and are identified by the chain on which the particular FR is located.

As used herein, the term "Fc region" refers to the Fc domain of an immunoglobulin. The Fc region refers to a protein that includes the heavy chain constant region 2 (CH2) and the heavy chain constant region 3 (CH3) of an immunoglobulin, but excludes the variable regions of the heavy and light chains of an immunoglobulin, the heavy chain constant region 1 (CH1), and the light chain constant region (CL).

The Fc region may be a wild-type Fc domain or a fragment of Fc domain. For example, the fragment of the Fc region may be one in which a lysine (K) is deleted from the C-terminus. Alternatively, the fragment of the Fc region may comprise only CH3. The immunoglobulin may be IgG, IgA, IgE, IgD, or IgM. In addition, it may be IgG1, IgG2, IgG3, or IgG4, which are subclasses of IgG, or IgA1 or IgA2, which are subclasses of IgA.

As used herein, the term "Fc region variant" may be a variant having a different glycosylation pattern than that of a wild-type Fc region, or may have increased glycosylation compared to a wild-type Fc region or decreased glycosylation compared to a wild-type Fc region, or may be a form in which glycosylation is removed (deglycosylated). In addition, aglycosylated Fc regions are also included. The Fc region or variant may have a modified number of sialic acids, fucosylation, or glycosylation levels, either through culture conditions or genetic engineering of the host.

In addition, the glycosylation patterns of the Fc region of an immunoglobulin may be modified using conventional methods, such as chemical methods, enzymatic methods, and genetic engineering using microorganisms. In addition, the Fc domain variant may be a mixed form of the Fc regions of immunoglobulins such as IgG, IgA, IgE, IgD, or IgM. In addition, the Fc region variant may be a form in which some amino acids of the Fc region are substituted.

The "amino acid" introduced by the substitution and/or addition may be any one selected from the group consisting of lysine (K), alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Specifically, the Fc region variant may have substitution of at least one amino acid, for example, substitutions of about 1 to about 10 amino acids, or about 1 to about 5 amino acid substitutions, compared to the native sequence Fc region. In addition. the Fc region variant may

have at least about 80% homology, at least about 90% homology, or at least about 95% homology to the native sequence Fc region. In one embodiment of the present invention, the Fc region or a variant thereof may comprise the amino acid sequence of SEQ ID NO: 54, SEQ ID NO: 63, or SEQ ID NO: 66.

As used herein, the term "antigen" refers to a structure capable of selectively binding to an antibody. A target antigen may be a polypeptide, carbohydrate, nucleic acid, lipid, hapten, or other naturally occurring or synthetic compound. Specifically, an antigen may be a polypeptide or a protein present on the cell surface or within a cell.

As used herein, the term "vector" refers to a material for transporting or expressing a nucleic acid sequence comprising a nucleic acid sequence encoding a multispecific fusion protein (e.g., an antibody) described herein. Specifically, vectors include expression vectors, plasmids, phage vectors, viral vectors, episomes, and artificial chromosomes.

As used herein, the term "polynucleotide," also referred to as "nucleic acid," refers to a polymer of nucleotides of any length. Specifically, the polynucleotide may be DNA or RNA.

As used herein, the term "bispecific antibody" refers to a material that binds to at least one target or antigen. In the present invention, the bispecific antibody may comprise an antigen binding site that specifically binds to Tie2 and a binding site that specifically binds to the VEGF protein.

Hereinafter, the present invention will be described in detail.

<Anti-Tie2 antibody or fragment thereof>

Antibody or fragment thereof that specifically binds to Tie2

The present invention provides an anti-Tie2 antibody or fragment thereof that specifically binds to Tie2.

As used herein, the term "Tie2" refers to a receptor-type tyrosine kinase specifically expressed in vascular endothelial cells. It is also known as TEK tyrosine kinase, angiopoietin-1 receptor, or cluster of differentiation 202B (CD202B), and is a protein encoded by the TEK gene in humans. The Tie2 protein is typically activated by phosphorylation by Ang-1, a Tie2 ligand. The Tie2 protein consists of an extracellular domain (ECD) composed of Ig-like domain 1, Ig-like domain 2, three EGF-like domains, Ig-like domain 3, and three fibronectin type-III domains; a transmembrane domain; and an intracellular tyrosine kinase domain (ITK). The Tie2 protein has been reported to play a crucial role in angiogenesis, structural/functional normalization, and stabilization of blood vessels in various physiological and pathological situations.

In the present invention, the Tie2 protein may be derived from mammals, including primates such as humans and monkeys, and rodents such as rats and mice, without limitation.

Specifically, the Tie2 protein may be the extracellular domain of a Tie protein. It generally refers to a polypeptide comprising the amino acid sequence of a Tie2 protein. The amino acid sequence and polynucleotide sequence for Tie2 may be obtained from known

databases, such as the GenBank of the National Institutes of Health (NCBI). In one embodiment, it may include the amino acid sequence of SEQ ID NO: 91.

In addition, the Tie protein may include a fragment thereof. The term "fragment" refers to a protein in which a portion of the N-terminus and/or C-terminus of the protein is deleted. Therefore, the fragment of Tie protein may be a Tie2 protein in which a portion of the N-terminus and/or C-terminus is deleted. Specifically, it may be a Tie protein in which the N-terminus and/or C-terminus of the extracellular domain is deleted.

In addition, as long as it has the same activity as the Tie2 protein or the gene encoding Tie2 is located at the same location on the chromosome, it may consist of a sequence in which one or more amino acids of the protein are added, deleted, or substituted. Specifically, the Tie2 protein may comprise or consist of an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity, or 100% identity to the amino acid sequence of SEQ ID NO: 91.

In the present invention, the antibody or fragment thereof may comprise (a) a heavy chain variable region (VH) comprising i) a heavy chain complementarity determining region 1 (HCDR1) represented by the amino acid sequence of SEQ ID NO: 84, ii) a heavy chain complementarity determining region 2 (HCDR2) represented by the amino acid sequence of SEQ ID NO: 85, and iii) a heavy chain complementarity determining region 3 (HCDR3) represented by any one amino acid sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 15, and SEQ ID NO: 86; and (b) a light chain variable region (VL) comprising iv) a light chain complementarity determining region 1 (LCDR1) represented by the amino acid sequence of SEQ ID NO: 87, v) a light chain complementarity determining region 2 (LCDR2) represented by the amino acid sequence of SEQ ID NO: 88, and vi) a light chain complementarity determining region 3 (LCDR3) represented by the amino acid sequence of SEQ ID NO: 89. In the amino acid sequence of $X_1X_2X_3FX_4X_5X_6X_7$ of SEQ ID NO: 84, X_1 is G or S, X_2 is F or D, X_3 is T or S, X_4 is N, T or A, X_5 is S or G, X_6 is Y, N, or Q, and X_7 is G, W, or A; in the amino acid sequence of $X_8X_9X_{10}DX_{11}X_{12}X_{13}X_{14}$ of SEQ ID NO: 85, X_8 is T or I, X_9 is S or Y, X_{10} is N, P, or W, X_{11} is G, D, or S, X_{12} is S or G, X_{13} is T, D, or G, and X_{14} is T or I; in the amino acid sequence of ARKVVRGY$X_{15}X_{16}$HDAFDI of SEQ ID NO: 86, X_{15} is S, H, or L, and X_{16} is Y or P; in the amino acid sequence of $QX_{17}X_{18}X_{19}SX_{20}$ of SEQ ID NO: 87, X_{17} is S, G, or D, X_{18} is V or I, X_{19} is S or D, and X_{20} is Y or N; in the amino acid sequence $X_{21}X_{22}S$ of SEQ ID NO: 88, X_{21} is G or A, and X_{22} is A or S; in the amino acid sequence of $QQX_{23}X_{24}X_{25}X_{26}PX_{27}T$ of SEQ ID NO: 89, X_{23} is Y, A, or G, X_{24} is G, N, or Y, X_{25} is T, S, or D, X_{26} is T or F, and X_{27} is Y, L, or W.

Specifically, the antibody or fragment thereof may comprise a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 86, and a light chain variable region comprising LCDR1 comprising

the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

More specifically, the antibody or fragment thereof may comprise a heavy chain variable region comprising HCDR1 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13, HCDR2 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 8, and SEQ ID NO: 14, and HCDR3 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 19, and SEQ ID NO: 20; and a light chain variable region comprising LCDR1 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 10, and SEQ ID NO: 16, LCDR2 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 11, and SEQ ID NO: 17, and LCDR3 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 12, and SEQ ID NO: 18.

In one embodiment, the antibody may comprise a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 3; and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

In one embodiment, it may comprise a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 7, HCDR2 comprising the amino acid sequence of SEQ ID NO: 8, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 9; and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 10, LCDR2 comprising the amino acid sequence of SEQ ID NO: 11, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 12.

In one embodiment, it may comprise a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 13, HCDR2 comprising the amino acid sequence of SEQ ID NO: 14, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 15; and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 16, LCDR2 comprising the amino acid sequence of SEQ ID NO: 17, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 18.

In one embodiment, it may comprise a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 19; and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6. In one embodiment, it may comprise a

heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 20; and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

At this time, the antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22; a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24; a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26; a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22; or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22.

In the present invention, the anti-Tie2 antibody or fragment thereof may bind to the Tie2 protein and activate Tie2. The binding of the anti-Tie2 antibody or fragment thereof to the Tie2 protein may be determined by measuring binding affinity.

As used herein, the term "binding affinity" refers to the strength of the sum of non-covalent interactions between a single binding site of a molecule and its binding partner. The affinity of molecule X for its partner Y may generally be expressed by the dissociation constant (K_D), which is the ratio of the dissociation and association rate constants (K_{off} and K_{on}, respectively).

The binding affinity of the anti-Tie2 antibody refers to the ability of the anti-Tie2 antibody or fragment thereof to bind to the Tie2 protein, and this binding may be measured using methods known to those of ordinary skill in the art. In one embodiment of the present invention, the anti-Tie antibody or fragment thereof may exhibit a dissociation constant of about 1.0E-11 M to about 1.0E-8 M for human-derived Tie2 protein. In one embodiment, it may exhibit a dissociation constant of about 4.75E-10 M to about 3.42E-09 M.

In addition, the anti-Tie2 antibody or fragment thereof according to the present invention may bind to Tie2 proteins derived from mammals, such as monkeys, mice, rats, dogs, and pigs, in addition to humans. In one embodiment of the present invention, the anti-Tie2 antibody or fragment thereof may exhibit a dissociation constant of about 1.0E-10 M to about 5.0E-7 M for mouse-derived Tie2 protein. In one embodiment, it may exhibit a dissociation constant of about 6.66E-9 M to about 1.44E-8 M.

Polynucleotide encoding an antibody or fragment thereof

In another aspect of the present invention, there is provided a polynucleotide encoding an anti-Tie2 antibody or fragment thereof. The anti-Tie 2 antibody and fragment thereof are the same as described above.

Specifically, the polynucleotide encoding the heavy chain variable region of the anti-Tie2 antibody may comprise a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or SEQ ID NO: 28. The polynucleotide encoding the light chain variable region of the anti-Tie2 antibody may comprise a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22, SEQ ID NO: 24, or SEQ ID NO: 26.

In addition, if the polynucleotide encodes the same polypeptide, one or more nucleotides may be mutated by substitution, deletion, insertion, or a combination thereof. When producing a polynucleotide sequence by chemical synthesis, synthetic methods well known in the art may be used, such as those described in Engels and Uhlmann, Angew Chem IntEd Engl., 37:73-127, 1988. Examples of such methods may include triester, phosphite, phosphoramidite, and H-phosphate methods, PCR and other autoprimer methods, and oligonucleotide synthesis on solid supports.

In one embodiment, the polypeptide encoding the heavy chain variable region may comprise a nucleotide sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identity to a polynucleotide encoding the amino acid sequence of SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or SEQ ID NO: 28.

In one embodiment, the polypeptide encoding the light chain variable region may comprise a nucleotide sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identity to a polynucleotide encoding the amino acid sequence of SEQ ID NO: 22, SEQ ID NO: 24, or SEQ ID NO: 26.

The polynucleotide may additionally comprise a signal sequence or a leader sequence. As used herein, the term "signal sequence" refers to a nucleic acid encoding a signal peptide that directs the secretion of a target protein. The signal peptide is cleaved after translation in the host cell. Specifically, the signal sequence of the present invention is a polynucleotide encoding an amino acid sequence that initiates the transports of a protein across the endoplasmic reticulum (ER) membrane.

Signal sequences are well characterized in the art and typically comprise 16 to 30 amino acid residues, although they may comprise more or fewer. A typical signal peptide consists of three regions: basically, a N-terminal region, a central hydrophobic region, and a more polar C-terminal region. The central hydrophobic region comprises 4 to 12 hydrophobic residues that anchor the signal sequence through the membrane lipid bilayer during transports of the immature polypeptide.

After initiation, the signal sequence is cleaved within the lumen of the ER by cellular enzymes commonly known as signal peptidases. At this time, the signal sequence may be a secretion signal sequence of tPa (tissue plasminogen activation), HSV gDs (signal sequence of herpes simplex virus glycoprotein D), or growth hormone. Preferably, a secretion signal sequence used in higher eukaryotic cells, including mammals, may be used. In addition, it may be substituted with a codon with a high expression frequency in the host cell. In one embodiment, the signal sequence may comprise the amino acid sequence of SEQ ID NO: 51.

Vector loaded with polynucleotide

In another aspect of the present invention, there is provided a vector loaded with a polynucleotide encoding the anti-Tie2 antibody or fragment thereof. Specifically, the vector may comprise a polynucleotide encoding the heavy chain of the anti-Tie2 antibody or fragment thereof. At this time, the polynucleotide may comprise a nucleotide sequence encoding a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or SEQ ID NO: 28. The vector may comprise a polynucleotide encoding the light chain of the anti-Tie2 antibody. At this time, the polynucleotide may comprise a nucleotide sequence encoding a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, SEQ ID NO: 24, or SEQ ID NO: 26. The polynucleotide encoding the heavy chain region and the polynucleotide comprising the light chain region may be loaded onto separate vectors or onto a single vector.

The vector may be introduced into a host cell and recombined and inserted into the host cell genome. Alternatively, the vector is understood to be a nucleic acid vehicle comprising a polynucleotide sequence capable of autonomously replicating as an episome. The vector includes linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors, and analogs thereof. Examples of viral vectors include, but are not limited to, retroviruses, adenoviruses, and adeno-associated viruses.

Specifically, the vector may be a plasmid DNA, phage DNA, etc., and may be a commercially developed plasmid (e.g., pUC18, pBAD, pIDTSAMRT-AMP, etc.), an *E. coli*-derived plasmid (e.g., pYG601BR322, pBR325, pUC118, pUC119, etc.), a *Bacillus subtilis*-derived plasmid (e.g., pUB110, pTP5, etc.), a yeast-derived plasmid (e.g., Yep13, Yep24, Ycp50, etc.), a phage DNA (e.g., Charon4A, Charon21A, EMBL3, EMBL4, λgt10, λgt11, λZAP, etc.), an animal virus vector (e.g., retrovirus, adenovirus, vaccinia virus, etc.), an insect virus vector (e.g., baculovirus). Since the vector exhibits different protein expression levels and

expression modifications depending on the host cell, it is desirable to select and use the host cell most suitable for the purpose.

As used herein, the term "gene expression" or "expression" of a target protein are understood to refer to the transcription of a DNA sequence, translation of an mRNA transcript, and secretion of an antibody product or fragment thereof. A useful expression vector may be RcCMV (Invitrogen, Carlsbad) or a variant thereof. The expression vector may comprise a human CMV (cytomegalovirus) promoter to promote continuous transcription of the target gene in mammalian cells, and a polyadenylation signal sequence to increase the steady-state level of RNA after transcription.

Transformed cell

In another aspect of the present invention, there is provided a transformed cell into which an expression vector comprising a polynucleotide encoding the anti-Tie2 antibody or fragment thereof has been introduced.

As used herein, the term "transformed cell" refers to a prokaryotic cell and a eukaryotic cell into which a recombinant expression vector may be introduced. The transformed cell may be constructed by introducing the vector into a host cell and transforming it. In addition, the polynucleotide included in the vector may be expressed to produce the antibody according to the present invention.

The transformation may be performed by various methods. As long as the antibody according to the present invention may be produced, it is not particularly limited thereto. Specifically, for the transformation method, $CaCl_2$ precipitation, Hanahan method whose efficiency has been increased by using a reducing agent such as dimethyl sulfoxide (DMSO) in $CaCl_2$ precipitation, electroporation, calcium phosphate precipitation, protoplast fusion, agitation using silicon carbide fiber, agrobacteria-mediated transformation, transformation using PEG, dextran sulfate-, lipofectamine-, or dry/inhibition-mediated transformation, or the like may be used. In addition, a target substance may be delivered into a cell using a virus particle by means of infection. In addition, a vector may be introduced into a host cell by gene bombardment, and the like.

In addition, the host cell used for constructing the transformed cell is not particularly limited thereto, as long as it may produce the antibody or fragment thereof according to the present invention. Specifically, the host cell may include, but is not limited to, a prokaryotic cell, a eukaryotic cell, and a cell of mammalian, plant, insect, fungal, or bacterial origin. As an example of the prokaryotic cell, *E. coli* may be used. In addition, as an example of the eukaryotic cell, yeast may be used. In addition, for the mammalian cells, CHO cells, F2N cells, COS cells, BHK cells, Bowes melanoma cells, HeLa cells, 911 cells, AT1080 cells, A549 cells, SP2/0 cells, human lymphoblastoid, NSO cells, HT-1080 cells, PERC.6 cells, HEK293 cells, HEK293T cells, or the like may be used, but is not limited thereto. Any cells which are known to those of ordinary skill in the art to be usable as mammalian host cells may be used.

In addition, in order to optimize the properties of the multispecific fusion protein according to the present invention as a therapeutic agent or for other purposes, sugar chain pattern of the antibody (e.g., sialic acids, fucosylations, glycosylations) may be adjusted by manipulating, through methods known to those of ordinary skill in the art, glycosylation-related genes possessed by host cells.

Method for producing an antibody or fragment thereof

In another aspect of the present invention, there is provided a method for producing the anti-Tie2 antibody or fragment thereof.

The method for producing the antibody may comprise: i) culturing the transformed cell; and ii) obtaining the antibody or fragment thereof from the culture solution. At this time, the anti-Tie2 antibody, fragment thereof, and transformed cell are the same as described above.

As used herein, the term "culture" refers to a method for growing a microorganism (e.g., the transformed cell) under appropriately artificially controlled environmental conditions.

The method for culturing the transformed cell may be carried out using a method well known in the art. Specifically, the culturing is not particularly limited thereto as long as the protein of the present invention may be expressed and produced. Specifically, the culturing may be carried out in a batch process, or carried out continuously in a fed batch or repeated fed batch process.

In addition, the step of obtaining the protein from the culture solution may be carried out by a method known in the art. Specifically, the obtaining method is not particularly limited thereto as long as the produced protein of the present invention may be obtained. Preferably, the obtaining method may be a method such as centrifugation, filtration, extraction, spraying, drying, evaporation, precipitation, crystallization, electrophoresis, differential dissolution (e.g., ammonium sulfate precipitation), chromatography (e.g., ion exchange, affinity, hydrophobicity, and size exclusion).

<Bispecific antibody>

In another aspect of the present invention, there is provided a bispecific antibody comprising the anti-Tie 2 antibody or fragment thereof; and a VEGF-specific binding site. The anti-Tie 2 antibody and fragment thereof are the same as described above.

At this time, at least one VEGF-specific binding site may be conjugated to the anti-Tie 2 antibody or fragment thereof. Preferably, two VEGF-specific binding sites may be conjugated to the anti-Tie 2 antibody or fragment thereof. In addition, the VEGF-specific binding site may be conjugated to the C-terminus of the heavy chain constant region of the anti-Tie 2 antibody, but is not limited thereto.

VEGF binding site

As used herein, the term "VEGF" refers to a vascular endothelial growth factor, which is produced by cells that stimulate angiogenesis. VEGF is a key signaling protein involved in angiogenesis by stimulating the proliferation and division of vascular endothelial cells and

increasing vascular permeability. VEGF binds to the VEGF receptor (VEGFR) to induce angiogenesis and lymphangiogenesis. VEGF may include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and/or PlGF (placental growth factor).

In the present invention, the VEGF protein may be included without limitation as long as it is derived from mammals, including primates such as humans and monkeys, and rodents such as rats and mice. In addition, the VEGF protein may be included without limitation as long as it binds to the VEGF receptor (VEGFR) and exhibits activity equivalent to or similar to that of VEGF. Here, "activity" may, for example, refer to specific binding to the VEGF receptor, and this specific binding may be measured using methods known to those of ordinary skill in the art.

In addition, the VEGF protein may include native VEGF and fragments thereof. The native VEGF protein refers to a natural VEGF protein. The amino acid sequence and polynucleotide sequence for the native VEGF protein may be obtained from known databases, such as the GenBank of the National Institutes of Health (NCBI). In one embodiment of the present invention, the VEGF protein may comprise the amino acid sequence of SEQ ID NO: 79, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, or SEQ ID NO: 95.

In addition, as long as it has the same activity as the VEGF protein or the gene encoding the VEGF protein is located at the same location on the chromosome, it may consist of a sequence in which one or more amino acids of the protein are added, deleted, or substituted. Specifically, VEGF may comprise or consist of an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identity to the amino acid sequence of SEQ ID NO: 79, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, or SEQ ID NO: 95.

In the present invention, the VEGF-specific binding site may be selected from the group consisting of a receptor, a ligand, an antibody, and a fragment thereof that bind to VEGF. However, the VEGF-specific binding site is not limited thereto, as long as it specifically binds to VEGF and inhibits VEGF activity. Specifically, the VEGF-specific binding site may be a receptor. At this time, the receptor may be a VEGF receptor (VEGFR).

The term "VEGF receptor (VEGFR)" refers to a transmembrane protein that binds to vascular endothelial growth factor (VEGF) and regulates angiogenesis. VEGFR is a tyrosine kinase receptor and exists in three types: VEGFR1, VEGFR2, and VEGFR3. VEGF binds to the extracellular domain of each VEGFR and activates the receptor. At this time, VEGF-A binds to both VEGFR1 and VEGFR2, both of which are expressed on vascular endothelial cells. In addition, VEGF-B and PlGF bind only to VEGFR1. In contrast, VEGF-C and VEGF-D are known to bind to VEGFR2 and VEGFR3. At this time, VEGFR3 is primarily expressed on lymphatic endothelial cells. The VEGFR comprises seven extracellular domains, a transmembrane domain, and an intracellular tyrosine kinase domain.

In the present invention, the VEGFR may be the extracellular domain of VEGFR.

As used herein, the term "extracellular domain of a VEGF receptor" refers to the domain of a VEGF receptor that binds to VEGF. Specifically, it refers to the portion of the VEGF receptor excluding the transmembrane domain and the intracellular tyrosine kinase domain. Specifically, it may be the extracellular domain of VEGFR1 (SEQ ID NO: 96) or VEGFR2 (SEQ ID NO: 80). In one embodiment, the VEGFR1 extracellular domain and the VEGFR2 extracellular domain may comprise the amino acid sequences of SEQ ID NO: 97 and SEQ ID NO: 98, respectively.

In the present invention, the receptor may comprise a fragment thereof. The "fragment" is the same as described above. Specifically, the receptor fragment may be one in which a portion of the N-terminus and/or C-terminus of the VEGFR is deleted. More specifically, it may be one in which a portion of the N-terminus and/or C-terminus of the extracellular domain of VEGFR is deleted. The "fragment of the extracellular domain of VEGFR" may comprise domains D1, D2, D3, or a combination thereof of VEGFR, which are VEGF-binding regions. Preferably, the fragment of VEGFR may comprise D2 and/or D3.

In addition, the VEGFR may be in the form of a fusion of extracellular domain fragments of VEGFR subtypes. In one embodiment, it may be in the form of a fusion of the extracellular domain fragment of VEGFR1 and the extracellular domain fragment of VEGFR2. In one embodiment, it may be in the form of a fusion of the extracellular domain fragment of VEGFR1 and the extracellular domain fragment of VEGFR3. In one embodiment, it may be in the form of a fusion of the extracellular domain fragment of VEGFR2 and the extracellular domain fragment of VEGFR3. At this time, the extracellular domains may be directly linked or linked via a linker. The linker is a peptide linker, and the peptide linker is described below. Preferably, the VEGFR may be in the form of a fusion of the D2 domain of VEGFR1 and the D3 domain of VEGFR2. In one embodiment of the present invention, the receptor may comprise the amino acid sequence of SEQ ID NO: 83.

Specifically, the VEGF-specific binding site may be an antibody or a fragment thereof. The antibody and fragment thereof are the same as described above.

More specifically, an antibody or fragment thereof that specifically binds to VEGF may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71, a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73, a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76, or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78. At this time, the antibody or fragment thereof that specifically binds to VEGF may be linked via a peptide linker. Here, the peptide linker is described below. In one embodiment, the peptide linker may

comprise the amino acid sequence of SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 70, or SEQ ID NO: 75.

In the present invention, the antibody or fragment thereof that specifically binds to VEGF may specifically bind to VEGF and inhibit VEGF activity.

Bispecific antibody structure

The bispecific antibody of the present invention may comprise an anti-Tie2 antibody and a VEGF-specific binding site. At this time, the VEGF-specific binding site may be bound to the Fc region of the anti-Tie2 antibody or a variant thereof. In addition, the binding may be linked via a linker.

More specifically, the bispecific antibody may be a fusion protein dimer comprising the following structural formulae (I) and (II):

N'-X'-[L1]p-Fc region or variant thereof-[L2]q-Y-C' (I); and

N'-X"-C' (II)

wherein, in the structural formulae (I) and (II),

N' is the N-terminus,

C' is the C-terminus,

- represents a bond,

X' is the heavy chain variable region (VH) and heavy chain constant region (CH1) of the anti-Tie2 antibody,

X" is the light chain variable region (VL) and light chain constant region (CL) of the anti-Tie2 antibody,

X' and X" combine to form a binding site (X) that specifically binds to Tie2,

Y is a VEGF-specific binding site,

L1 and L2 are peptide linkers, and

p and q are each independently 0 or 1.

The anti-Tie2 antibody, VEGF-specific binding site, Fc region or variant thereof, heavy chain variable region (VH), heavy chain constant region (CH), light chain variable region (VL), and light chain constant region (CL) are the same as described above.

As used herein, the term "peptide linker" refers to a peptide consisting of one or more amino acids. Typically, the peptide linker may consist of 1 to 100 consecutive amino acids, 5 to 80 consecutive amino acids, 7 to 70 consecutive amino acids, 10 to 60 consecutive amino acids, or 12 to 50 amino acids. The peptide linkers are known in the art or described herein. In one embodiment, the peptide linker L1 or L2 may consist of 30 amino acids. Specifically, the linker L1 may comprise at least one cysteine. Specifically, it may comprise one, two, or three cysteines. In addition, the peptide linker L1 may be derived from the hinge of an immunoglobulin. In one embodiment, the linker L1 may comprise the amino acid sequence of SEQ ID NO: 53 or SEQ ID NO: 65. The linker L2 may comprise (G4S)n and may further comprise (G)n or 1 to 10 amino acids. In addition, the linker L2 may be (G)n or (GS3)n. At

this time, n may be each independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In one embodiment, the linker L2 may comprise the amino acid sequence of SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 70, or SEQ ID NO: 75.

At this time, the VEGF-specific binding site is the same as described above.

Specific examples of the bispecific antibody are as follows.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise the amino acid sequence of SEQ ID NO: 83.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ

ID NO: 24, and the VEGF-specific binding site may comprise the amino acid sequence of SEQ ID NO: 83.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26, and the VEGF-specific binding site may comprise the amino acid sequence of SEQ ID NO: 83.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise the amino acid sequence of SEQ ID NO: 83.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ

ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise the amino acid sequence of SEQ ID NO: 83.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76.

In one embodiment, the anti-Tie2 antibody or fragment thereof of the bispecific antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22, and the VEGF-specific binding site may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

Polynucleotide encoding a bispecific antibody

In another aspect of the present invention, there is provided a polynucleotide encoding the bispecific antibody. The bispecific antibody is the same as described above.

Specifically, the polynucleotide may comprise a nucleotide sequence encoding a heavy chain comprising the amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46. In addition, the polynucleotide may comprise a nucleotide sequence encoding a light chain comprising the amino acid sequence of SEQ ID NO: 47.

In addition, if the polynucleotide encodes the same polypeptide, one or more nucleotides may be mutated by substitution, deletion, insertion, or a combination thereof. When producing a polynucleotide sequence by chemical synthesis, synthetic methods well known in the art may be used, such as those described in Engels and Uhlmann, Angew Chem IntEd Engl., 37:73-127, 1988. Examples of such methods may include triester, phosphite, phosphoramidite, and H-phosphate methods, PCR and other autoprimer methods, and oligonucleotide synthesis on solid supports.

In one embodiment, the polynucleotide encoding the heavy chain may comprise a nucleotide sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identity to a polynucleotide encoding the amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46.

In one embodiment, the polynucleotide encoding the light chain may comprise a nucleotide sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identity to a polynucleotide encoding the amino acid sequence of SEQ ID NO: 47.

The polynucleotide may additionally comprise a signal sequence or a leader sequence. The signal sequence is the same as described above.

Vector loaded with a polynucleotide

In another aspect of the present invention, there is provided a vector loaded with a polynucleotide encoding the bispecific antibody. At this time, the polynucleotide is the same as described above. Specifically, the vector may comprise a polynucleotide encoding a heavy chain comprising the amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46. The vector may comprise a polynucleotide encoding a light chain comprising the amino acid sequence of SEQ ID NO: 47.

At this time, each polynucleotide may be loaded into a separate vector or may be loaded into the same vector.

Transformed cell

In another aspect of the present invention, there is provided a cell transformed with a vector loaded with a polynucleotide encoding the bispecific antibody. The bispecific antibody is the same as described above.

Method for producing a bispecific antibody

In another aspect of the present invention, there is provided a method for producing the bispecific antibody.

The method for producing the bispecific antibody may comprise: i) culturing the transformed cell; and ii) obtaining the bispecific antibody from the culture solution.

<Pharmaceutical composition>

In another aspect of the present invention, there is provided a pharmaceutical composition for preventing or treating a vascular abnormality-related disease, comprising the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof and a VEGF-specific binding site as an active ingredient. The anti-Tie2 antibody or fragment thereof and bispecific antibody are the same as described above.

As used herein, the term "vascular abnormality-related disease" refers to a disease related to an angiogenesis abnormality or a disease related to structural and/or functional alterations of blood vessels.

The vascular abnormality-related disease may be a disease related to an angiogenesis abnormality an angiogenesis abnormality or a disease related to structural and/or functional alterations of blood vessels. The "angiogenesis" refers to the formation or growth of new blood vessels from pre-existing blood vessels, and the "angiogenesis-related disease" refers to a disease related to the occurrence or progression of angiogenesis. Specifically, the "angiogenesis abnormality" may be caused by an excessive increase, decrease, or deficiency. The "disease related to structural and/or functional alterations of blood vessels" may be caused by any one abnormality selected from the group consisting of structural abnormalization of blood vessels, vascular dysfunction, and vascular injury.

The vascular abnormality-related disease may be specifically caused by vascular leakage, inflammation, or local ischemia, but is not limited thereto. For example, it may be any one selected from the group consisting of acute kidney injury, chronic renal failure, macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vessel occlusion, proliferative retinopathy, retinopathy of prematurity, corneal graft rejection, glaucoma, critical limb ischemia, diabetic erectile dysfunction, sepsis, acute respiratory distress syndrome, vasculitis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, thrombosis, occlusion, cancer, systemic erythremia, psoriasis, hemophilic arthritis, associated sclerosis, capillary formation of atherosclerotic plaques, keloid, wound granulation, vascular adhesion,

rheumatoid arthritis, osteoarthritis, autoimmune disease, Crohn's disease, restenosis, atherosclerosis, intestine adhesion, cat scratch disease, ulcer, liver cirrhosis, nephritis, diabetic nephropathy, diabete mellitus, inflammatory disease, and neurodegenerative disease, but is not limited thereto.

The term "prevention" refers to any action intended to suppress or delay the onset of a disease. In addition, the term "treatment" refers to any action intended to improve or alleviate the symptoms of an existing disease. Desirable effects of such treatment include preventing the occurrence or recurrence of the disease, alleviating symptoms, reducing any direct or indirect pathological consequences of the disease, preventing metastasis, slowing the progression of the disease, improving or alleviating the disease condition, and achieving remission or improving prognosis.

The preferred dosage of the pharmaceutical composition may vary depending on the patient's condition and body weight, the severity of the disease, the drug form, the route of administration, and the duration of the treatment, but may be appropriately selected by those of ordinary skill in the art. In the pharmaceutical composition for treating or preventing a disease of the present invention, the active ingredient may be included in any amount (effective amount) depending on the intended use, formulation, and formulation purpose, as long as it exhibits therapeutic activity against the disease. Here, the term "effective amount" refers to the amount of the active ingredient capable of improving the disease condition or inducing a therapeutic effect. Such effective amount may be determined experimentally within the capabilities of those of ordinary skill in the art.

Pharmacokinetic parameters such as bioavailability and underlying parameters such as clearance rate may also affect efficacy. Therefore, "enhanced efficacy" (for example, improved efficacy) may be due to enhanced pharmacokinetic parameters and enhanced efficacy, and may be measured by comparing parameters such as clearance rate and treatment or improvement of a disease in laboratory animals or human subjects.

In addition to the active ingredient, the pharmaceutical composition may further comprise a pharmaceutically acceptable carrier or additive. The term "pharmaceutically acceptable" means that it does not inhibit the activity of the active ingredient and does not exhibit toxicity beyond what is acceptable to the applied (prescribed) subject. The carrier refers to a compound that facilitates the addition of the antibody or antigen binding fragment thereof according to the present invention into cells or tissues. The additive refers to any ingredient necessary for preparing the pharmaceutical composition into the required dosage form, such as an excipient, a disintegrant, a binder, a flavoring agent, a preservative, a lubricant, a stabilizer, a viscosifier, etc.

When the pharmaceutical composition is prepared as a parenteral formulation, it may be formulated in the form of an injection, a transdermal preparation, a nasal inhalant, and a suppository together with suitable carriers according to methods known in the art. When it is

formulated as an injection, as a suitable carrier, sterile water, ethanol, polyol such as glycerol or propylene glycol, or a mixture thereof may be used, and Ringer's solution, PBS (Phosphate Buffered Saline) containing triethanolamine, or sterile water for injection, an isotonic solution such as 5% dextrose, and the like may be preferably used. Methods for formulating pharmaceutical compositions are known in the art, and specific references may be made to literature [Remington's Pharmaceutical Sciences (19th ed., 1995)], etc. The above literature is considered a part of the present specification.

The active ingredient of the present invention or a pharmaceutical composition comprising the active ingredient may be administered to a subject in a pharmaceutically effective amount. The term "pharmaceutically effective amount" refers to an amount sufficient to produce a preventive or therapeutic effect at a reasonable benefit/risk ratio applicable to all medical treatments. In addition, the "pharmaceutically effective amount" varies depending on various factors, including the severity of the disease to be treated, the patient's age and sex, the type of disease, the activity of the drug, the sensitivity to the drug, the time of administration, the route of administration, the rate of secretion, the duration of treatment, and other parameters known in the art.

The subject may be a mammal, preferably a human. In addition, the term "subject" refers to a subject suffering from, or likely to suffer from, a vascular abnormality-related disease.

As used herein, the term "administration" refers to introducing a given substance into a subject through an appropriate method. The composition may be administered via any conventional route as long as it may reach the target tissue. Specifically, parenteral administration may be used. Examples of parenteral administration include, but are not limited to, intravenous administration, subcutaneous administration, intraperitoneal administration, intrauterine administration, and intracerebrovascular administration. In addition, it may be administered directly locally to the diseased area.

The preferred dosage of the pharmaceutical composition may be in the range of 0.01 μg/kg to 10 g/kg per day, or in the range of 0.01 mg/kg to 1 g/kg per day, depending on the patient's condition, body weight, sex, and age, the severity of the patient, and the route of administration. The administration may be performed once a day or divided into several times per day. This dosage should not be construed as limiting the scope of the present invention in any way.

The pharmaceutical composition may be administered alone or in combination with other treatments. In such cases, it may be administered sequentially or simultaneously with conventional treatments. In addition, the composition may be administered as a single dose or in multiple divided doses. Considering these factors fully, it is important to administer the minimum dose sufficient to achieve maximum effect without side effects, and this dosage may be readily determined by a specialist in the field. The dosage of the pharmaceutical composition

is not particularly limited, but may vary depending on various factors, including the patient's health and body weight, the severity of the disease, the type of drug, the route of administration, and the duration of administration.

In addition, the active ingredient of the present invention or a pharmaceutical composition comprising the active ingredient may be administered in combination with other therapeutic agents for vascular abnormality-related diseases, anticancer agents, and the like. At this time, the active ingredient of the present invention or the pharmaceutical composition may be administered simultaneously or sequentially with other therapeutic agents, such as therapeutic agents for vascular abnormality-related disease, anti-inflammatory drugs, anticancer agents, and the like. For example, other therapeutic agents, such as therapeutic agents for vascular abnormality-related disease, anti-inflammatory drugs, anticancer agents, and the like, may be administered to the subject first, followed by the additional administration of the antibody or antigen binding fragment thereof according to the present invention, or the pharmaceutical composition to the subject. Alternatively, the antibody or antigen binding fragment thereof according to the present invention, or the pharmaceutical composition, may be administered first, followed by the additional administration of other therapeutic agents, such as therapeutic agents for vascular abnormality-related disease, anti-inflammatory drugs, anticancer agents, and the like. In addition, in some cases, the antibody or antigen binding fragment thereof according to the present invention, or the pharmaceutical composition may be administered simultaneously to a subject with other therapeutic agents, such as therapeutic agents for vascular abnormality-related disease, anti-inflammatory drugs, anticancer agents, and the like.

In another aspect of the present invention, there is provided a use of the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof and a VEGF-specific binding site for the prevention or treatment of a vascular abnormality-related disease. In another aspect of the present invention, there is provided a method for preventing or treating a vascular abnormality-related disease, comprising administering to a subject the anti-Tie2 antibody or fragment thereof; or a bispecific antibody comprising the anti-Tie2 antibody or fragment thereof and a VEGF-specific binding site.

The anti-Tie2 antibody, fragment thereof, VEGF, bispecific antibody, subject, prevention, treatment, and vascular abnormality-related disease are the same as described above.

Mode for Carrying out the Invention

Hereinafter, the present invention will be described in more detail by way of the following examples. However, the following examples are intended to illustrate the present invention, and the scope of the present invention is not limited to these examples.

I. Antibody that specifically binds to human Tie2 protein

Example 1. Production of antibody that specifically binds to human Tie2 protein

Example 1.1. Selection of antibody that specifically binds to human Tie2 protein

In order to identify effective antibodies included in the present invention, phage display panning was performed using a proprietary human antibody library (diversity: 2.5 x 10^{11}) and a recombinant human Tie2 extracellular region (hTie2-ECD-mFc) protein (SEQ ID NO: 99) conjugated to the Fc region (mFc) of mouse IgG.

Specifically, hTie2-ECD-mFc, used as an antigen, was immobilized in an immunotube at a concentration of 10 μg/mL. The phage molecules expressed from the antibody library were allowed to bind for 1 hour, and then washed seven times with PBST (PBS/0.05% Tween 20) buffer. The antigen-bound phage molecules were recovered by treating with a 100 mM triethylamine solution for 10 minutes. TG1 *E. coli* hosts were infected and plated on solid medium with 2x YT/ampicillin/2% glucose to obtain TG1 hosts infected with antigen-binding phages.

Thereafter, phage molecules were expressed again from the obtained TG1 hosts using the M13KO7 helper phage and used in subsequent panning. A total of three panning rounds were performed using the above-described method. In order to recover antibody-expressing phages with high binding affinity, washing processes were performed 15 times in the second round and 20 times in the third round. Thereafter, single clones recovered in the third round were seeded into 150 μL/well of 2x YT/ampicillin/2% glucose liquid medium dispensed in 96-well plates and incubated with agitation at 37 °C for 16 hours. A master plate was prepared by adding 50 μL of 2x YT/50% glycerol solution to the culture solution. 10 μL was then taken from the master plate and seeded into 150 μL/well of liquid medium with 2x YT/ampicillin/2% glucose. The plate was then incubated with agitation at 37 °C for approximately 3 hours. After incubation, the plate was centrifuged at 4,000 rpm for 15 minutes to remove the supernatant. The *E. coli* pellet was resuspended in 150 μL of liquid medium with 2x YT/ampicillin/1 mM IPTG and incubated at 30 °C for 16 hours.

The culture solution cultured using the above method was centrifuged, and then the *E. coli* pellet was treated with 150 μL of periplasmic extraction solution (30 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0) and left on ice for 30 minutes. Thereafter, the periplasmic proteins were recovered from the supernatant by centrifugation at 4,000 rpm for 10 minutes. The periplasmic proteins were applied to 96-well ELISA plates coated with 100 ng/well of hTie2-ECD-mFc or mouse Tie2 extracellular site (mTie2-ECD-mFc) to screen for periplasmic scFv antibodies.

Thereafter, scFv antibodies that cross-bind to hTie2-ECD (SEQ ID NO: 91) and mTie2-ECD (SEQ ID NO: 100) were screened. Sequence analysis of the antibodies that exhibited binding affinity identified five distinct antibodies (designated 'MT-100', 'MT-110',

'MT-120', 'MT-101', and 'MT-111', respectively) possessing complementarity determining regions (CDRs) within the heavy chain and heavy chain variable regions (Table 1).

The heavy chain variable region (VH) and light chain variable region (VL) sequences of each antibody are shown in Tables 2 to 6. The antibodies were cloned into pcDNA3.4-based heavy and light chain expression vectors (Invitrogen) containing the IgG1 heavy chain constant region (CH1-hinge-CH2-CH3) and light chain constant region (CL), respectively, for transient expression in IgG form. These vectors were used for transient expression and purification. The antibodies described below can be used interchangeably as MT-100 or MT-100 antibodies, MT-110 or MT-110 antibodies, MT-120 or MT-120 antibodies, MT-101 or MT-101 antibodies, and MT-111 or MT-111 antibodies.

[Table 1]

Antibody Name	CDR	Amino acid sequence	SEQ ID NO
MT-100	MT-100-HCDR1	GFTFNSYG	1
	MT-100-HCDR2	TSNDGSTT	2
	MT-100-HCDR3	ARKVVRGYSYHDAFDI	3
	MT-100-LCDR1	QSVSSY	4
	MT-100-LCDR2	GAS	5
	MT-100-LCDR3	QQYGTTPYT	6
MT-110	MT-110-HCDR1	SDSFTSNW	7
	MT-110-HCDR2	IYPDDSDT	8
	MT-110-HCDR3	ARQIDTGYFDY	9
	MT-110-LCDR1	QGISSN	10
	MT-110-LCDR2	AAS	11
	MT-110-LCDR3	QQANSFPLT	12
MT-120	MT-120-HCDR1	GFTFAGQA	13
	MT-120-HCDR2	ISWDSGGI	14
	MT-120-HCDR3	ARGSTAFPRYFEY	15
	MT-120-LCDR1	QDIDSY	16
	MT-120-LCDR2	ASS	17
	MT-120-LCDR3	QQGYDFPWT	18
MT-101	MT-101-HCDR1	GFTFNSYG	1
	MT-101-HCDR2	TSNDGSTT	2
	MT-101-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-101-LCDR1	QSVSSY	4
	MT-101-LCDR2	GAS	5

	MT-101-LCDR3	QQYGTTPYT	6
MT-111	MT-111-HCDR1	GFTFNSYG	1
	MT-111-HCDR2	TSNDGSTT	2
	MT-111-HCDR3	ARKVVRGYLPHDAFDI	20
	MT-111-LCDR1	QSVSSY	4
	MT-111-LCDR2	GAS	5
	MT-111-LCDR3	QQYGTTPYT	6

[Table 2]

MT-100		Amino acid sequence	SEQ ID NO
MT-100 HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQW VRQAPGKGLEWVAVTSNDGSTTYYADSVKGRFTISRD NSKNTLYLQMNSLRSEDTAVYYCARKVVRGYSYHDA FDIWGQGTMVTVSS	21
	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG	54
MT-100 LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWYRQ KPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFTLTISRL EPEDFAVFYCQQYGTTPYTFGQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 3]

MT-110		Amino acid sequence	SEQ ID NO
MT-110 HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	QMQLVQSGAEVKKPGESLKISCKGSSDSFTSNWIAWV RQKPGKGLEWMGIIYPDDSDTRYSPSFQGQVTMSADK STSTAYLQWSSLKASDTAMYYCARQIDTGYFDYWGQ GTLITVSS	23
	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPG	54
MT-110 LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQYPSSLAASTGDRVTITCRASQGISSNLAWYQQ KPGNSPKLLIYAASSLQSGVPSKFSGSGSGTDFTLTISSL QPEDFATYYCQQANSFPLTFGPGTKVEIK	24
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 4]

MT-120		Amino acid sequence	SEQ ID NO
MT-120 HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	QLQLVQSGGDLVQPGRSLRLSCAASGFTFAGQAMHWV RQAPGKGLEWVSSISWDSGGIGYADSVKGRFTISRDNA KNSLYLQMNSLRAEDTAVYYCARGSTAFPRYFEYWGQ GTLVTVSS	25

	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	54
MT-120 LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQSPSSVSASIGDKVTITCRSSQDIDSYLAWYQQR PGQTPNLLIYASSTLQDGVPSRFSGSGSGTHFTLTITNLQ PEDSATYHCQQGYDFPWTFGQGTKVEIK	26
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 5]

MT-101		Amino acid sequence	SEQ ID NO
MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWV RQAPGKGLEWVAVTSNDGSTTYYADSVKGRFTISRDNS KNTLYLQMNSLRSEDTAVYYCARKVVRGYHYHDAFDI WGQGTMVTVSS	27
	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	54

MT-101 LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWYRQK PGQAPRLLIYGASIRATGIPDRFSGSGSGTDFTLTISRLEP EDFAVFYCQQYGTTPYTFGQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 6]

MT-111		Amino acid sequence	SEQ ID NO
MT-111 HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWV RQAPGKGLEWVAVTSNDGSTTYYADSVKGRFTISRDNS KNTLYLQMNSLRSEDTAVYYCARKVVRGYLPHDAFDI WGQGTMVTVSS	28
	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	54
MT-111 LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWYRQK PGQAPRLLIYGASIRATGIPDRFSGSGSGTDFTLTISRLEP EDFAVFYCQQYGTTPYTFGQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

Example 1.2. Design of IgG1-LALA and IgG4 mutant antibodies based on MT-101

Since IgG1 antibodies can bind to the Fc-gamma receptor (FcγR) family and induce nonspecific immune cell activation side effects, two mutant antibodies with reduced Fc reactivity were produced and evaluated from an IgG1 Tie2-binding antibody (MT-101). Specifically, a mutant antibody was produced in which the 234th amino acid and the 235th amino acid of the CH2 region of IgG1, leucine, were substituted with alanine. Specifically, primers capable of inducing DNA sequence mutations such that leucine at positions 234 and 235 in the Fc region of MT-101 (IgG1) is substituted with alanine were produced, and point mutations (site-directed mutagenesis) were induced in the polynucleotide encoding the heavy chain of MT-101. As a result, an expression vector loaded with a polynucleotide encoding the MT-101 (IgG1-LALA) heavy chain (Table 9, SEQ ID NO: 29) was produced. Furthermore, in order to produce an isotype MT-101 (IgG4) mutant antibody with low binding affinity to FcγR in a natural state, the heavy chain variable region of MT-101 was cloned into a pcDNA3.4 vector into which a human IgG4 constant region was introduced, to produce an expression vector for the MT-101 (IgG4) heavy chain (Table 9, SEQ ID NO: 30). The two mutant antibodies described above were transiently expressed and purified using their respective heavy chain expression vectors and the MT-101 (IgG1) light chain expression vector.

[Table 7]

MT-101 (IgG1-LALA)		Amino acid sequence	SEQ ID NO
MT-101 (IgG1-LALA) HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWV RQAPGKGLEWVAVTSNDGSTTYYADSVKGRFTISRDNS KNTLYLQMNSLRSEDTAVYYCARKVVRGYHYHDAFDI WGQGTMVTVSS	27
	CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKV	52
	first linker	EPKSCDKTHTCPPCP	53
	Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	63
	signal peptide	MGWSCIILFLVATATGVHS	51

MT-101 (IgG1-LALA) LC	VL	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWYRQK PGQAPRLLIYGASIRATGIPDRFSGSGSGTDFTLTISRLEP EDFAVFYCQQYGTTPYTFGQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 8]

MT-101 (IgG4)		Amino acid sequence	SEQ ID NO
MT-101 (IgG4) HC	signal peptide	MGWSCIILFLVATATGVHS	51
	VH	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWV RQAPGKGLEWVAVTSNDGSTTYYADSVKGRFTISRDNS KNTLYLQMNSLRSEDTAVYYCARKVVRGYHYHDAFDI WGQGTMVTVSS	27
	CH1	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTK TYTCNVDHKPSNTKVDKRV	90
	first linker	ESKYGPPCPPCP	65
	Fc(CH2+CH3)	APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQE DPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPR EPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLSLGK	66
MT-101 (IgG4) LC	signal peptide	MGWSCIILFLVATATGVHS	51
	VL	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWYRQK PGQAPRLLIYGASIRATGIPDRFSGSGSGTDFTLTISRLEP EDFAVFYCQQYGTTPYTFGQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	82

[Table 9]

Sequence information	Sequence	SEQ ID NO
MT-101 (IgG1-LALA)-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWVRQAPG KGLEWVAVTSNDGSTTYYADSVKGRFTISRDNSKNTLYLQMN SLRSEDTAVYYCARKVVRGYHYHDAFDIWGQGTMVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPG	29
MT-101 (IgG4)-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGMQWVRQAPG KGLEWVAVTSNDGSTTYYADSVKGRFTISRDNSKNTLYLQMN SLRSEDTAVYYCARKVVRGYHYHDAFDIWGQGTMVTVSSAST KGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSN TKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCS VMHEALHNHYTQKSLSLSLGK	30

Example 1.3. Design of control antibody

For comparative analysis of the MT-101 of the present invention with previously filed Tie2-activating antibodies, a total of five control Tie2-activating antibodies were designed and produced. Specifically, the 2F2 and 4E2 antibodies included in International Patent Publication No. WO 2021-029746 A2, the TIE-1-Igγ1 antibody included in U.S. Patent Registration No. US 9902782 B2, the IGT-427 antibody included in U.S. Patent Publication No. US 2023-0287109 A1, and the #3 antibody included in International Patent Publication No. WO 2021-102173 A1 were set as controls. Reverse translation was performed with reference to the heavy and light chain variable region amino acid sequences of each antibody above, and then a codon optimization process for production in Expi293F cells was performed to synthesize the genes. Each synthesized heavy and light chain variable region gene was cloned into the pcDNA3.4 vector containing the IgG1 heavy and light chain constant regions, respectively, to produce

heavy and light chain expression vectors for a total of five antibodies. The amino acid sequences of the heavy and light chains of the control antibody are shown in Table 10, and were used in the experiments after transient expression and purification processes.

[Table 10]

Antibody Name	Class	Amino acid sequence	SEQ ID NO
2F2	2F2-HC	QVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMTWVRQAP GKGLEWVSRISGSGGSTNYADSVKGRFTISRDNSKNTLYLQ MNSLRAEDTAVYYCARGGLHHGFDIWGQGTMVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	31
	2F2-LC	SYELTQPPSLSVAPGKTARITCDGDNIESKSVHWYQQKPGQA PVLVIYYDNDRPSGIPERFSGSNSGNTATLTISRVEAGDEADY YCQVWDTYTDQPVFGGGTKLTVLGQPKAAPSVTLFPPSSEE LQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPS KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTV APTECS	32
4E2	4E2-HC	QVQLVQSGGGLIQPGGSLRLSCAASGFTFSDYAMHWVRQA PGKGLEWVALISFDGNNQYYADSVKGRFTISRDNSKNTIYL QMNSLRAEDTAVYYCTTDTMSGYDWEDAFDIWGQGTMIT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP G	33

	4E2-LC	DIQMTQSPSSLSASIGDRVTITCRASQSIGRWLAWYQQKPGK APKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATY YCQQSYSTPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGT ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN RGEC	34
TIE-1-Igγ1	TIE-1-Igγ1-HC	EVQLVESGGGLVQPGGSLRLSCAASGFTFDDYAMHWVRQA PGKGLEWVAGISWNSGSIVYADSVKGRFTISRDNSKNTLYL QMNSLRAEDTAVYYCAKDIREQLVEDAFDIWGQGTLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPASIEKTISKAKGQPREPQVYTLPPSRDELTKNQVS LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG K	35
	TIE-1-Igγ1-LC	DIQMTQSPSSLSASVGDRVTITCRSSQSLLHSHRYNYLDWY QQKPGKAPKLLIYLGSNRASGVPSRFSGSGSGTDFTLTISSV QPEDFATYYCMQTLQTPLTFGQGTKVEIKRTVAAPSVFIFPPS DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS SPVTKSFNRGEC	36
	TIE-1-Igγ1-tetravalent-HC	EVQLVESGGGLVQPGGSLRLSCAASGFTFDDYAMHWVRQA PGKGLEWVAGISWNSGSIVYADSVKGRFTISRDNSKNTLYL QMNSLRAEDTAVYYCAKDIREQLVEDAFDIWGQGTLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDKKVEPKSCGSEVQLVESGGGLVQPGGSLRLS CAASGFTFDDYAMHWVRQAPGKGLEWVAGISWNSGSIVYA DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDIRE QLVEDAFDIWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV	37

		VSVLTVLHQDWLNGKEYKCKVSNKALPASIEKTISKAKGQP REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGK	
IGT-427	IGT-427-HC	EVQLVQSGAEVKKPGASVKVSCKASGYSFTSYWMNWVRQ APGQGLEWMGMIHPSDSETRLNQKFMDRVTMTRDTSTSTV YMELSSLRSEDTAVYYCARGLYGNSWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA KTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKA LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	38
	IGT-427-LC	DIQMTQSPSSLSASVGDRVTITCRASQDIGISLNWYQQEPGK AIKRLIYATSSLDSGVPKRFSGSRSGTEYTLTISSLESEDFADY YCLQYASSPYTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGT ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN RGEC	39
p2.3	p2.3-HC	DVQLVESGGGVVRPGESLRLSCTASGFTFDSYGMSWVRQA PGKGLEWVSSINVGDNTYYADSVRGRFIISRDSSRNTLYLQM NSLTAEDTAVYYCANWNSFFDYWGLGTLVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP IEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	40
	p2.3-LC	EIVLTQSPGTLSLSPGEIATLSCRASQNVRSDLAWYQQKPGQ APRLLIYDASNRATGIPDRFSGSGSGTDFTLTISSLQSEDFAVY YCQQYSNWPPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKS GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD	41

		SKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC	

Example 1.4. Production of antibody that specifically binds to human Tie2 protein

A pcDNA3.4 vector (Invitrogen) loaded with a polynucleotide encoding the heavy and light chains of the antibodies produced using the methods described in Examples 1.1 to 1.3 above was introduced into Expi293F or ExpiCHO cells, and transient expression and purification of the antibodies were performed. Specifically, for transient expression of the antibodies in Expi293 cells, 50 μg each of the heavy and light chain expression vectors (100 μg in total) were diluted in Opti-MEM medium (6 mL). 320 μL of the ExpiFectamine™ 293 Transfection Kit reagent (ExpiFectamine™ 293 Reagent) was diluted in Opti-MEM medium (6 mL), and the cells were allowed to stand for 5 minutes. The medium containing the vector and the medium containing the reagent were mixed and allowed to stand for an additional 10 minutes. The mixture was then added to 100 mL of Expi293F cells (2.0 x 10^6 cells/mL) and cultured in suspension for 24 hours.

The cells cultured using the above-described method were further cultured for 4 days with ExpiFectamine™ 293 Transfection Enhancer. Thereafter, the cells were centrifuged at 10,000 x g for 30 minutes and filtered through a 0.2 μm filter to recover the supernatant. In order to express ExpiCHO-based antibodies, 40 μg each of the heavy and light chain expression vectors (80 μg total) were diluted in OptiPRO™ SFM medium (4 mL). 320 μL of the ExpiFectamine™ CHO Transfection Kit reagent (ExpiFectamine™ CHO Reagent) was diluted in 3.7 mL of OptiPRO™ SFM medium, and the mixture was allowed to stand for 4 minutes. The medium containing the vector and the medium containing the reagent were mixed and allowed to stand for 5 minutes. Thereafter, 100 mL of ExpiCHO cells (3.0 x 10^6 cells/mL) were added and cultured in suspension for 22 hours.

The cells cultured as described above were treated with 600 μL of ExpiFectamine™ CHO Transfection Enhancer 1 and 16 mL of ExpiCHO™ Feed solution. Five days later, the cells were further treated with 16 mL of ExpiCHO™ Feed solution and cultured for 12 days. Thereafter, The culture solution was centrifuged at 10,000 x g for 40 minutes and filtered through a 0.2 μm filter to recover the supernatant. The supernatant recovered from Expi293F or ExpiCHO were allowed to bind to an affinity column (MabSelect PrismA, Cytiva) using an AKTA Avant 150 instrument (Cytiva) and then eluted with an acidic eluent (100 mM glycine buffer, pH 3.5). The eluted antibody was finally purified into PBS buffer using a desalting column (Hiprep 26/10). The concentration of the purified antibody was measured by absorbance or BCA assay, followed by SDS-PAGE analysis under non-reducing and reducing conditions. As a result, the antibody was identified as having a total molecular weight of approximately 150 kDa, comprising a heavy chain (approximately 50 kDa) and a light chain (approximately 25 kDa).

Example 2. Confirmation of binding affinity of anti-Tie2 antibody

The binding affinity of the antibodies prepared in Example 1.4 above to human Tie2 protein was confirmed.

The antibody affinity was measured using an Octet K2 (ForteBio) instrument based on the BLI (biolayer interferometry) technique. Specifically, the AR2G biosensor tip was hydrated in third-pass distilled water (deionized water) for 10 minutes and then activated using a buffer comprising 20 mM EDC and 10 mM Sulfo-NHS. Thereafter, 60 nM hTie2-ECD-6xHis protein (SEQ ID NO: 101) or mTie2-ECD-mFc protein as an antigen was diluted in immobilization buffer (10 mM sodium acetate; pH 6.0) and reacted with the activated AR2G tip for 10 minutes to immobilize the antigen on the AR2G tip.

Thereafter, the antigen immobilization reaction was terminated by treatment with a 1 M ethanolamine (pH 8.5) solution, and the AR2G tip was reacted in PBS buffer for 60 seconds to establish a baseline of antigen-antibody binding. Thereafter, MT-100 (IgG1), MT-111 (IgG1), MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA) were diluted three times in PBS buffer at a concentration ranging from 5 nM to 100 nM and allowed to associate with the antigen-immobilized AR2G tip for 800 seconds, followed by dissociation for 800 seconds. Based on the reaction curves identified through the association and dissociation reaction analysis, the K_{on}, K_{off}, and K_D values were calculated through global fitting analysis using the Data Analysis HT 12.0 program, thereby measuring the affinity of each antibody.

[Table 11]

Antigen	Antibody	K_D (M)	K_{on} (1/Ms)	K_{off} (1/s)
human Tie2	MT-100	3.42E-09	3.38E05	1.15E-03
	MT-111	6.41E-10	1.04E05	6.67E-05
	MT-101 (IgG1)	4.71E-10	1.93E05	9.12E-05
	MT-101 (IgG4)	7.21E-10	7.40E05	5.34E-04
	MT-101 (IgG1-LALA)	4.75E-10	7.87E05	3.74E-04
mouse Tie2	MT-100	1.44E-08	5.84E05	8.44E-03
	MT-111	1.95E-09	2.43E05	4.75E-04
	MT-101 (IgG1)	6.66E-09	2.73E05	1.82E-03

As a result, as shown in Table 11 above, among MT-100, MT-111, and MT-101, MT-101 (IgG1) was confirmed to have the highest affinity for human Tie2 protein. In addition, it was confirmed that the MT-101 (IgG4) and MT-101 (IgG1-LALA) variants, which have the same variable region as MT-101 (IgG1), also exhibited affinities for human Tie2 protein ranging from 0.47 nM to 0.72 nM, similar to MT-101 (IgG1).

Example 3. Confirmation of Tie2 activation by anti-Tie2 antibody

In order to confirm the Tie2 activating ability of the antibodies (MT-100, MT-101, MT-110, and MT-111) having the IgG1-type Fc backbone prepared in Example 1.4 above, as well as the homologous antibody (MT-101 (IgG4)) and the mutant antibody (MT-101 (IgG1-LALA)) of MT-101, the phosphorylation of Tie2 protein (human or mouse) and its downstream signaling factors (AKT, ERK, eNOS, or FOXO1) (P-Tie2, P-AKT, P-ERK, P-eNOS, or P-FOXO1) was confirmed through western blotting.

Specifically, HEK293 cells (1 x 10^6 cells) expressing human Tie2 protein or 3T3-J2 cells (5 x 10^5 cells) expressing mouse Tie2 protein were cultured in a 60 mm culture dish in DMEM medium at a temperature of 37 °C. The cultured cells were maintained in serum-free DMEM medium for 6 hours to achieve serum starvation. Thereafter, the cells were treated with the antibody (MT-100) at concentrations ranging from 0.1 μg/mL to 20 μg/mL for 15 minutes. The cells were then lysed with lysis buffer to obtain cell lysates.

The cell lysates obtained as described above were subjected to SDS-PAGE and then transferred to the PVDF membranes. Thereafter, the membranes were treated with 0.1% Tween 20 TBST buffer containing 3% BSA and reacted at room temperature for 2 hours. Then, each was treated with primary antibodies diluted 1:1,000 and reacted at room temperature for 2 hours. At this time, the primary antibodies used were anti-phospho-tyrosine antibody (Sigma, 05-321), anti-Tie2 antibody (R&D systems, AF313), anti-phospho-AKT antibody (Cell Signaling, 9271), anti-AKT antibody (Cell Signaling, 9272), anti-phospho-ERK antibody (Cell Signaling, 9106), anti-ERK antibody (Cell Signaling, 9102), anti-phospho-eNOS antibody (Cell Signaling, 9571), anti-eNOS antibody (Cell Signaling, 5880), anti-phsopho-FOXO1 antibody (Cell Signaling, 9461), and anti-FOXO1 antibody (Cell Signaling, 2880). After the primary antibody reaction, it was treated with a peroxidase-conjugated secondary antibody diluted 1:5,000 and reacted at room temperature for 1 hour. At this time, the secondary antibody used was anti-rabbit IgG antibody (Invitrogen, 31460) or anti-mouse IgG antibody (Abcam, ab97023). After completion of the secondary antibody reaction, the protein expression was confirmed by treatment with ECL solution.

As a result, it was confirmed that MT-100 induced the phosphorylation of human or mouse Tie2, AKT, or ERK proteins (Figures 1a and 1b).

In addition, human umbilical vein endothelial cells (HUVECs) were used instead of HEK293 cells, and EGM-2 medium and serum-free M199 medium were used as media. The cells were lysed to confirm the phosphorylation of Tie2. Thereafter, the phosphorylation of Tie2 protein and the phosphorylation of the signaling factors were confirmed in human Tie2-expressing HUVECs in the same manner as described above, except that immunoprecipitation was used.

As a result, it was confirmed that, similar to HEK293 cells, the antibodies (MT-100, MT-101, MT-110, MT-111, MT-101 (IgG4), or MT-101 (IgG1-LALA)) induced the phosphorylation of Tie2, AKT, ERK, eNOS, or FOXO1 proteins in HUVECs (Figures 1c to 3).

Example 4. Confirmation of specificity of anti-Tie2 antibody for Tie2 antigen protein

We confirmed whether the anti-Tie2 antibodies obtained in Example 1.4 above specifically phosphorylated only the Tie2 protein in vascular endothelial cells, or whether the anti-Tie2 antibodies also phosphorylated other receptor proteins in addition to the Tie2 protein.

Specifically, HUVECs (3 x 10^6 cells) were cultured in a 100 mm culture dish a temperature of 37 °C in EGM-2 medium. The cultured HUVECs were maintained in M199 medium containing 1% serum for 6 hours to achieve serum starvation, and then treated with MT-100 at a concentration of 5 μg/mL for 30 minutes. The cells were treated with lysis buffer and lysed to obtain cell lysates. The cell lysates obtained by the above method were quantified using the BCA protein quantification method, and then applied to the membranes containing antibodies against 49 human tyrosine receptor proteins (receptor tyrosine kinases (RTKs); ALK/CD246, Axl, DDR1, DDR2, Dtk, EGF R, EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA10, EphB1, EphB2, EphB3, EphB4, EphB6, ErbB2, ErbB3, ErbB4, FGF R1, FGF R2 alpha, FGF R3, FGF R4, Flt-3/Flk-2, HGF R/c-MET, IGF-I R, Insulin R/CD220, M-CSF R, Mer, MSP R/Ron, MuSK, PDGF R alpha, PDGF R beta, c-Ret, ROR1, ROR2, Ryk, SCF R/c-kit, Tie-1, Tie-2, TrkA, TrkB, TrkC, VEGF R1/Flt-1, VEGF R2/KDR, and VEGF R3/Flt-4) and reacted at 4 °C for 12 hours. The membranes were treated with anti-tyrosine phosphorylation antibody (anti-phospho-tyrosine-HRP) and reacted at room temperature for 1 hour. Thereafter, the phosphorylation of 49 RTKs was confirmed by treatment with ECL solution.

As a result, it was confirmed that MT-100 selectively phosphorylated only the Tie2 protein among the 49 RTKs in HUVECs (Figure 4).

Example 5. Confirmation of cross-species binding affinity of anti-Tie2 antibody to Tie2 antigen protein

We confirmed whether the anti-Tie2 antibodies (MT-100, MT-101 (IgG1), MT-101 (IgG4), and MT-101 (IgG1-LALA)) that bind to cross-species Tie2 proteins also exhibit cross-species binding to the Tie2 proteins derived from other species.

Therefore, the Tie2 genes from monkey, mouse, rat, dog, and pig were synthesized and cloned into the pcDNA3.4 vector to produce the Tie2 expression vectors for each species (SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, and SEQ ID NO: 106). HEK293T cells (2.0 x 10^6 cells) were treated with 10 μg of each Tie2 expression vector and 20 μL of Lipofectamine 2,000 (Invitrogen) diluted in 1 mL of Opti-MEM medium and cultured

for 2 days. The culture solution was centrifuged (300 x g for 5 minutes), the supernatant was removed, and the cells were washed with FACS buffer.

The Tie2-expressing cells for each species obtained as described above were treated with anti-Tie2 antibodies (MT-100, MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA)) at a concentration of 1 μg/mL. After reaction for 1 hour at room temperature, the cells were washed twice with FACS buffer. Then, 100 μL of 1:200 diluted goat anti-human IgG-FITC (FITC-conjugated goat anti-human IgG, Ivitrogen) was added and reacted at room temperature for 30 minutes. Thereafter, the cells were again washed twice with FACS buffer, and the antibody binding was confirmed using a NovoCyte Flow Cytometer (Agilent).

As a result, it was confirmed that the anti-Tie2 antibodies (MT-100, MT-101 (IgG1), MT-101 (IgG4), MT-101 (IgG1-LALA)) were bound not only to Tie2 proteins derived from human but also monkey, mouse, rat, dog, and pig (Figures 5a to 5d).

Example 6. Confirmation of vascular endothelial cell activation effect of anti-Tie2 antibody

Example 6.1. Analysis of angiogenesis and network structure stabilization effect of anti-Tie2 antibody

We analyzed whether the anti-Tie2 antibodies induce angiogenesis (tube formation) and network stabilization in vascular endothelial cells using Matrigel.

Specifically, serum-starved HUVECs (1 x 10^5 cells) cultured in M199 medium containing 1% serum for 6 hours were seeded onto a 24-well culture dish coated with Growth Factor Reduced (GFR)-Matrigel. The HUVECs were treated with MT-100 at a concentration of 10 μg/mL and the control substance, Ang-1 (R&D systems, 923-AN), at a concentration of 500 ng/mL. The capillary-like tube formation was confirmed during 18 hours of incubation.

As a result, it was confirmed that the vascular network in the MT-100 treatment group was increased and maintained for a long period of time, similar to the positive control, Ang-1 treatment group (Fig. 6).

Example 6.2. Analysis of vascular endothelial cell injury inhibitory effect of anti-Tie2 antibody

The inhibitory efficacy of anti-Tie2 antibody on vascular endothelial cell injury was confirmed by analyzing apoptosis of vascular endothelial cells induced when cultured under serum-free and hypoxic conditions.

Specifically, HUVECs (5 x 10^4 cells) were seeded in a 24-well plate and cultured in EGM-2 medium at 37 °C for 48 hours. The following day, HUVECs were treated with anti-Tie2 antibody (MT-101 (IgG1)) at the concentrations shown in Figure 7a (0.01 nM, 0.1 nM, 1 nM, 10 nM, or 100 nM) in serum-free M199 medium and cultured for 40 hours under hypoxic conditions (<1% oxygen) at 37 °C. The cells cultured as described above were immobilized

and stained with Crystal Violet reagent for 10 minutes. The cells were washed four times with distilled water, and then the stained reagent was eluted with distilled water containing 1% SDS, and the absorbance value (OD 570 nm) was measured.

In addition, the cells were treated with anti-Tie2 antibodies (MT-101 (IgG1), MT-101 (IgG4), and MT-101 (IgG1-LALA)) at the concentrations (0.5 nM or 5 nM) shown in Figure 7b in the same manner as above, and the absorbance value (OD 570 nm) was measured.

As a result, it was confirmed that the MT-101 (IgG1) treatment group inhibited apoptosis in a concentration-dependent manner compared to the control (Figure 7a). In addition, it was confirmed that MT-101 (IgG4) and MT-101 (IgG1-LALA) also had the same apoptosis inhibitory efficacy as MT-101 (IgG1) (Figure 7b).

Example 6.3. Analysis of vascular endothelial permeability (leakage) and inflammation inhibitory effect of anti-Tie2 antibody

The inhibitory effect on increased vascular endothelial permeability (also known as leakage phenomenon) due to vascular abnormalization was confirmed using cell immunochemical staining.

Specifically, HUVECs (1 x 10^5 cells) were seeded in the upper chamber of a 12-well Transwell plate (0.4 μm pore), cultured for 3 days, and then maintained in a serum-starved state for 6 hours. Thereafter, in order to artificially increase vascular permeability, the cells were treated with 50 ng/mL of VEGF (R&D systems, 293-VE), while the cells were treated with MT-100 at a concentration of 20 μg/mL for 5 hours. Thereafter, fluorescently labeled dextran (FITC-Dextran, 70 kDa) was added to the culture solution, and dextran permeated through the HUVECs was analyzed using a fluorometer (SpectraMAx i3x) after 30 minutes.

As a result, it was confirmed that the vascular permeability was increased in cells treated with VEGF alone, whereas the vascular permeability was completely inhibited in cells treated with VEGF and MT-100 (Figure 8a).

In addition, HUVECs (1 x 10^5 cells) were seeded in a 35 mm confocal dish (SPL) and cultured for 2 days, and then maintained in a serum-starved state for 6 hours. Then, the cells were treated with VEGF (50 ng/mL) or VEGF (50 ng/mL) and MT-100 (10 μg/mL) and then reacted for 3 hours. Thereafter, the cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with PBS buffer containing 0.2% Triton X-100 for 10 minutes, and then blocked with PBS buffer containing 1% BSA for 1 hour at room temperature. The anti-VE-cadherin antibody (Santacruz, sc-9989) was diluted 1:50 as the primary antibody, and the cells were treated therewith and then reacted at room temperature for 2 hours. Then, fluorescently labeled secondary antibody (Alexa Fluor 488 donkey anti-goat, Invitrogen) was diluted 1:200, and the cells were were treated therewith and then reacted at room temperature for 1 hour. Finally, the cells were treated with DAPI diluted 1:1,000, and the cell nuclei were stained for 3 minutes and observed under a fluorescence microscope.

As a result, it was confirmed that in the cells treated with VEGF alone, cell-cell junctions were significantly weaker, whereas in the cells treated with MT-100, cell-cell junctions were well maintained and the interfaces developed strongly (Figure 8b).

In addition, the inhibitory effect of anti-Tie2 antibody on vascular endothelial inflammation was analyzed by assessing the expression of proteins that mediate the adhesion between vascular endothelial cells and inflammatory cells (leukocytes): intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

Specifically, HUVECs (5×10^5 cells) were seeded in a 60 mm culture dish and cultured for 24 hours. The cells were then maintained in serum-free M199 medium for 6 hours to achieve serum starvation. Thereafter, the cells were treated with 20 ng/mL of VEGF alone and/or 10 μg/mL of MT-100, and reacted for 6 hours. The cells were then lysed to obtain cell lysates.

Western blotting was performed using the cell lysate obtained as described above as a sample using the same method as in Example 3 above. At this time, anti-ICAM-1 antibody (Santacruz, sc-8439) or anti-VCAM-1 antibody (Santacruz, sc-13160) was used, and anti-mouse IgG antibody (Abcam, ab97023) was used as a secondary antibody to confirm the expressions of ICAM-1 and VCAM-1.

As a result, it was confirmed that the MT-100 treatment group showed a decrease in the expression of ICAM-1 and VCAM-1 induced by VEGF treatment (Figure 8c).

Example 7. Confirmation of inhibitory effect of anti-Tie2 antibody on Ang-2 binding to Tie2

The binding inhibitory effect of anti-Tie2 antibody on the mutual binding of Tie2 and Ang-2 in pathological conditions with increased Ang-2 was confirmed using the same method as in Example 2 above.

Specifically, the AR2G biosensor tip was hydrated in third-pass distilled water (deionized water) for 10 minutes and then activated using a buffer comprising 20 mM EDC and 10 mM Sulfo-NHS. Thereafter, 100 nM hTie2-ECD-6x His protein as an antigen was diluted in immobilization buffer (10 mM sodium acetate, pH 6.0) and reacted with the activated AR2G tip for 10 minutes to immobilize the antigen on the AR2G tip.

Thereafter, the antigen immobilization reaction was terminated by treatment with a 1 M ethanolamine (pH 8.5) solution. First, in order to measure the baseline antigen-antibody binding, the AR2G tip was reacted in PBS buffer for 60 seconds. Thereafter, 200 nM MT-101 (IgG1) or PBS buffer was associated with the antigen-immobilized AR2G tip for 600 seconds, and then 40 nM recombinant human Ang-2 protein was associated and dissociated for 600 seconds, respectively. The association and dissociation reaction values were analyzed through global fitting using the Data Analysis HT 12.0 program

As a result, it was confirmed that Ang-2 did not bind to Tie2 when MT-101 (IgG1) was bound to Tie2 (Figures 9a and 9b).

Example 8. Confirmation of Tie2 activation-inducing effect of anti-Tie2 antibody in the presence of Ang-2

We analyzed whether the anti-Tie2 antibody could induce the phosphorylation of Tie2 expressed in vascular endothelial cells in the presence of Ang-2.

Specifically, HUVECs (3 x 10^6 cells) were seeded in a 100 mm culture dish and cultured for 24 hours. The cells were then maintained in serum-free M199 medium for 6 hours to achieve serum starvation. Thereafter, the cells were pretreated with Ang-2 (5 nM) 20 minutes prior to treatment with anti-Tie2 antibody (MT-101 (1 nM)). Then, the cells were treated with Ang-2 (5 nM) and MT-101 (1 nM) alone or in combination and reacted for 20 minutes. After completion of the reaction, the cells were lysed with lysis buffer to obtain cell lysates.

The anti-Tie2 antibody (R&D systems, AF313) was added at a concentration of 1 μg/mL to the cell lysate obtained as described above, and the reaction was performed at 4 °C for 12 hours. 30 μL of protein G beads (Millipore, 16-266) were added to obtain a sample by immunoprecipitation. At this time, whole cell lysate (WCL) was also obtained and used as a sample.

Western blotting was performed using the same method as in Example 3 above using the sample prepared as described above. At this time, the primary antibody used was anti-phospho-tyrosine antibody (Sigma, 05-321), anti-Tie2 antibody, anti-phospho-AKT antibody, anti-AKT antibody, anti-phospho-ERK antibody, or anti-ERK antibody.

As a result, it was confirmed that MT-101 strongly induced the phosphorylation of Tie2, AKT, or ERK proteins even in the presence of Ang-2 (Figure 10).

Example 9. Confirmation of inhibitory effect of anti-Tie2 antibody on acute kidney injury

Example 9.1. Analysis of effect of anti-Tie2 antibody in animal model of acute kidney injury induced by ischemia and reperfusion

The inhibitory efficacy of the anti-Tie2 antibody prepared in Example 1.4 above on acute kidney injury was confirmed using an animal model of acute kidney injury induced by ischemia and reperfusion.

Specifically, 100 μL of Control IgG (10 mg/kg) or anti-Tie2 antibodies ((MT-101 (IgG1), MT-101 (IgG4), and MT-101 (IgG1-LALA)) were administered into the tail vein of 8-week-old C57BL/6J mice in the normal group (Sham) or ischemia reperfusion (I/R) group at the concentrations (5 mg/kg or 10 mg/kg) shown in Figures 11b and 11c. Thirty minutes after injection, the mice were anesthetized by intraperitoneal injection (0.2 μL/g) of a solution containing a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg), a general anesthetic for

animals. Thereafter, the abdominal skin of the mice was incised approximately 1.5 cm. Through the incision site, the right kidney was secured using a thin cotton swab soaked in PBS buffer and precision iris forceps, avoiding the perirenal fat. The artery and vein connected to the kidney were secured using a microclamp. Ischemia was induced in the left kidney using the same method (Figure 11a).

In addition, the body temperature of the mice was maintained at 35 °C to 36 °C using a heating pad and a rectal thermometer. After 30 minutes, the microclamp was removed to allow reperfusion of blood to the kidneys. Then, the incised abdomen was closed with sutures, and the external wound was closed with metal staples. The mice were observed and allowed to recover at a temperature of 22 °C to 25 °C. Twenty-four hours after surgery, blood was collected from the inferior vena cava of anesthetized mice and centrifuged at 4 °C to obtain serum. Serum creatinine (s-creatinine) and blood urea nitrogen (BUN) were analyzed using an automated chemical analyzer (Hitachi 7180).

As a result, it was confirmed that in the Control IgG group, serum creatinine and BUN concentrations were increased due to I/R, whereas they were significantly reduced in the anti-Tie2 antibody (MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA)) administration groups (Figures 11b and 11c).

Example 9.2. Analysis of inhibitory effect of anti-Tie2 antibody on renal tubular injury

In order to confirm the inhibitory effect of anti-Tie2 antibody on acute kidney injury, the degree of renal tubular injury was analyzed through histological analysis.

Specifically, the kidneys were extracted from the laboratory mice in Example 9.1 above, fixed in 10% formalin for 24 hours, and embedded in paraffin to produce blocks. The paraffin blocks were sectioned into 4 μm thick sections and mounted and immobilized on slides to produce tissue section slides. Thereafter, the section slides were treated with xylene to remove paraffin and stained with hematoxylin-eosin (Figure 12a) to observe renal tubular injury. The injury was evaluated using an injury score as follows: 0: Normal, 1: 25% or less injury, 2: 25% to 50% injury, 3: 50% to 75% injury, 4: 75% or more injury.

As a result, it was confirmed that the acute kidney injury-induced group (Control IgG) showed more pronounced tubular injury compared to the normal group (Sham), whereas the anti-Tie2 antibody (MT-101 (IgG1), MT-101 (IgG4), or MT-101 (IgG1-LALA)) administration groups showed a concentration-dependent inhibition of tubular injury (Figures 12a and 12b).

Example 9.3. Analysis of effects of anti-Tie2 antibody on inducing Tie2 activation and inhibiting vascular endothelial cell injury

In order to confirm whether the acute kidney injury inhibitory effect of anti-Tie2 antibody, identified in Example 9.1 above, was induced by Tie2 activation and inhibition of

renal endothelial cell injury, Tie2 phosphorylation and immunostaining of renal vascular endothelial cells and vascular endothelial pericytes were performed.

Specifically, the mouse kidney of Example 9.1 above was extracted, fixed in 4% paraformaldehyde for 2 hours, dehydrated in a PBS buffer containing 15% sucrose at 4 °C for 2 hours, and then in a PBS buffer containing 30% sucrose at 4 °C for 24 hours. Then, a frozen block was prepared using a cryogenic embedding agent (OCT). The frozen block was sectioned into 7 μm thick sections, fixed on slides with 4% paraformaldehyde for about 5 minutes, washed three times with a washing buffer (2% FBS + 0.1% sodium azide in PBS), and then blocked with a nonspecific protein blocking buffer (1% BSA in PBS) for 1 hour.

The sections were treated with primary antibody (anti-CD31 antibody (Sigma, MAB1398Z, 1:200), anti-p-Tie2 antibody (R&D system, AF2720, 1:100), or anti-PDGFRβ (Invitrogen, 14-1402-82, 1:100)) and reacted at 4 °C for 16 hours. After completion of the reaction, the sections were washed three times with washing buffer, and then treated with secondary antibodies labeled with FITC or Alexa flour 594 (FITC-labeled anti-rabbit IgG, Invitrogen, A21206; Alexa flour 594-labeled anti-hamster IgG, Invitrogen, A21113; FITC-labeled anti-hamster IgG, Invitrogen, A21110; Alexa flour 594-labeled anti-rat IgG, Invitrogen, A21209, each 1:1,000). The sections were reacted at room temperature for 2 hours. Thereafter, the sections were again washed twice with washing buffer, treated with DAPI (4',6-diamidino-2-phenylindole; 1 μg/mL), and reacted at room temperature for 1 minute to stain cell nuclei. After completion of the reaction, the sections were washed twice with washing buffer and tissue sections were observed under a fluorescence microscope.

As a result, it was confirmed that the phosphorylation of Tie2 protein was not induced in the acute kidney injury-induced group (Control IgG), whereas the phosphorylation of Tie2 protein was increased in renal blood vessels in the MT-101 (IgG1-LALA) administration group (Figure 13a). In addition, it was confirmed that in the MT-101 (IgG1-LALA) administration group, vascular endothelial cells and vascular endothelial pericytes were maintained similar to those in the normal group (Sham) (Figure 13b).

Example 9.4. Analysis of inflammation inhibitory efficacy of anti-Tie2 antibody

One of the major factors in acute kidney injury caused by ischemia and reperfusion is an excessive inflammatory response. When reactive oxygen species (ROS) increase due to ischemia and reperfusion, the expression of proteins (e.g., VCAM-1, vascular cell adhesion molecule-1) that mediate the adhesion of inflammatory cells, such as neutrophils, increases on the surface of renal vascular endothelial cells. This increases neutrophil adhesion to the vessel wall and influx into the tubular interstitium, inducing a persistent inflammatory response and exacerbating tubular injury.

In order to analyze whether the inhibitory effect of anti-Tie2 antibody on acute kidney injury in Example 9.1 above was induced by inflammation inhibition, the expression of CD31,

a vascular endothelial cell marker protein, VCAM-1, an inflammatory cell adhesion mediator protein, and Gr-1, a neutrophil marker, was analyzed through immunostaining.

Specifically, the kidneys were extracted from the mice of Example 9.1 above, and tissue staining was performed using the same method as in Example 9.3 above. At this time, primary antibodies (anti-CD31 antibody (Sigma, MAB1398Z, 1:200), anti-VCAM-1 antibody (Santacruz, sc-13160, 1:100), or anti-Gr-1 antibody (Abcam, ab25377, 1:100) were used, and FITC- or Alexa flour 594-labeled secondary antibodies (FITC-labeled anti-mouse IgG, Invitrogen, A21202; FITC-labeled anti-rat IgG, Invitrogen, A21208; Alexa flour 594-labeled anti-hamster IgG, Invitrogen, A21113, each 1:1,000) were used.

As a result, it was confirmed that the expression of VCAM-1 in renal vascular endothelial cells was increased in the acute renal injury-induced group (Control IgG) compared to the normal group (Sham). On the other hand, in the MT-101 (IgG1-LALA) administration group, it was decreased similar to the normal group (Figure 14a). At this time, it was confirmed that the influx of neutrophils into the tubular interstitium was also reduced (Figure 14b).

Example 9.5. Analysis of inhibitory effect of anti-Tie2 antibody on expression of inflammatory response inducer

Changes in the expression of cytokines (CCL2, IL-6, or IL-1β) that induce inflammatory responses in mouse kidney tissue treated with anti-Tie2 antibody (MT-101 (IgG1-LALA)) were confirmed through real-time polymerase chain reaction (PCR) analysis.

Specifically, the kidneys were extracted from the mice of Example 9.1 above and homogenized in 1 mL of TRIzol (Invitrogen) solution using a homogenizer. Thereafter, it was allowed to stand for 5 minutes to completely dissociate the nucleoprotein complex. 200 μL of chloroform was added, and the mixture was shaken for 15 seconds and reacted at room temperature. The mixture was centrifuged at 14,000 rpm at 4 °C for 15 minutes to separate the supernatant. Approximately 400 μL was transferred to a new tube, and an equal volume of isopropanol was added to precipitate the RNA. The precipitate was centrifuged at 14,000 rpm at 4 °C for 10 minutes, washed with 1 mL of 75% ethanol, and centrifuged for 5 minutes under the same conditions to collect the RNA pellet. The pellet was dried, and then 50 to 100 μL of RNase-free water was added. The pellet was dissolved at 55 °C for 10 minutes to prepare the RNA.

The RNA prepared for cDNA synthesis was diluted to a concentration of 1 to 1.5 μg/μL, and then RNA (2 μg), oligo dT primer (1 μL), and RNase-free water were mixed to make a total volume of 10 μL, reacted at 70 °C for 10 minutes, and cooled on ice for 5 minutes. Thereafter, a cDNA synthesis mixture was prepared and reacted at 42°C for 1 hour, followed by treatment at 70 °C for 10 minutes to inactivate RTase. qPCR was performed using Solg 2X Real-Time PCR Smart mix (solgent) on a Bio-rad instrument (96-well PCR plate). The PCR reaction was performed with enzyme activation at 95 °C for 15 minutes, followed by 35 cycles

(95 °C, 20 seconds; 58 °C, 40 seconds; 72 °C, 30 seconds), and finally, a PCR melting curve step was performed.

As a result, it was confirmed that the MT-101 (IgG1-LALA) administration group showed inhibition of the expression of inflammatory inducers, CCL2 (C-C motif chemokine ligand 2), IL-6, and IL-1β, compared to the control IgG treatment group (Figure 14c).

Example 10. Confirmation of inhibitory effect of anti-Tie2 antibody on sepsis-induced acute kidney injury

Example 10.1. Analysis of effect of anti-Tie2 antibody in lipopolysaccharide-induced sepsis-induced acute kidney injury animal model

The inhibitory efficacy of the anti-Tie2 antibody prepared in Example 1.4 above on acute kidney injury was also confirmed in a lipopolysaccharide (LPS)-induced sepsis-induced acute kidney injury mouse model.

Specifically, 8-week-old C57BL/6J mice were randomly divided into groups and administered with a single intraperitoneal injection of LPS (Sigma, L3024) at a dose of 10 mg/kg. One hour before or one hour after LPS administration, 100 μL of Control IgG or MT-101 (IgG1-LALA) at a concentration of 10 mg/kg was injected into the tail vein. Twenty-four hours later, blood was collected from the inferior vena cava of anesthetized mice and centrifuged at 4 °C to obtain serum. Serum creatinine (s-creatinine) and blood urea nitrogen (BUN) were analyzed using an automated chemical analyzer (Hitachi 7180) (Figure 15a).

As a result, it was confirmed that s-creatinine and BUN concentrations were increased in the Control IgG administration group, but were significantly reduced in the group administered with MT-101 (IgG1-LALA) 1 hour before or after the treatment (Figures 15b and 15c).

Example 10.2. Analysis of inhibitory effect of anti-Tie2 antibody on renal vascular endothelial cell and renal tubular injury in sepsis-induced acute kidney injury animal model

The inhibitory effect of anti-Tie2 antibody on acute kidney injury in Example 10.1 above was analyzed through immunostaining of KIM-1, a renal tubular injury marker protein, and CD31, a vascular endothelial cell marker protein, in renal tissue.

Specifically, the kidneys were extracted from the mice of Example 10.1 above, and tissue staining was performed using the same method as in Example 9.3 above. At this time, the primary antibody (anti-CD31 antibody (Sigma, MAB1398Z, 1:200) or anti-KIM-1 antibody (R&D system, MAB18171, 1:200)) was used, and FITC- or Alexa flour 594-labeled secondary antibody (FITC-labeled anti-rabbit IgG (Invitrogen, A21206) and Alexa flour 594-labeled anti-hamster IgG (Invitrogen, A21113), 1:1,000, respectively) were used.

As a result, it was confirmed that renal tubular injury and renal vascular injury were significantly reduced in the group administered with MT-101 (IgG1-LALA) 1 hour before or after (Figure 15d).

Example 11. Confirmation of therapeutic effect of anti-Tie2 antibody on chronic renal failure

Example 11.1. Analysis of effect of anti-Tie2 antibody in animal model of chronic renal failure induced by ischemia and reperfusion

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above on chronic renal failure was analyzed in an animal model of chronic renal failure induced by ischemia and reperfusion.

Specifically, 8-week-old C57BL/6J mice were randomly divided into groups. A solution containing a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg), a general anesthetic for animals, was injected intraperitoneally (0.2 μL/g) to anesthetize the mice. Thereafter, the abdominal skin of the mice was incised approximately 1.5 cm. Through the incision site, the right kidney was secured using a thin cotton swab soaked in PBS buffer and precision iris forceps, avoiding the perirenal fat. The artery and vein connected to the kidney were secured using a microclamp. Ischemia was induced in the left kidney using the same method (Figure 16a).

In addition, the body temperature of the mice was maintained at 35 °C to 36 °C using a heating pad and a rectal thermometer. After 27 minutes, the microclamp was removed to allow reperfusion of blood to the kidneys. Then, the incised abdomen was closed with sutures, and the external wound was closed with metal staples. The mice were observed and allowed to recover at a temperature of 22 °C to 25 °C. On days 2, 4, and 6, 100 μL of Control IgG or anti-Tie2 antibody (MT-101 (IgG-LALA)) was injected into the tail vein at a concentration of 5 mg/kg. Blood was collected from the inferior vena cava on days 3, 7, or 14 and centrifuged at 4 °C to obtain serum. On day 14, the mouse kidneys were extracted, and tissue staining was performed using hematoxylin-eosin (H&E) reagent using the same method as in Example 9.2 above. The renal tubular injury was observed, and the injury score was evaluated as follows: 0: Normal, 1: 25% or less injury, 2: 25% to 50% injury, 3: 50% to 75% injury, 4: 75% or more injury.

As a result, it was confirmed that the chronic renal failure-induced group (Control IgG) showed more pronounced tubular injury compared to the normal group (Sham), whereas the MT-101 (IgG1-LALA) administration group showed a marked inhibition of renal tubular injury (Figure 16b).

Example 11.2. Analysis of fibrosis inhibitory effect of anti-Tie2 antibody in animal model of chronic renal failure induced by ischemia and reperfusion

The efficacy of MT-101 in inhibiting renal fibrosis was confirmed through Masson's trichrome staining, sirus red staining, and immunostaining of collagen IV or α-SMA, which are fibrosis marker proteins.

Specifically, the kidneys were extracted from the 14-day mice used in Example 11.1, and paraffin blocks were prepared using the same method as in Example 9.2. In order to confirm the degree of fibrosis using Masson's trichrome method, paraffin-embedded kidney tissue was sectioned into 4 μm thick sections and attached to slides. Thereafter, deparaffinization and rehydration with distilled water were performed. Bouin's solution was preheated to 60 °C in a hood, and the slides were immersed in the solution for 60 minutes and cooled for 10 minutes. The slides were rinsed with tap water to completely clear the tissue, and then washed once with distilled water.

Weigert's Iron Hematoxylin Solutions A and B were mixed in equal proportions, and the slides were stained therewith for 5 minutes. The sections were then rinsed under running water for 2 minutes. Thereafter, Biebrich Scarlet/Acid Fuchsin solution was applied to the slides. The sections were stained for 15 minutes and washed with distilled water. After differentiation treatment with a phosphomolybdic/phosphotungstic acid solution for 15 minutes or until collagen no longer appeared red, the slides were immersed in an aniline blue solution for 5 to 10 minutes without washing, and then very rapidly dehydrated twice sequentially with 95% alcohol. Finally, the slides were cleared with xylene and embedded in a synthetic resin.

In addition, in order to confirm the fibrosis using Sirius red, paraffin-embedded kidney tissue was sectioned into 4 μm thick sections and attached to slides. Thereafter, the sections were deparaffinized by immersing them thre times in xylene for three minutes each. They were then rehydrated three times in 100% ethanol for two minutes each, 95% to 50% ethanol for two minutes each, and deionized water for five minutes each. The slides were stained by immersing them in a Sirius red solution for 60 minutes, washed twice with an acetic acid solution, dehydrated five times by tapping in 100% ethanol, cleared three times for 3 minutes each in xylene, and embedded using Permount.

In addition, in order to identify fibrosis marker proteins, collagen IV (abcam, ab6586) and α-SMA (abcam, ab7817), paraffin-embedded kidney tissues were sectioned into 4 μm thick sections and attached to slides. Thereafter, the tissues were deparaffinized by immersing them twice in xylene for 5 minutes each, and rehydrated twice in 100% ethanol for 5 minutes each, in 95% or 50% ethanol for 2 minutes, and in deionized water for 5 minutes. Antigen retrieval was performed in a pH 6.0 sodium citrate buffer at 95 °C for 20 minutes, followed by washing with 1x TBST buffer for 5 minutes. In order to permeabilize the cell membrane, the sections were treated with 0.1 M TBS buffer containing 0.2% Triton X-100 for 20 minutes, then blocked with a solution containing 0.1 M TBST, 2% BSA, 2% serum, and 10% Avidin D for 20 minutes, and washed with 0.1 M TBST buffer. The primary antibody was diluted in 0.1 M TBST buffer containing 2% BSA, 2% serum, and 10% Biotin and incubated overnight at 4 °C in a wet

chamber. The sections were then washed twice with 1x TBS buffer for 10 minutes each. Thereafter, the sections were treated with 0.1 M TBS and 0.3% H_2O_2 solution for 15 minutes and washed twice.

The sections were treated with the secondary antibody diluted in 0.1 M PBS buffer and reacted at room temperature for 1 hour. After completion of the reaction, the sections were washed twice with 0.1 M TBS buffer. The sections were incubated with diluted Vectastain Elite ABC reagent (Vector laboratories) at room temperature for 1 hour, washed twice with 0.1 M TBS buffer for 5 minutes each, treated with DAB solution, and reacted for 5 minutes. Finally, the sections were washed twice with distilled water, counterstained with hematoxylin, and washed with distilled water for 10 minutes. Dehydration was performed in 70% ethanol for 1 minute, 95% ethanol for 2 minutes three times, 100% ethanol for 2 minutes three times, and then treated in xylene for 3 minutes three times. Finally, the slides were embedded using the diaminobenzidine (DAB) method with Permount.

As a result, the chronic renal failure-induced group (Control IgG) showed a marked progression of fibrosis compared to the normal group (Sham). On the other hand, it was confirmed that the MT-101 (IgG1-LALA) administration group showed a significant reduction in fibrosis progression and expression of marker proteins compared to the chronic renal failure-induced group (Figure 16c).

Example 11.3. Analysis of effect of anti-Tie2 antibody in adenine-induced chronic renal failure animal model

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above was also analyzed in a chronic renal failure animal model induced by administrating adenine.

Specifically, 8-week-old C57BL/6J mice were randomly divided into five groups, and each group was administered with adenine (Sigma, A5665) orally daily at a dose of 50 mg/kg for 15 days. At this time, Control IgG (10 mg/kg) or MT-101 (2 mg/kg, 5 mg/kg, or 10 mg/kg) was administered intravenously via the tail vein at 100 μL on days 3, 8, 13, and 18. Twenty-one days after the first adenine administration, the mice were sacrificed, and the serum was obtained using the same method as in Example 9.1 above, and serum creatinine levels were measured. In addition, in order to confirm the inhibition of the expression of KIM-1 and NGAL (neutrophil gelatinase-associated lipocalin), factors associated with renal tubular injury, real-time polymerase chain reaction was performed using the same method as in Example 9.5 above (Figure 17a).

As a result, serum creatinine concentrations were reduced in the MT-101 (IgG1-LALA) administration group (Figure 17b), and the gene expression analysis of KIM-1 or NGAL confirmed that tubular injury was inhibited (Figure 17c).

In addition, the inhibitory effect on kidney and tubular injury was assessed through histological analysis.

Specifically, the kidneys were extracted from the mice sacrificed on day 21, the section slides were prepared using the same method as in Example 9.2 above, and then renal tubular injury was observed using hematoxylin-eosin staining (Figure 17d).

Basement membrane injury in the glomeruli and tubules was confirmed using the following method.

The kidney tissue section slides were deparaffinized, hydrated in distilled water, and then immersed in a periodic acid solution for 10 minutes. Thereafter, the sections were washed four times with distilled water and immersed in Schiff's solution for 30 minutes. Thereafter, the section slides were washed twice with distilled water, stained with hematoxylin for 1 minute, dehydrated with ethanol, and then embedded in xylene (Figure 17e). Vacuolization of the renal cortex, leukocyte infiltration in the tubules/proximal tubules, and proximal tubule simplification were assessed using the following tubular injury score: 0: Normal, 1: 25% or less injury, 2: 25% to 50% injury, 3: 50% to 75% injury, and 4: 75% or more injury.

As a result, it was confirmed that adenine-induced kidney injury was evident in the Control IgG administration group, while the MT-101 (IgG1-LALA) administration group showed concentration-dependent inhibition of renal cortex and tubular injury (Figures 17d and 17e).

Example 11.4. Analysis of fibrosis inhibitory efficacy of anti-Tie2 antibody in adenine-induced chronic renal failure model

The renal fibrosis inhibitory efficacy of anti-Tie2 antibody was confirmed through Sirus red staining and immunostaining for fibrosis marker proteins (collagen IV, α-SMA). Specifically, the kidneys were extracted from the laboratory mice in Example 11.3 above, and Sirus red staining (Figure 18a) and immunostaining for fibrosis marker proteins, collagen IV (Figure 18b) or α-SMA (Figure 18c), were performed using the same method as in Example 11.2 above.

As a result, it was confirmed that in the Control IgG administration group, where chronic renal failure was induced by adenine, fibrosis in the renal glomeruli and tubules was increased compared to the normal group (sham), whereas fibrosis was reduced in the MT-101 (IgG1-LALA) administration group (Figures 18a to 18c).

In addition, the antifibrotic efficacy of MT-101 (IgG1-LALA) was analyzed using real-time polymerase chain reaction.

Specifically, the kidneys were extracted from the laboratory mice of Example 11.3 above, and the gene expression levels of Col1a1, Col3a1, ACTA2, and TGF-β in each group were measured through polymerase chain reaction using the same method as in Example 9.5 above.

As a result, it was confirmed that consistent with the results observed in the immunostaining (Figures 18a to 18c), the MT-101 (IgG1-LALA) administration group showed a concentration-dependent decrease in the expression of fibrosis marker proteins (Figure 18d).

Example 11.5. Analysis of the vascular injury inhibition and stabilization efficacy of anti-Tie2 antibody in adenine-induced chronicrenal failure model

In order to confirm the inhibitory efficacy of anti-Tie2 antibody on chronic renal failure, immunostaining was performed for LTL, a tubular marker protein, and CD31, a vascular endothelial cell marker protein, in kidney tissue.

Specifically, the kidneys were extracted from the laboratory mice of Example 11.3 above and fixed in 4% paraformaldehyde for 2 hours. Thereafter, the sections were immersed in a PBS buffer containing 15% sucrose and reacted at 4 °C for 2 hours. The sections were then dehydrated by immersing in a PBS buffer containing 30% sucrose at 4 °C for 24 hours. Then, a frozen block was prepared using a cryogenic embedding agent (OCT). The frozen block was sectioned into 7 μm thick sections, fixed on slides with 4% paraformaldehyde for about 5 minutes, washed three times with a washing buffer (2% FBS + 0.1% sodium azide in PBS), and then blocked with a nonspecific protein blocking buffer (1% BSA in PBS) for 1 hour.

Thereafter, the sections were treated with primary antibody (anti-LTL antibody (Verot laboratories, RL-1321-2, 1:400) or anti-CD31 antibody (Sigma, MAB1398Z, 1:200)) and reacted at 4 °C for 16 hours. After completion of the reaction, the sections were washed three times with washing buffer, and then treated with anti-LTL antibody (1:1,000) or secondary antibody labeled with Alexa Flour594 (Alexa Flour594-labeled anti-hamster IgG (Invitrogen, A21113), 1:1,000). The sections were reacted at room temperature for 2 hours. Thereafter, the sections were again washed twice with washing buffer, treated with DAPI (1 μg/mL), and reacted at room temperature for 1 minute to stain cell nuclei. After completion of the reaction, the sections were washed twice with washing buffer and tissue sections were observed under a fluorescence microscope

As a result, it was confirmed that renal tubular injury and renal vascular injury was inhibited in the MT-101 (IgG1-LALA) administration group (Figure 19a).

Example 11.6. Analysis of inflammation inhibitory efficacy of anti-Tie2 antibody

Chronic renal failure and fibrosis are exacerbated by persistent inflammatory responses in the kidney. Therefore, real-time polymerase chain reaction (PCR) was performed to confirm whether the expression of inflammation-related factors was inhibited in the MT-101 (IgG1-LALA) administration group.

Specifically, the kidneys were extracted from the laboratory mice of Example 11.3 above, and the gene expression levels of inflammatory inducers MCP-1 (Monocyte chemoattractant protein-1), TNF-α (Tumor necrosis factor-α), and IL-1β; and immune cell

markers CD4, CD11c, F4/80, and CD206 were measured through polymerase chain reaction using the same method as in Example 9.5 above.

As a result, it was confirmed that the MT-101 (IgG1-LALA) group showed a significant decrease in the expression of inflammatory inducers and immune cell markers compared to the Control IgG administration group (Figure 19b).

Example 11.7. Analysis of therapeutic efficacy of anti-Tie2 antibody in unilateral ureteral obstruction-induced kidney injury animal model

The anti-Tie2 antibody prepared in Example 1.4 above was further confirmed to have an inhibitory efficacy on kidney injury in a complete unilateral ureteral obstruction (UUO) mouse model.

Specifically, 8-week-old C57BL/6J mice were randomly divided into groups. A solution containing a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg), a general anesthetic for animals, was injected intraperitoneally (0.2 μL/g) to anesthetize the mice. Under anesthesia, the abdominal skin of the mice was incised approximately 1.5 cm. The left ureter was exposed through a lateral incision using a thin cotton swab soaked in PBS and precision iris forceps, and ligated using two sutures at the level of the inferior pole of the kidney. Control IgG (5 mg/kg) or MT-101 (5 mg/kg) was intravenously injected at 100 μL each on days 2, 4, and 6 after surgery. On day 7 after surgery, the mice were sacrificed, blood and urine samples were collected, and kidney tissue was extracted (Figure 20a).

The extracted kidney tissues were stained with hematoxylin-eosin reagent using the same method as in Example 9.2 above. Thereafter, the renal tubular injury was observed, and the injury score was evaluated as follows: 0: Normal, 1: 25% or less injury, 2: 25% to 50% injury, 3: 50% to 75% injury, 4: 75% or more injury.

As a result, it was confirmed that the tubular injury was significantly more pronounced in the Control IgG administration group compared to the normal group (Sham), whereas the tubular injury was reduced in the MT-101 (IgG1-LALA) administration group (Figure 20b).

Example 11.8. Analysis of fibrosis inhibitory efficacy of anti-Tie2 antibody in unilateral ureteral obstruction-induced kidney injury animal model

The renal fibrosis inhibitory efficacy of MT-101 was confirmed through immunostaining in an animal model of kidney injury induced by unilateral ureteral obstruction (UUO). Specifically, Masson's trichrome staining (Figure 20c) and immunostaining for collagen IV (Figure 20d) or α-SMA (Figure 20e) were performed on the kidney tissues extracted from the laboratory mice of Example 11.7 above using the same method as in Example 11.2 above.

As a result, it was confirmed that fibrosis was increased and the expression of collagen IV and α-SMA was increased in the Control IgG administration group compared to the normal

group (sham), whereas it was reduced in the MT-101 (IgG1-LALA) administration group compared to the Control IgG administration group (Figures 20c to 20e).

Example 12. Confirmation of therapeutic effect of anti-Tie2 antibody on sepsis

The sepsis therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above was confirmed in a lipopolysaccharide (LPS)-induced sepsis mouse model.

Specifically, 8-week-old C57BL/6J mice were randomly divided into groups and administered with a single intraperitoneal injection of LPS (Sigma, L3024) at a dose of 10 mg/kg. One hour before or one hour after LPS administration, 100 μL of Control IgG (10 mg/kg) or MT-101 (IgG1-LALA) (5 mg/kg or 10 mg/kg) was injected into the tail vein. The mice were then analyzed for survival for up to 168 hours (7 days) (Figure 21a).

As a result, in the group administered with Control IgG 1 hour after LPS administration, the mortality rate was increased over time, and by day 7 (168 hours) after LPS-induced sepsis, the mouse survival rate was reduced to 43.75%. On the other hand, all mice in the group administered with MT-101 (IgG1-LALA) (10 mg/kg) 1 hour before LPS injection survived. In addition, it was confirmed that in the groups administered with 5 mg/kg or 10 mg/kg of MT-101 (IgG1-LALA) 1 hour after LPS injection, the mouse survival rate was increased to 93.75% and 100%, respectively (Figure 21b).

Example 13. Confirmation of therapeutic effect of anti-Tie2 antibody on critical limb ischemia

Example 13.1. Analysis of therapeutic effect in limb ischemia animal model

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above was confirmed in a limb ischemia animal model.

Specifically, 6-week-old male BALB/CA-nu/nu mice (average weight: 20 g to 24 g) were anesthetized using a respiratory anesthesia system, and the legs of the mice were secured with tape. Using forceps and scissors, a skin incision of approximately 1 cm was made from the knee to the mid-thigh, and a retractor was used to secure visibility of the surgical site. Through the above surgical site, the proximal and distal portions between the femoral artery and the popliteal artery were each ligated with two sutures under a dissecting microscope, occluded, and then resected to block blood flow into the lower extremity.

Thereafter, HBSS solution, Control IgG (5 mg/kg), or anti-Tie2 antibody (MT-100 or MT-101) (1 mg/kg or 5 mg/kg) were injected into the tissue surrounding the ischemic limbs, respectively. The bloodstream flow in the lower extremities of mice was measured using laser doppler perfusion imaging (LDPI, Moor Instruments Ltd). The bloodstream flow was measured using laser doppler imaging on the day of surgery (day 0), days 7, 14, 21, and 28. Perfusion of the ischemic and non-ischemic limbs was calculated based on pixel types generated in the color histogram. At this time, red and blue represent high and low perfusion levels, respectively.

Blood perfusion is expressed as the LDPI index, which represents the ratio of bloodstream to the ischemic and non-ischemic limbs. A preoperative ratio of 100 indicates equal blood perfusion in both paws. The extent of ischemic hindlimb necrosis was measured 28 days after surgery, and the necrosis score was assessed as follows: 0, limb recall; 1, toe amputation; 2, foot amputation; 3, limb amputation.

As a result, it was confirmed that bloodstream flow was significantly improved in the lower extremities of mice administered with MT-100 or MT-101 compared to the lower extremities of the HBSS or Control IgG (5 mg/kg) administration groups (Figures 22a and 22b). In addition, it was confirmed that necrosis was inhibited in the MT-101 treatment group (Figure 22c).

Example 13.2. Confirmation of induction of functional angiogenesis in limb ischemic area

In order to confirm whether the improvement in limb ischemia in Example 13.1 above was due to the induction of functional angiogenesis in the limb ischemic area, the expression of CD31, a vascular endothelial cell marker protein, and α-SMA, a vascular endothelial pericyte marker protein, was confirmed through immunostaining.

Specifically, after ischemia was induced in the laboratory mouse of Example 13.1 above, the lower limb muscles were extracted, and then the section slides were prepared using the same method as in Example 9.3 above. At this time, the produced section slides had a thickness of 7 μm. The section slides were fixed by treating them with 4% paraformaldehyde and reacting for approximately 5 minutes. Thereafter, the sections were washed three times with washing buffer (2% FBS + 0.1% sodium azide in PBS) and then blocked with nonspecific protein blocking buffer (5% BSA in PBS) for 1 hour. The tissue section slides prepared as described above were subjected to immunostaining using the same method as in Example 9.3 above.

At this time, the primary antibody used were anti-CD31 antibody (Sigma, MAB1398Z, 1:200) or anti-α-SMA antibody (Abcam, ab5694, 1:200). The section slides were treated with the primary antibody, reacted at 4 °C for 16 hours, washed three times with washing buffer, and then treated with the secondary antibody at a 1:1,000 dilution, followed by reaction at room temperature for 2 hours. At this time, Cy3-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch, 128-165-160) or TRITC-labeled anti-hamster IgG antibody (Jackson ImmunoResearch, 101-025-165) was used as the secondary antibody. Capillary (CD31) density and α-SMA cell counts were assessed for each mouse using three randomly selected microscopic fields from three consecutive sections of each tissue block.

As a result, it was confirmed that increased angiogenesis was observed in the lower extremities of mice administered with MT-100 or MT-101 compared to the lower extremities of the HBSS solution or Control IgG (5 mg/kg) administration groups. At this time, it was

confirmed that the newly formed blood vessels were well surrounded by vascular pericytes (Figure 22d).

Example 14. Confirmation of therapeutic effect of anti-Tie2 antibody on erectile dysfunction

Example 14.1. Analysis of therapeutic effect in diabetic erectile dysfunction animal model

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above on erectile dysfunction was confirmed using an animal model of diabetic erectile dysfunction induced by streptozotocin (STZ).

Specifically, 8-week-old male C57BL/6J mice (average weight: 20 g to 25 g) were injected intraperitoneally with STZ (0.1 M citrate buffer, pH 4.5, Sigma-Aldrich) for 5 days. After 8 weeks of administration, fasting and postprandial blood glucose levels of mice were measured using an Accu-Chek blood glucose meter (Roche Diagnostics), and mean systolic blood pressure (MSBP) was measured using a noninvasive tail cuff system (Visitech system). In order to confirm the effect of anti-Tie2 antibody (MT-100, MT-101) on improving erectile function, 20 μL of PBS, Control IgG (10 μg), MT-100 (1 μg or 10 μg), or MT-101 (1 μg or 10 μg) were each administered to the corpus cavernosum penis (intracavernous) of STZ-induced diabetic mice using a 30-gauge insulin syringe.

Two weeks later, in order to measure erectile power, the left superficial portion of the lower abdomen of each mouse was opened, allowing for visualization of the corpus cavernosum nerve (penile nerve), located posterolateral to the prostate. In order to electrically stimulate the penile nerve, a platinum electrode was placed on the nerve. Thereafter, electrical stimulation was applied at 5 V and 12 Hz for approximately 1 minute to induce penile erection. At this time, during erection, the intracavernosal pressure (ICP) was measured using a pressure transmitter (BioSpec system) connected to a computer via a catheter inserted into the corpus cavernosum penis.

In Figure 23a, the ICP on the vertical axis represents the pressure inside the penis during erection, and is generally an indicator of erectile power. The horizontal axis represents the time after electrical stimulation, and the 1-minute electrical stimulation period is indicated by a black bar on the horizontal axis. In addition, Figure 23b shows the value calculated by dividing the maximal intracavernosal pressure (maximal ICP) by the mean systolic blood pressure (MSBP). Figure 23c shows the value obtained by dividing the area under the intracavernosal pressure curve (Total ICP (area under the curve)) by the mean systolic blood pressure (MSBP). At this time, blood pressure is a value checked to measure erectile power because it can affect the intracavernosal pressure.

As a result, it was confirmed that the group administered with MT-100 (10 μg) or MT-101 (1 μg or 10 μg) showed a higher erectile power improvement effect compared to the PBS

or Control IgG treatment group, and erectile power was restored to approximately 90% or more of the level of the non-diabetic group (Normal). In particular, the maximum intracavernosal pressure and the area of the penile pressure curve related to penile erection were improved to normal levels in the anti-Tie2 antibody (MT-100 and MT-101) administration groups (Figures 23a to 23c).

Example 14.2. Analysis of efficacy of inducing functional angiogenesis and stabilization in corpus cavernosum penis

The penis is composed of corpus cavernosum tissue, which is responsible for penile erection, and small blood vessels that supply nutrients to it. However, under pathological conditions such as diabete mellitus, hyperlipidemia, or nerve injury, these blood vessels become dysfunctional and unstable, leading to erectile dysfunction.

In order to confirm whether the erectile-improving effect of the anti-Tie2 antibody described in Example 14.1 above was induced by functional angiogenesis and stabilization in the corpus cavernosum penis, the level of blood vessels in the corpus cavernosum penis was assessed using immunostaining for the expression of CD31, a vascular endothelial cell marker protein, and NG2, a vascular endothelial pericyte marker protein.

Specifically, the dorsal penile nerve and corpus cavernosum penis were isolated from penile tissue of each mouse, and then each tissue was fixed in 4% paraformaldehyde at 4 °C for 24 hours. The fixed tissue was fixed using a cryogenic embedding agent and then sectioned into 7 μm thick sections using a cryosectioner to prepare tissue section slides. Next, the prepared tissue sections were fixed on slides with 4% paraformaldehyde for approximately 5 minutes and then washed three times with washing buffer (2% FBS + 0.1% sodium azide in PBS). The tissue section slides were then treated with nonspecific protein blocking buffer (5% BSA in PBS) and blocked for 1 hour.

Thereafter, the sections were treated with anti-CD31 antibody (Sigma, MAB1398Z, 1:200) or NG2 antibody (Sigma, ZRB5320, 1:100) as a primary antibody and reacted at 4 °C for 16 hours. After completion of the reaction, the sections were washed three times with washing buffer, and then treated with secondary antibodies labeled with FITC or TRITC (FITC-labeled anti-hamster IgG (Invitrogen, A21110), Alexa Fluor 594-labeled anti-rabbit IgG (Invitrogen, A21207), each 1:1,000). The sections were reacted at room temperature for 2 hours. Thereafter, the sections were again washed twice with washing buffer, treated with DAPI (4',6-diamidino-2-phenylindole; 2 μg/mL), and reacted at room temperature for 5 minutes to stain cell nuclei. After completion of the reaction, the sections were washed twice with washing buffer and tissue sections were observed under a fluorescence microscope.

As a result, among the group of mice with diabete mellitus (DM) induced by streptozotocin (STZ), it was confirmed that the blood vessel distribution and coverage of vascular pericytes in the corpus cavernosum penis of the group administered with PBS or

Control IgG (10 μg) were reduced compared to the normal group (Normal), whereas in the group administered with MT-100 (10 μg) or MT-101 (1 μg or 10 μg), the blood vessel distribution and coverage of vascular pericytes were restored to similar levels as in the normal group (Figures 24a to 24c).

Example 14.3. Analysis of efficacy of inducing angiogenesis and stabilizing network structure under hyperglycemic conditions

Angiogenesis is induced by the proliferation, mutual migration, and differentiation of vascular endothelial cells into tubular structures, ultimately maintaining the network. At this time, when vascular endothelial cells are exposed to various conditions (hypoxia or hyperglycemia), they become abnormal, inhibiting their differentiation into lumen and disrupting cell-cell junctions, which are crucial for network formation.

In order to verify the effect of inducing functional angiogenesis by the anti-Tie2 antibody identified in Example 14.2 above, vascular endothelial cells were exposed to hyperglycemia, and the tube formation-inducing and stabilizing efficacy of MT-100 or MT-101 were analyzed.

Specifically, HUVECs were treated with 5 mM glucose, similar to normal blood glucose level, and 30 mM glucose, corresponding to hyperglycemia level, and cultured for 48 hours. At this time, the cells were treated with PBS, Control IgG (5 μg/mL), or anti-Tie2 antibody (MT-100 (1 μg/mL, 10 μg/mL) or MT-101 (0.1 μg/mL, 1 μg/mL, 5 μg/mL) along with the glucose. Thereafter, each HUVEC (2 x 10^5 cells) was seeded onto a 24-well plate coated with matrigel and observed under a microscope while incubating for 16 hours at 37 °C in a 5% CO_2 incubator.

As a result, the degree of blood vessel formation (tube formation) was significantly reduced in the high-concentration glucose (HG)-only treatment group (HG-PBS or HG-Control IgG) compared to the normal concentration glucose (NG) treatment group. On the other hand, it was confirmed that the high-concentration glucose (HG) and MT-100 (10 μg/mL) or MT-101 (1 μg/mL or 5 μg/mL) combination treatment group showed a significant increase in blood vessel formation compared to the normal concentration glucose (NG) treatment group, and that the network was well maintained (Figures 25a and 25b).

Example 15. Confirmation of therapeutic effect of anti-Tie2 antibody on retinal disease

Example 15.1. Analysis of therapeutic effect in laser-induced choroidal neovascularization animal model

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above for age-related macular degeneration (AMD) was confirmed using a laser-induced neovascularization (CNV) mouse model.

Specifically, 7-week-old C57BL6 mice were locally anesthetized with 0.5% proparacaine hydrochloride (Alcon) in the eyes. Then, the pupils were dilated with 1% tropicamide (Santen Pharmaceuticals). Using a laser photocoagulator, laser photocoagulation (wavelength 532 nm, diameter 50 μm, duration 80 mS, power level 200 mW) was used to rupture Bruch's membrane in four areas (3, 6, 9, and 12 o'clock positions on the posterior pole) of each eye. When the laser was irradiated onto the retinal surface, bubbles formed on the retinal surface due to burns to the retinal tissue. Mice in which no bubbles were observed were excluded from the study.

Three days after laser photocoagulation, 1 μL each of Control IgG (10 μg), MT-100 (10 μg), MT-101 (10 μg), or the control drug EYLEA (10 μg) was injected intravitreally into the mouse vitreous cavity. Seven days after laser photocoagulation, indocyanine green angiography (ICGA) was performed to observe abnormal choroidal neovascularization. At this time, fluorescein angiography (FA) was also performed to analyze choroidal neovascular leakage. Eight days after laser photocoagulation, each mouse eye was extracted, fixed in 4% paraformaldehyde at room temperature for 1 hour, and then washed three times with PBS buffer (Figure 26a).

Thereafter, under a dissecting microscope, a radial incision was made through the cornea using a scalpel. Starting from the incision site, the sclera was carefully peeled away toward the optic nerve with forceps, and the lens was finally removed. The retina was cut into a four-leaf clover shape and permeabilized in PBST (0.5% Triton X-100 in PBS) buffer for 16 hours at 4 °C to prepare tissue slides. Immunohistochemical staining was performed.

The permeabilized retinas were washed three times with PBS buffer and then blocked in PBST-BSA (0.5% Triton X-100 + 3% BSA in PBS) solution for 16 hours at 4 °C. The retinas were treated with anti-endomucin antibody (Santacruz, SC-65495, 1:200) used as the primary antibody and reacted at 4 °C for 16 hours. Thereafter, the retinas were washed four times with PBST buffer at 20-minute intervals, treated with secondary antibody (Cy3-labeled anti-antibody, 1:100), and reacted at room temperature for 1 hour. The tissue slides were washed four times with PBST buffer at 20-minute intervals, treated with hydrosoluble mounting medium, and then observed using a confocal microscope.

The yellow dotted area in Figure 27a represents the mass of CNV, and the white dotted area represents the area of CNV. The volume of CNV can be calculated using the following mathematical equation 1:

[Mathematical equation 1]

CNV Volume = CNV Mass size x z-Stack height (z-Scan 1 μm) x 2/3 (Hemisphere coefficient)

As a result, the CNV area and choroidal vascular leakage were significantly reduced in the MT-100 or MT-101 administration group. In particular, it was confirmed that the reduction effect was similar to that in the control drug EYLEA administration group (Figures

26b and 26c). In addition, tissue Endomucin expression results confirmed that the MT-100 or MT-101 administration group shows a decrease in the area, mass, or volume of CNV (Figures 27a to 27d).

Example 15.2. Analysis of therapeutic effect in oxygen-induced retinopathy animal model

The therapeutic efficacy of the anti-Tie2 antibody prepared in Example 1.4 above in ischemic retinal diseases including retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and retinal vein occlusion (RVO) was analyzed using an oxygen-induced retinopathy (OIR) mouse model.

Specifically, 7-day-old C57BL/6J mice were exposed to a hyperbaric oxygen chamber maintained at 75% oxygen for 5 days. On day 12, the mice were removed from the hyperbaric oxygen chamber and then injected intravitreally with 1 μL of Control IgG (10 μg), MT-101 (10 μg), or the control drug EYLEA (10 μg). The mice were maintained under normal atmospheric conditions (21% oxygen concentration) for 5 days (Figure 28a). On day 17, the eyes were extracted from the mice, fixed in 4% paraformaldehyde at room temperature for 1 hour, and then washed three times with PBS buffer. The retinas were extracted using the same method as in Example 15.1 above, and ocular tissue slides were prepared and immunohistochemical staining was performed. At this time, anti-CD31 antibody was used as the primary antibody. The tissues stained using the above method were observed using a confocal microscope.

As a result, it was confirmed that the MT-101 (10 μg) administration group showed a significant reduction in the avascular area (Figure 28b) and neovascular tuft area (Figure 28c) within the retina compared to the control drug EYLEA administration group.

Example 15.3. Analysis of efficacy of inhibiting retinal vascular leakage

In retinal diseases associated with angiogenesis, abnormalities in retinal blood vessels increase permeability, leading to leakage into surrounding tissues. Therefore, the inhibitory effect of the anti-Tie2 antibody according to the present invention on retinal vascular leakage was confirmed using Evans Blue staining in the mouse model described in Example 15.2 above.

Specifically, Control IgG or MT-101 antibody was injected intraocularly into mice using the same method as described in Example 15.2 above. On day 17, Evans Blue dye was administered into the femoral vein under a microscope. Two hours later, the eyes were extracted and fixed in 4% paraformaldehyde for 1 hour. The fixed tissue was treated with a cryogenic embedding agent (OCT) to prepare a frozen block. The frozen block was sectioned into 10 μm thick sections to prepare tissue section slides. The tissue section slides were then fixed by treatment with 4% paraformaldehyde for approximately 5 minutes. The tissue section slides were observed using a laser scanning confocal microscope to analyze the sensitivity of Evans Blue dye leakage around retinal blood vessels.

As a result, it was confirmed that leakage around retinal blood vessels was significantly reduced in the MT-101- administration group compared to the Control IgG administration group (Figures 29a and 29b).

II. Bispecific antibody

Example 16. Production of bispecific antibody for Tie2 activation and VEGF signaling pathway inhibition

A bispecific antibody with triple functions was produced, activating vascular endothelial cell-specific Tie2 and its downstream signaling factors, while inhibiting Ang-2 binding to Tie2 and VEGF or VEGFR2 signaling pathways.

Example 16.1. Design of bispecific antibody construct

In order to activate Tie2 activation, inhibit Ang-2 binding, and inhibit the VEGF signaling pathway, a fusion protein or bispecific antibody construct was designed by introducing five types of VEGF signaling pathway inhibitors (SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46) into the C-terminal site of the Fc region using the MT-101 antibody as a backbone (Figure 30).

Specifically, the following antibodies were designed: MT-103-1 fusion protein (SEQ ID NO: 42) in which Aflibercept VEGFR1-D2 and VEGFR2-D3 domains were introduced; MT-103-2 bispecific antibody (SEQ ID NO: 43) in which Bevacizumab heavy chain variable region and light chain variable region were introduced in scFv form; MT-103-3 bispecific antibody (SEQ ID NO: 44) in which Ranibizumab heavy chain variable region and light chain variable region were introduced in scFv form; MT-103-4 bispecific antibody (SEQ ID NO: 45) in which Brolucizumab light chain variable region and heavy chain variable region were introduced in scFv form; and MT-103-5 (SEQ ID NO: 46) in which Ramucirumab heavy chain variable region and light chain variable region were introduced in scFv form.

At this time, reverse translation was performed with reference to the amino acid sequence of each of the five VEGF signaling pathway inhibitors, and then the codon optimization process was performed to express it in Expi293F cells, and then the coding gene was synthesized. The five synthesized VEGF signaling pathway inhibitor genes were cloned into the C-terminal site of the pcDNA3.4 (Invitrogen)-based MT-101 heavy chain expression vector and introduced as a linker comprising the amino acid sequence of SEQ ID NO: 49 or SEQ ID NO: 50 into MT-101 IgG. As a control bispecific antibody, the heavy chain variable region (SEQ ID NO: 40) of p2.3 was cloned into the heavy chain variable region (SEQ ID NO: 27) of MT-101-1 to construct a heavy chain expression vector.

Table 12 shows the CDR amino acid sequences of the bispecific antibodies, and Tables 13 to 18 show the amino acid sequences of the heavy chains of MT-103-1, MT-103-2, MT-103-3, MT-103-4, and MT-103-5, the amino acid sequence of the common MT-103 light chain, and

the amino acid sequence of the heavy chain of the control p2.3-1. The light chain of the control p2.3-1 fusion protein is described in SEQ ID NO: 41. Transient expression of the fusion protein or bispecific antibody was performed using the constructed heavy and light chain expression vectors, respectively.

[Table 12]

Antibody	Sequence information	Sequence	SEQ ID NO
MT-103-1	MT-103-1-HCDR1	GFTFNSYG	1
	MT-103-1-HCDR2	TSNDGSTT	2
	MT-103-1-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-103-1-LCDR1	QSVSSY	4
	MT-103-1-LCDR2	GAS	5
	MT-103-1-LCDR3	QQYGTTPYT	6
MT-103-2	MT-103-2-HCDR1	GFTFNSYG	1
	MT-103-2-HCDR2	TSNDGSTT	2
	MT-103-2-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-103-2-LCDR1	QSVSSY	4
	MT-103-2-LCDR2	GAS	5
	MT-103-2-LCDR3	QQYGTTPYT	6
MT-103-3	MT-103-3-HCDR1	GFTFNSYG	1
	MT-103-3-HCDR2	TSNDGSTT	2
	MT-103-3-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-103-3-LCDR1	QSVSSY	4
	MT-103-3-LCDR2	GAS	5
	MT-103-3-LCDR3	QQYGTTPYT	6
MT-103-4	MT-103-4-HCDR1	GFTFNSYG	1
	MT-103-4-HCDR2	TSNDGSTT	2
	MT-103-4-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-103-4-LCDR1	QSVSSY	4
	MT-103-4-LCDR2	GAS	5
	MT-103-4-LCDR3	QQYGTTPYT	6
MT-103-5	MT-103-5-HCDR1	GFTFNSYG	1
	MT-103-5-HCDR2	TSNDGSTT	2
	MT-103-5-HCDR3	ARKVVRGYHYHDAFDI	19
	MT-103-5-LCDR1	QSVSSY	4

	MT-103-5-LCDR2	GAS	5
	MT-103-5-LCDR3	QQYGTTPYT	6

[Table 13]

MT-103-1			Amino acid sequence	SEQ ID NO
MT-103-1 HC	MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
		VH1	EVQLVESGGGVVQPGRSLRLSCAASGFTFNS YGMQWVRQAPGKGLEWVAVTSNDGSTTYY ADSVKGRFTISRDNSKNTLYLQMNSLRSEDT AVYYCARKVVRGYHYHDAFDIWGQGTMVT VSS	27
		CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKV	52
		first linker	EPKSCDKTHTCPPCP	53
		Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	63
	VEGF specific binding receptor fragment	second linker	GGGGSGGGGSGGGGS	49
		D2 domain	SDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVT SPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIIS NATYKEIGLLTCEATVNGHLYKTNYLTHRQT NTIID	67
		D3 domain	VVLSPSHGIELSVGEKLVLNCTARTELNVGID FNWEYPSSKHQHKKLVNRDLKTQSGSEMKK FLSTLTIDGVTRSDQGLYTCAASSGLMTKKNS TFVRVHEK	68
	signal peptide		MGWSCIILFLVATATGVHS	51

MT-103-1 LC	VL1	DIQMTQSPATLSLSPGERATLSCRASQSVSSYL AWYRQKPGQAPRLLIYGASIRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVFYCQQYGTTPYTF GQGTKVEIK	22
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	82

[Table 14]

MT-103-2			Amino acid sequence	SEQ ID NO
MT-103-2 HC	MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
		VH1	EVQLVESGGGVVQPGRSLRLSCAASGFTFNS YGMQWVRQAPGKGLEWVAVTSNDGSTTYY ADSVKGRFTISRDNSKNTLYLQMNSLRSEDT AVYYCARKVVRGYHYHDAFDIWGQGTMVT VSS	27
		CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKV	52
		first linker	EPKSCDKTHTCPPCP	53
		Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	63
	VEGF specific binding antibody fragment	second linker	GGGGSGGGGSGGGGSKL	50
		VH2	EVQLVESGGGLVQPGGSLRLSCAASGYTFTN YGMNWVRQAPGKGLEWVGWINTYTGEPTY AADFKRRFTFSLDTSKSTAYLQMNSLRAEDT AVYYCAKYPHYYGSSHWYFDVWGQGTLVT VSS	69

		third linker	SGGGGSGGGGSGGGGS	70
		VL2	DIQMTQSPSSLSASVGDRVTITCSASQDISNYL NWYQQKPGKAPKVLIYFTSSLHSGVPSRFSG SGSGTDFTLTISSLQPEDFATYYCQQYSTVPW TFGQGTKVEIK	71
MT-103-2 LC	signal peptide		MGWSCIILFLVATATGVHS	51
	VL1		DIQMTQSPATLSLSPGERATLSCRASQSVSSYL AWYRQKPGQAPRLLIYGASIRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVFYCQQYGTTPYTF GQGTKVEIK	22
	CL		RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	82

[Table 15]

MT-103-3			Amino acid sequence	SEQ ID NO
MT-103-3 HC	MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
		VH1	EVQLVESGGGVVQPGRSLRLSCAASGFTFNS YGMQWVRQAPGKGLEWVAVTSNDGSTTYY ADSVKGRFTISRDNSKNTLYLQMNSLRSEDT AVYYCARKVVRGYHYHDAFDIWGQGTMVT VSS	27
		CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKV	52
		first linker	EPKSCDKTHTCPPCP	53
		Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	63

	VEGF specific binding antibody fragment	second linker	GGGGSGGGGSGGGGSKL	50
		VH2	EVQLVESGGGLVQPGGSLRLSCAASGYDFTH YGMNWVRQAPGKGLEWVGWINTYTGEPTY AADFKRRFTFSLDTSKSTAYLQMNSLRAEDT AVYYCAKYPYYYGTSHWYFDVWGQGTLVT VSS	72
		third linker	GGGGSGGGGSGGGGS	49
		VL2	DIQLTQSPSSLSASVGDRVTITCSASQDISNYL NWYQQKPGKAPKVLIYFTSSLHSGVPSRFSG SGSGTDFTLTISSLQPEDFATYYCQQYSTVPW TFGQGTKVEIK	73
MT-103-3 LC	signal peptide		MGWSCIILFLVATATGVHS	51
	VL1		DIQMTQSPATLSLSPGERATLSCRASQSVSSYL AWYRQKPGQAPRLLIYGASIRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVFYCQQYGTTPYTF GQGTKVEIK	22
	CL		RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	82

[Table 16]

MT-103-4			Amino acid sequence	SEQ ID NO
MT-103-4 HC	MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
		VH1	EVQLVESGGGVVQPGRSLRLSCAASGFTFNS YGMQWVRQAPGKGLEWVAVTSNDGSTTYY ADSVKGRFTISRDNSKNTLYLQMNSLRSEDT AVYYCARKVVRGYHYHDAFDIWGQGTMVT VSS	27
		CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKV	52
		first linker	EPKSCDKTHTCPPCP	53

		Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	63
	VEGF specific binding antibody fragment	second linker	GGGGSGGGGSGGGGS	49
		VH2	EVQLVESGGGLVQPGGSLRLSCTASGFSLTDY YYMTWVRQAPGKGLEWVGFIDPDDDPYYAT WAKGRFTISRDNSKNTLYLQMNSLRAEDTAV YYCAGGDHNSGWGLDIWGQGTLVTVSS	74
		third linker	GGGGGSGGGGSGGGGSGGGGS	75
		VL2	EIVMTQSPSTLSASVGDRVIITCQASEIIHSWL AWYQQKPGKAPKLLIYLASTLASGVPSRFSG SGSGAEFTLTISSLQPDDFATYYCQNVYLAST NGANFGQGTKLTVL	76
MT-103-4 LC	signal peptide		MGWSCIILFLVATATGVHS	51
	VL1		DIQMTQSPATLSLSPGERATLSCRASQSVSSYL AWYRQKPGQAPRLLIYGASIRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVFYCQQYGTTPYTF GQGTKVEIK	22
	CL		RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	82

[Table 17]

MT-103-5			Amino acid sequence	SEQ ID NO
MT-103-5 HC	MT-101 HC	signal peptide	MGWSCIILFLVATATGVHS	51
		VH1	EVQLVESGGGVVQPGRSLRLSCAASGFTFNS YGMQWVRQAPGKGLEWVAVTSNDGSTTYY ADSVKGRFTISRDNSKNTLYLQMNSLRSEDT AVYYCARKVVRGYHYHDAFDIWGQGTMVT VSS	27

		CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKV	52
		first linker	EPKSCDKTHTCPPCP	53
		Fc(CH2+CH3)	APEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	63
	VEGF specific binding antibody fragment	second linker	GGGGSGGGGSGGGGSKL	50
		VH2	EVQLVQSGGGLVKPGGSLRLSCAASGFTFSS YSMNWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNAKNSLYLQMNSLRAEDTAV YYCARVTDAFDIWGQGTMVTVSS	77
		third linker	GGGGSGGGGSGGGGS	49
		VL2	DIQMTQSPSSVSASIGDRVTITCRASQGIDNW LGWYQQKPGKAPKLLIYDASNLDTGVPSRFS GSGSGTYFTLTISSLQAEDFAVYFCQQAKAFP PTFGGGTKVDIK	78
MT-103-5 LC	signal peptide		MGWSCIILFLVATATGVHS	51
	VL1		DIQMTQSPATLSLSPGERATLSCRASQSVSSYL AWYRQKPGQAPRLLIYGASIRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVFYCQQYGTTPYTF GQGTKVEIK	22
	CL		RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	82

[Table 18]

Antibody Name	Class	Amino acid sequence	SEQ ID NO

MT-103-1	MT-103-1-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGM QWVRQAPGKGLEWVAVTSNDGSTTYYADSVKGR FTISRDNSKNTLYLQMNSLRSEDTAVYYCARKVVR GYHYHDAFDIWGQGTMVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGGGGGSGGGGSGGGGSSDTGRPFVEMYSEIPEII HMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGK RIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTN YLTHRQTNTIIDVVLSPSHGIELSVGEKLVLNCTAR TELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGS EMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKN STFVRVHEK	42
	MT-103-1-LC	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWY RQKPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVFYCQQYGTTPYTFGQGTKVEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C	47
MT-103-2	MT-103-2-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGM QWVRQAPGKGLEWVAVTSNDGSTTYYADSVKGR FTISRDNSKNTLYLQMNSLRSEDTAVYYCARKVVR GYHYHDAFDIWGQGTMVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK	43

		GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGGGGGSGGGGSGGGGSKLEVQLVESGGGLVQPG GSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWV GWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQM NSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQG TLVTVSSSGGGGSGGGGSGGGGSDIQMTQSPSSLS ASVGDRVTITCSASQDISNYLNWYQQKPGKAPKV LIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYCQQYSTVPWTFGQGTKVEIK	
	MT-103-2-LC	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWY RQKPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVFYCQQYGTTPYTFGQGTKVEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C	47
MT-103-3	MT-103-3-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGM QWVRQAPGKGLEWVAVTSNDGSTTYYADSVKGR FTISRDNSKNTLYLQMNSLRSEDTAVYYCARKVVR GYHYHDAFDIWGQGTMVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGGGGGSGGGGSGGGGSKLEVQLVESGGGLVQPG GSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWV GWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQM NSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQG TLVTVSSGGGGSGGGGSGGGGSDIQLTQSPSSLSAS VGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIY	44

		FTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATY YCQQYSTVPWTFGQGTKVEIK	
	MT-103-3-LC	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWY RQKPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVFYCQQYGTTPYTFGQGTKVEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C	47
MT-103-4	MT-103-4-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGM QWVRQAPGKGLEWVAVTSNDGSTTYYADSVKGR FTISRDNSKNTLYLQMNSLRSEDTAVYYCARKVVR GYHYHDAFDIWGQGTMVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGGGGGSGGGGSGGGGSKLEIVMTQSPSTLSASV GDRVIITCQASEIIHSWLAWYQQKPGKAPKLLIYLA STLASGVPSRFSGSGSGAEFTLTISSLQPDDFATYYC QNVYLASTNGANFGQGTKLTVLGGGGGSGGGGS GGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCTAS GFSLTDYYYMTWVRQAPGKGLEWVGFIDPDDDP YYATWAKGRFTISRDNSKNTLYLQMNSLRAEDTAV YYCAGGDHNSGWGLDIWGQGTLVTVSS	45
	MT-103-4-LC	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWY RQKPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVFYCQQYGTTPYTFGQGTKVEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C	47

MT-103-5	MT-103-5-HC	EVQLVESGGGVVQPGRSLRLSCAASGFTFNSYGM QWVRQAPGKGLEWVAVTSNDGSTTYYADSVKGR FTISRDNSKNTLYLQMNSLRSEDTAVYYCARKVVR GYHYHDAFDIWGQGTMVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGGGGGSGGGGSGGGGSKLEVQLVQSGGGLVKPG GSLRLSCAASGFTFSSYSMNWVRQAPGKGLEWVS SISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNS LRAEDTAVYYCARVTDAFDIWGQGTMVTVSSGGG GSGGGGSGGGGSDIQMTQSPSSVSASIGDRVTITCR ASQGIDNWLGWYQQKPGKAPKLLIYDASNLDTG VPSRFSGSGSGTYFTLTISSLQAEDFAVYFCQQAKA FPPTFGGGTKVDIK	46
	MT-103-5-LC	DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWY RQKPGQAPRLLIYGASIRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVFYCQQYGTTPYTFGQGTKVEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C	47
p2.3	p2.3-VEGFR1D2-VEGFR2D3-HC	DVQLVESGGGVVRPGESLRLSCTASGFTFDSYGMS WVRQAPGKGLEWVSSINVGDN TYYADSVRGRFIISRDSSRNTLYLQMNSLTAEDTAV YYCANWNSFFDYWGLGTLVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF PEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV	48

		VVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGGGGG SGGGGSGGGGSKLSDTGRPFVEMYSEIPEIIHMTE GRELVIPCRVTSPNITVTLKK FPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEA TVNGHLYKTNYLTHRQTN TIIDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDF NWEYPSSKHQHKKLVNRDL KTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSG LMTKKNSTFVRVHEK	

Hereinafter, a bispecific fusion protein comprising a VEGF receptor is also described as a bispecific antibody.

Example 16.2. Expression and purification of bispecific antibody

The produced MT-103-1, MT-103-2, MT-103-3, MT-103-4 or MT-103-5 heavy chain expression vector and MT-103 light chain expression vector were introduced into Expi293F or ExpiCHO cells using the same method as in Example 1.4 above, thereby carrying out transient expression and purification of antibodies. The final concentration of the purified bispecific antibody in PBS buffer was measured by absorbance or BCA assay, and then analyzed by SDS-PAGE under non-reducing and reducing conditions.

As a result, it was confirmed that MT-103-1 had a heavy chain of about 80 kDa, a light chain of about 25 kDa, and a total molecular weight of about 210 kDa. In addition, it was confirmed that MT-103-2, MT-103-3, MT-103-4, or MT-103-5 had a heavy chain of about 75 kDa, a light chain of about 25 kDa, and a total molecular weight of about 200 kDa, respectively.

Example 16.3. Confirmation of target affinity of bispecific antibody

The target affinity of the bispecific antibody (MT-103-1 or MT-103-4) was analyzed using the BLI technique using the same method as in Example 2 above.

Specifically, in order to measure the affinity for Tie2, the AR2G biosensor tip was hydrated in third-pass distilled water (deionized water) for 10 minutes and then activated using a buffer comprising 20 mM EDC and 10 mM Sulfo-NHS. Thereafter, 100 nM hTie2-ECD-6x His protein was diluted in immobilization buffer (10 mM sodium acetate, pH 6.0) and reacted

with the activated AR2G tip for 10 minutes to immobilize the antigen on the AR2G tip. Thereafter, the antigen immobilization reaction was terminated by treatment with a 1 M ethanolamine (pH 8.5) solution, and the AR2G tip was reacted in PBS buffer for 60 seconds to establish a baseline of antigen-antibody binding.

Thereafter, 60 nM MT-103-1 or MT-103-4 was allowed to associate and dissociate with the hTie2-ECD-6xHis antigen-immobilized AR2G tip for 800 seconds, respectively. In order to measure the affinity for VEGF, 200 nM MT-103-1 or MT-103-4 was immobilized on the AR2G tip as described above, and then 60 nM recombinant human VEGF (hVEGF) protein was allowed to associate and dissociate with the bispecific antibody-immobilized tip for 800 seconds, respectively. Based on the reaction curves identified through the association and dissociation reaction analysis, the K_{on}, K_{off}, and K_D values were calculated through global fitting analysis using the Data Analysis HT 12.0 program, thereby measuring the affinity of MT-103-1 or MT-103-4 to each antigen. The results are shown in Table 1.

As a result, it was confirmed that MT-103-1 and MT-103-4 antibodies exhibited high affinity for hTie2 and hVEGF (Table 19).

[Table 19]

Antigen	Antibody	K_D (M)	K_{on} (1/Ms)	K_{off} (1/s)
human Tie2	MT-103-1	7.92E-11	1.92E05	1.52E-05
	MT-103-4	5.58E-10	1.49E05	8.32E-05
human VEGF	MT-103-1	<1.00E-12	1.78E05	<1.00E-07
	MT-103-4	<1.00E-12	1.02E05	<1.00E-07

Example 17. Confirmation of activation of bispecific antibody

The activation of the bispecific antibody was confirmed through analysis of phosphorylation-inducing activity of Tie2 and its downstream signaling factors in vascular endothelial cells, inhibition of apoptosis and vascular endothelial permeability, inhibition of VEGF-VEGFR2 signaling pathway, and inhibition of Ang-2 binding to Tie2.

Example 17.1. Confirmation of VEGF signaling pathway inhibitory efficacy of bispecific antibody

The VEGF signaling pathway inhibitory efficacy of the bispecific antibody prepared by the method of Example 16.2 above was analyzed by comparing the level of phosphorylation of VEGFR2 and its downstream signaling factors by VEGF in vascular endothelial cells with that of the control drug (Aflibercept: EYLEA, Bevacizumab: Avastin, or Brolucizumab: Beovu).

Specifically, HUVECs (5 x 10^5 cells) were cultured in a 60 mm culture dish at a temperature of 37 °C in EGM-2 medium. The HUVECs cultured as described above were cultured in M199 medium containing 1% serum for 6 hours to achieve serum starvation.

Thereafter, bispecific antibodies (MT-103-1, MT-103-2, MT-103-3, MT-103-4) or control drugs (EYLEA, Avastin, or Beovu) and VEGF (at different concentrations as shown in Figures 31a to 31e) were mixed and reaction for 20 minutes. The mixture was then applied to the HUVECs and reacted for 15 minutes.

Thereafter, the cells were lysed using lysis buffer, proteins were extracted, and SDS-PAGE was performed on these proteins. Then, the proteins were transferred to PVDF membranes, and the membranes were blocked with 0.1% Tween-20 TBST buffer containing 3% BSA for 2 hours at room temperature. Thereafter, the membrane was treated with primary antibodies diluted 1:1,000 and reacted at room temperature for 2 hours. At this time, the primary antibodies used were anti-phospho-VEGFR2 antibody (Cell signaling, 3770), anti-phospho-AKT antibody, and anti-phospho-ERK antibody. Thereafter, the membrane was treated with secondary antibody (peroxidase-conjugated) diluted 1:3,000 and reacted at room temperature for 1 hour. After completion of the reaction, the membrane was treated with ECL solution to confirm the phosphorylation level of VEGF signaling pathway factors.

As a result, MT-103-1 or MT-103-4 significantly reduced the VEGFR2 phosphorylation to a level equivalent to that of the respective control drugs, EYLEA or Beovu (Figures 31a and 31d). In the MT-103-2, MT-103-3, or MT-103-5 treatment groups, the VEGFR2 phosphorylation was reduced in a concentration-dependent manner (Figures 31b, 31c, and 31e). In addition, in the bispecific antibody treatment group, the phosphorylation of AKT or ERK was significantly increased, indicating that the bispecific antibody activated the Tie2 signaling pathway (Figures 31a to 31e).

Example 17.2. Confirmation of Tie2 signaling pathway activation by bispecific antibody

We compared and analyzed whether the bispecific antibodies prepared using the method described in Example 16.2 above induced the phosphorylation of Tie2 and its downstream signaling factors in vascular endothelial cells. The experiment was conducted using the same method as in Example 3 above.

Specifically, HUVECs (3 x 10^6 cells) were cultured in a 100 mm culture dish at a temperature of 37 °C in EGM-2 medium. The HUVECs cultured as described above were cultured in M199 medium containing 1% serum for 6 hours to achieve serum starvation. Thereafter, the cells were treated with anti-Tie2 antibody (MT-101) or bispecific antibody (MT-103-1 or MT-103-4) at the concentrations (0.5 nM, 1 nM, 5 nM, or 10 nM) shown in Figure 32a and then reacted for 20 minutes. Thereafter, the cells were lysed with lysis buffer to obtain cell lysates. Using the cell lysates as samples, immunoprecipitation and western blotting were performed using the same method as in Example 3 above.

As a result, the bispecific antibody MT-103-1 induced the phosphorylation of Tie2, AKT, or ERK proteins, similar to the monoclonal antibody MT-101 (Figure 32a). In addition,

MT-103-4 exhibited phosphorylation-inducing activity similar to that of MT-103-1 (Figure 32b).

Example 17.3. Confirmation of efficacy of bispecific antibody in inhibiting vascular endothelial cell injury and permeability

The vascular endothelial cell activation efficacy of the bispecific antibody prepared in Example 16.2 above was confirmed by analyzing apoptosis of endothelial cells induced when cultured under serum-free and hypoxic conditions and VEGF-induced vascular endothelial cell permeability.

Specifically, HUVECs (5 x 10^4 cells) were cultured in a 24-well plate in EGM-2 medium at 37 °C for 48 hours. The following day, HUVECs were treated with the anti-Tie2 antibody (MT-101) and the bispecific antibody (MT-103-1 or MT-103-4) at the concentrations shown in Figure 24a (1 nM or 10 nM) in serum-free M199 medium and cultured for 40 hours under hypoxic conditions (<1% oxygen) at 37 °C. Thereafter, the surviving cells were immobilized and stained with Crystal Violet reagent for 10 minutes. The cells were washed four times with distilled water, and then the stained reagent was eluted with distilled water containing 1% SDS, and the absorbance value was measured.

As a result, it was confirmed that the MT-103-1 or MT-103-4 treatment group inhibited apoptosis to a level equivalent to or greater than that of the MT-101 treatment group (Figure 33a).

HREC cells (1 x 10^5 cells) were seeded in a 35 mm confocal dish (SPL), cultured for 2 days, and then maintained in a serum-starved state for 4 hours. Thereafter, the cells were co-treated with VEGF (1 nM) and Control IgG (2 nM) or bispecific antibody (MT-103-1 or MT-103-4) at different concentrations (0.125 nM, 0.5 nM, 2 nM, 8 nM, and 32 nM) and then reacted for 30 minutes. Thereafter, the cells were fixed with 4% paraformaldehyde for 10 minutes and the cell membrane was permeabilized for 30 minutes using a PBS solution containing 0.2% Triton X-100. Thereafter, the cells were blocked with a PBS solution containing 3% BSA for 1 hour at room temperature. The cells were treated with anti-VE-cadherin antibody (R&D, 1:100) and reacted at room temperature for 2 hours. Thereafter, the cells were treated with a fluorescently labeled secondary antibody (Alexa Fluor 488 donkey anti-goat, Invitrogen) diluted 1:200, and then reacted at room temperature for 1 hour. The cells were treated with DAPI diluted 1:1,000 to stain the cell nuclei for 3 minutes, and then observed under a fluorescence microscope.

As a result, it was confirmed that cell-to-cell junctions were formed significantly weaker in the groups treated with VEGF and Control IgG. On the other hand, it was confirmed that the groups treated with MT-103-1 or MT-103-4 in combination with VEGF showed well-maintained cell-cell junctions and strong development of cell-cell interfaces (Figure 33b). In

addition, it was confirmed that this pattern was increased with increasing treatment concentration for both bispecific antibodies (Figure 33c).

In addition, HREC cells (1 x 10^5 cells) were seeded in a 12-transwell plate, cultured in EGM-2 medium at 37 °C for 72 hours, and then cultured in EGM-2 medium without VEGF at 37 °C for 48 hours. The cells were treated with a 40 kDa FITC-dextran solution at a concentration of 200 ng/mL and reacted at 37 °C for 30 minutes. Thereafter, the initial fluorescence leakage was measured. The cells were treated with VEGF, Ang-2, and Control IgG (5 nM) or bispecific antibody (MT-103-1 or MT-103-4) at different concentrations (0.05 nM, 0.5 nM, and 5 nM). At the completion of treatment, the cells were treated with a 40 kDa FITC-dextran solution at a concentration of 200 ng/mL and reacted at 37 °C for 30 minutes. Thereafter, the fluorescence leakage was measured 24 hours later, and the change compared to the initial measurement value was calculated.

As a result, it was confirmed that all groups treated with MT-103-1 or MT-103-4 showed a decrease in relative fluorescence leakage compared to the VEGF, Ang-2, and Control IgG treatment groups. At this time, the group treated with 5 nM bispecific antibody, VEGF and Ang-2 exhibited the highest inhibitory efficacy. In particular, it was confirmed that the inhibitory efficacy of MT-103-4 was superior to that of MT-103-1 (Figure 33d).

Example 18. Confirmation of triple efficacy of bispecific antibody

In a pathological situation where both VEGF and Ang-2 were increased, the triple efficacy (Tie2 binding, inhibition of Ang-2 binding to Tie2, and VEGF binding) of MT-103-1 or MT-103-4 was confirmed using the BLI technique described in Example 2 above.

Specifically, the AR2G biosensor tip was hydrated in third-pass distilled water (deionized water) for 10 minutes and then activated using a buffer comprising 20 mM EDC and 10 mM Sulfo-NHS. Thereafter, 200 nM MT-103-1, MT-103-4, or the p2.3-1 control bispecific antibody was reacted for 10 minutes to immobilize the antibody on the tip. In order to analyze the Tie2 binding affinity after antibody immobilization, 100 nM hTie2-ECD-6xHis protein was allowed to associate and dissociate for 600 seconds, respectively. In order to analyze the Ang-2 binding inhibition capacity, the tip was again allowed to associate and dissociate with 40 nM recombinant Ang-2 protein for 600 seconds, respectively. In the final step, to evaluate the VEGF binding affinity, the tip was treated with 60 nM hVEGF protein and allowed to associate and dissociate for 600 seconds, respectively. The association and dissociation reactions were analyzed through global fitting using the Data Analysis HT 12.0 program.

As a result, MT-103-1 and MT-103-4 bound to Tie2 and inhibited the binding of Ang-2 to Tie2. In addition, they also bound to hVEGF. The control p2.3-1 bispecific antibody bound to Tie2 and hVEGF, but did not inhibit the binding of Ang-2 to Tie2 (Figure 34).

Example 19. Confirmation of efficacy of bispecific antibody in activating the Tie2 signaling pathway and inhibiting the VEGF signaling pathway

We confirmed that the bispecific antibody activates Tie2 expressed in vascular endothelial cells while inhibiting VEGF, under conditions where Ang-1, Ang-2, or VEGF is expressed.

Specifically, HUVECs (3 x 10^6 cells) were seeded in a 100 mm culture dish and cultured for 24 hours, then maintained in serum-free M199 medium for 6 hours. Then, MT-103-1 (1 nM or 5 nM) or MT-103-4 (1 nM or 5 nM) was added to the medium containing Ang-2 (5 nM) and VEGF (1 nM) or Ang-1 (5 nM), Ang-2 (5 nM) and VEGF (1 nM), and the cells were allowed to react for 10 minutes. The cells were treated for 15 minutes under the same conditions as in Figure 35a, and then lysed with lysis buffer to obtain cell lysates.

The cell lysates obtained as described above were treated with anti-Tie2 antibody (R&D) at a concentration of 1 μg/mL and reacted at 4 °C for 12 hours. Thereafter, immunoprecipitation was performed by adding 30 μL of protein G beads. Western blotting was performed using the same method as in Example 3 above using the sample prepared as described above. At this time, the primary antibody used was anti-phospho-tyrosine antibody, anti-phospho-VEGFR2 antibody, anti-phospho-AKT antibody, or anti-phospho-ERK antibody diluted at a ratio of 1:1,000.

As a result, it was confirmed that MT-103-1 or MT-103-4 strongly induced the phosphorylation of Tie2, AKT, or ERK proteins in the presence of Ang-1, Ang-2, and VEGF, while inhibiting the phosphorylation of VEGFR2 (Figure 35b).

Example 20. Comparison of Tie2 activation and VEGF inhibition efficacy of bispecific antibody and control drug

The efficacy of the bispecific antibody prepared in Example 16.2 above and the bispecific antibody (Faricimab: Vabysmo), currently used as a therapeutic agent, that simultaneously inhibits Ang-2 and VEGF, was compared.

Specifically, HUVECs (3 x 10^6 cells) were seeded in a 60 mm culture dish, cultured for 24 hours, and then maintained in serum-free M199 medium for 6 hours. Then, the cells were treated with MT-103-1, MT-103-4, or Vabysmo at different concentrations (0.5 nM, 1 nM, 2 nM, 5 nM, or 10 nM) for 15 minutes. Thereafter, the cells were lysed with lysis buffer, and western blotting was performed using the same method as in Example 17.1 above.

As a result, the control drug, Vabysmo, did not induce the phosphorylation of Tie2 downstream signaling pathway factors (AKT and ERK). On the other hand, MT-103-1 or MT-103-4 induced the phosphorylation of Tie2 downstream signaling pathway factors (AKT and ERK). In addition, it was confirmed that the phosphorylation of VEGFR2 was equally inhibited at a concentration about 8 times lower for MT-103-1 and about 20 times lower for MT-103-4 compared to Vabysmo (Figure 36a).

In addition, as a result of analyzing the apoptosis inhibition efficacy using the same method as in Example 17.3 above, it was confirmed that MT-103-1 or MT-103-4 effectively reduced the apoptosis of HUVECs induced when cultured in serum-free and hypoxic conditions. On the other hand, no apoptotic effect was observed by Vabysmo (Figure 36b).

Example 21. Confirmation of therapeutic effect of bispecific antibody on retinal disease

Example 21.1. Analysis of choroidal neovascularization formation inhibitory efficacy in laser-induced choroidal neovascularization animal model

The therapeutic efficacy for age-related macular degeneration (AMD) was confirmed using a laser-induced neovascularization (CNV) mouse model. In this mouse model, neovascularization was quantitatively analyzed using VE-cad-creERT^2-tdTomato mice (Max Plank Institute and Jackson Laboratories), which express tdTomato fluorescent labels in vascular endothelial cells by tamoxifen.

Specifically, 7-week-old VE-cad-creERT^2-tdTomato mice were locally anesthetized with 0.5% proparacaine hydrochloride (Alcon) in the eyes. Then, the pupils were dilated with 1% tropicamide (Santen Pharmaceuticals). Using a laser photocoagulator, laser photocoagulation (wavelength 532 nm, diameter 50 μm, duration 80 mS, power level 200 mW) was used to rupture Bruch's membrane in four areas (3, 6, 9, and 12 o'clock positions on the posterior pole) of each eye. When the laser was irradiated onto the retinal surface, bubbles formed on the retinal surface due to burns to the retinal tissue. Mice in which no bubbles were observed were excluded from the study.

Three days after laser photocoagulation, 1 μL each of Control IgG (20 μg), MT-101 (20 μg), MT-103-1 (20 μg), MT-103-4 (20 μg), or the control drug EYLEA (10 or 20 μg) was injected intravitreally into the mouse vitreous cavity. Seven days after laser photocoagulation, indocyanine green angiography (ICGA) was performed to observe abnormal choroidal neovascularization. At this time, fluorescein angiography (FA) was also performed to analyze choroidal neovascular leakage (Figure 37a).

Eight days after laser photocoagulation, each mouse eye was extracted, fixed in 4% paraformaldehyde at room temperature for 1 hour, and then washed three times with PBS buffer. Thereafter, under a dissecting microscope, a radial incision was made through the cornea using a scalpel. Starting from the incision site, the sclera was carefully peeled away toward the optic nerve with forceps, and the lens was finally removed. The retina was cut into a four-leaf clover shape. After some tissue was collected for Western blot analysis, the remaining tissue was permeabilized in PBST (0.5% Triton X-100 in PBS) at 4 °C for 16 hours to prepare tissue slides. The permeabilized retinas were washed three times with PBS buffer, treated with hydrosoluble mounting medium, and then observed using a confocal microscope.

As a result, choroidal vascular leakage was significantly reduced in the anti-Tie2 antibody (MT-101) or bispecific antibody (MT-103-1 or MT-103-4) administration groups. In particular, it was confirmed that this reduction was more pronounced than in the group administered with two concentrations of the control drug EYLEA (Figure 37b). In addition, it was confirmed that the CNV vascular area expressing the dtTomato fluorescent label within the tissue was significantly reduced in the bispecific antibody administration group (MT-103-1 or MT-103-4) compared to the control drug EYELA administration group (Figure 37c).

Example 21.2. Analysis of inhibitory efficacy of pre-formed choroidal neovascularization in laser-induced choroidal neovascularization animal model

The inhibitory efficacy of pre-formed choroidal neovascularization was confirmed in a laser-induced neovascularization (CNV) mouse model using the same method as in Example 21.1 above. In this mouse model, neovascularization was quantitatively analyzed using VE-cad-creERT^2-tdTomato mice, which express the tdTomato fluorescent label in vascular endothelial cells by tamoxifen.

In this experiment, seven days after laser photocoagulation, indocyanine green angiography (ICGA) was performed to observe abnormal choroidal neovascularization. At this time, fluorescein angiography (FA) was also performed to analyze choroidal neovascular leakage. Thereafter, 1 μL of Control IgG (20 μg), MT-103-1 antibody (20 μg), MT-103-4 antibody (20 μg), or the control drug EYLEA (10 or 20 μg) was injected intravitreally into the mouse vitreous cavity. ICGA and FA were performed 14 days after laser photocoagulation. Data were compared with data obtained 7 days after photocoagulation to assess the degree of inhibition of leakage caused by unstable neovascularization after laser photocoagulation and its efficacy in reducing the area of pre-formed choroidal neovascularization.

As a result, choroidal vascular leakage and choroidal neovascularization were significantly reduced in the bispecific antibody (MT-103-1 or MT-103-4) administration group. In particular, it was confirmed that the reduction effect was equivalent to or greater than that of the control drug EYLEA (two concentrations) administration group (Figure 38b). In addition, it was confirmed that the CNV vascular area expressing the dtTomato fluorescent label in the tissue was significantly reduced in the bispecific antibody (MT-103-1 or MT-103-4) administration group compared to the control drug EYELA administration group (Figure 38c).

Example 21.3. Analysis of therapeutic effect in oxygen-induced retinopathy animal model

The therapeutic efficacy of the bispecific antibody (MT-103-1 or MT-103-4) in ischemic retinal diseases including retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and retinal vein occlusion (RVO) was analyzed using a high oxygen-induced retinopathy (OIR) mouse model.

Specifically, 7-day-old C57BL/6J mice were exposed to hyperoxia by maintaining the oxygen concentration at 75% in a hyperbaric oxygen chamber for 5 days. On day 12, the mice were removed from the hyperbaric oxygen chamber and then injected intravitreally with 1 μL of Control IgG (20 μg), MT-103-1 antibody (20 μg), MT-103-4 antibody (20 μg), or the control drug EYLEA (10 μg or 20 μg). The mice were maintained under general atmospheric conditions (21% oxygen concentration) for 5 days. On day 17, the eyes were extracted from the mice, fixed in 4% paraformaldehyde at room temperature for 1 hour, and then washed three times with PBS buffer. The retinas were extracted using the same method as in Example 15.1 above, and ocular tissue slides were prepared and immunostaining was performed. At this time, anti-CD31 antibody was used as the primary antibody. The tissues stained using the above method were observed using a confocal microscope (Figure 39a).

As a result, the area of intraretinal ischemic neovascularization was significantly reduced in the MT-103-1 or MT-103-4 administration group compared to the control drug EYELA administration group. In addition, it was confirmed that the area of pathological neovascular tuft was significantly reduced in the MT-103-1 administration group compared to EYELA (Figures 39b and 39c).

Claims

[Claim 1]

An antibody or fragment thereof that specifically binds to Tie2, comprising:

(a) a heavy chain variable region (VH) comprising i) a heavy chain complementarity determining region 1 (HCDR1) represented by the amino acid sequence of SEQ ID NO: 84; ii) a heavy chain complementarity determining region 2 (HCDR2) represented by the amino acid sequence of SEQ ID NO: 85; and iii) a heavy chain complementarity determining region 3 (HCDR3) represented by any one amino acid sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 15, and SEQ ID NO: 86; and

(b) a light chain variable region (VL) comprising iv) a light chain complementarity determining region 1 (LCDR1) represented by the amino acid sequence of SEQ ID NO: 87; v) a light chain complementarity determining region 2 (LCDR2) represented by the amino acid sequence of SEQ ID NO: 88; and vi) a light chain complementarity determining region 3 (LCDR3) represented by the amino acid sequence of SEQ ID NO: 89:

$X_1X_2X_3FX_4X_5X_6X_7$ (SEQ ID NO: 84)

wherein X_1 is G or S, X_2 is F or D, X_3 is T or S, X_4 is N, T or A, X_5 is S or G, X_6 is Y, N, or Q, and X_7 is G, W, or A;

$X_8X_9X_{10}DX_{11}X_{12}X_{13}X_{14}$ (SEQ ID NO: 85)

X_8 is T or I, X_9 is S or Y, X_{10} is N, P, or W, X_{11} is G, D, or S, X_{12} is S or G, X_{13} is T, D, or G, and X_{14} is T or I;

$ARKVVRGYX_{15}X_{16}HDAFDI$ (SEQ ID NO: 86)

X_{15} is S, H, or L, and X_{16} is Y or P;

$QX_{17}X_{18}X_{19}SX_{20}$ (SEQ ID NO: 87)

X_{17} is S, G, or D, X_{18} is V or I, X_{19} is S or D, and X_{20} is Y or N;

$X_{21}X_{22}S$ (SEQ ID NO: 88)

X_{21} is G or A, and X_{22} is A or S;

$QQX_{23}X_{24}X_{25}X_{26}PX_{27}T$ (SEQ ID NO: 89)

X_{23} is Y, A, or G, X_{24} is G, N, or Y, X_{25} is T, S, or D, X_{26} is T or F, and X_{27} is Y, L, or W.

[Claim 2]

The antibody or fragment thereof that specifically binds to Tie2 according to claim 1, wherein the antibody or fragment thereof comprises:

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 86; and

a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6:

ARKVVRGYX$_{15}$X$_{16}$HDAFDI (SEQ ID NO: 86)

wherein X$_{15}$ is S, H, or L, and X$_{16}$ is Y or P.

[Claim 3]

The antibody or fragment thereof that specifically binds to Tie2 according to claim 1, wherein the antibody comprises:

a heavy chain variable region comprising HCDR1 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13, HCDR2 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 8, and SEQ ID NO: 14, and HCDR3 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 19, and SEQ ID NO: 20; and

a light chain variable region comprising LCDR1 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 10, and SEQ ID NO: 16, LCDR2 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 11, and SEQ ID NO: 17, and LCDR3 comprising any one amino acid sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 12, and SEQ ID NO: 18.

[Claim 4]

The antibody or fragment thereof that specifically binds to Tie2 according to claim 1, wherein the antibody comprises:

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6;

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 7, HCDR2 comprising the amino acid sequence of SEQ ID NO: 8, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 10, LCDR2 comprising the amino acid sequence of SEQ ID NO: 11, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 12;

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 13, HCDR2 comprising the amino acid sequence of SEQ ID NO: 14, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 15, and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 16, LCDR2 comprising the amino acid sequence of SEQ ID NO: 17, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 18;

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 19, and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

a heavy chain variable region comprising HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, and HCDR3 comprising the amino acid sequence of SEQ ID NO: 20, and a light chain variable region comprising LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

[Claim 5]

The antibody or fragment thereof that specifically binds to Tie2 according to claim 4, wherein the antibody comprises:

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 21 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 26;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 28 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 22.

[Claim 6]

A bispecific antibody comprising the anti-Tie2 antibody or fragment thereof according to claim 1; and a VEGF-specific binding site.

[Claim 7]

The bispecific antibody according to claim 6, wherein the VEGF-specific binding site is any one selected from the group consisting of a receptor, a ligand, an antibody, and a fragment thereof.

[Claim 8]

The bispecific antibody according to claim 7, wherein the VEGF-specific binding site is a receptor.

[Claim 9]

The bispecific antibody according to claim 8, wherein the receptor comprises the amino acid sequence of SEQ ID NO: 83.

[Claim 10]

The bispecific antibody according to claim 7, wherein the VEGF-specific binding site is an antibody or a fragment thereof.

[Claim 11]

The bispecific antibody according to claim 10, wherein the antibody or fragment thereof comprises:

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 69 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 71;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 72 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 74 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 76; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 77 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78.

[Claim 12]

The bispecific antibody according to claim 6, wherein the anti-Tie2 antibody or fragment thereof, and the VEGF-specific binding site are linked via a linker.

[Claim 13]

The bispecific antibody according to claim 6, wherein the bispecific antibody is a fusion protein dimer comprising the following structural formulae (I) and (II):

N'-X'-[L1]p-Fc region or variant thereof-[L2]q-Y-C' (I); and

N'-X''-C' (II)

wherein, in the structural formulae (I) and (II),

N' is the N-terminus,

C' is the C-terminus,

- represents a bond,

X' is the heavy chain variable region (VH) and heavy chain constant region (CH1) of the anti-Tie2 antibody according to claim 1,

X" is the light chain variable region (VL) and light chain constant region (CL) of the anti-Tie2 antibody according to claim 1,

X' and X" combine to form a binding site (X) that specifically binds to Tie2,

Y is a VEGF-specific binding site,

L1 and L2 are peptide linkers, and

p and q are each independently 0 or 1.

[Claim 14]

A polynucleotide encoding the antibody or fragment thereof according to claim 1; or the bispecific antibody according to claim 6.

[Claim 15]

An expression vector comprising the polynucleotide according to claim 14.

[Claim 16]

A cell transformed with the vector according to claim 15.

[Claim 17]

A method for producing an anti-Tie2 antibody or fragment thereof, or a bispecific antibody, comprising:

i) culturing the transformed cell according to claim 16; and

ii) obtaining an anti-Tie2 antibody or fragment thereof, or a bispecific antibody from the cell culture solution.

[Claim 18]

A pharmaceutical composition for preventing or treating a vascular abnormality-related disease, comprising the anti-Tie2 antibody or fragment thereof according to claim 1, or the bispecific antibody according to claim 6 as an active ingredient.

[Claim 19]

The pharmaceutical composition according to claim 18, wherein the vascular abnormality-related disease is an angiogenesis abnormality-related disease or a disease related to structural and/or functional alterations of blood vessels.

[Claim 20]

The pharmaceutical composition according to claim 19, wherein the angiogenesis abnormality-related disease is caused by an excessive increase, decrease, or deficiency.

[Claim 21]

The pharmaceutical composition according to claim 19, wherein the disease related to structural and/or functional alterations of blood vessels is caused by any one abnormality selected from the group consisting of structural abnormalization of blood vessels, vascular dysfunction, and vascular injury.

[Claim 22]

The pharmaceutical composition according to claim 18, wherein the vascular abnormality-related disease is any one selected from the group consisting of acute kidney injury, chronic renal failure, macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vessel occlusion, proliferative retinopathy, retinopathy of prematurity, corneal graft rejection, glaucoma, critical limb ischemia, diabetic erectile dysfunction, sepsis, acute respiratory distress syndrome, vasculitis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, thrombosis, occlusion, cancer, systemic erythremia, psoriasis, hemophilic arthritis, associated sclerosis, capillary formation of atherosclerotic plaques, keloid, wound granulation, vascular adhesion, rheumatoid arthritis, osteoarthritis, autoimmune disease, Crohn's disease, restenosis, atherosclerosis, intestine adhesion, cat scratch disease, ulcer, liver cirrhosis, nephritis, diabetic nephropathy, diabete mellitus, inflammatory disease, and neurodegenerative disease.

[Claim 23]

A use of the anti-Tie2 antibody or fragment thereof according to claim 1; or the bispecific antibody according to claim 6 for the prevention or treatment of a vascular abnormality-related disease.

[Claim 24]

A method for preventing or treating a vascular abnormality-related disease, comprising administering to a subject the anti-Tie2 antibody or fragment thereof according to claim 1; or the bispecific antibody according to claim 6.