CANCER TREATMENT COMPOSITION FOR INHIBITING TUMOR ANGIOGENESIS, CONTAINING VEGF DEEP BLOCKER, AND PREPARATION METHOD THEREFOR

The present invention relates to a cancer treatment composition for inhibiting angiogenesis, and a preparation method therefor. An angiogenesis inhibitor according to the present invention is a cancer treatment composition containing a fusion protein comprising a vascular endothelial growth factor-binding domain of vascular endothelial growth factor receptor 1 (VEGFR1) and a b1 domain of neuropilin-1 (NRP1). The novel fusion protein is an angiogenesis inhibitor for blocking the binding of VEGF to a receptor thereof in the cell membrane, and has an effect of inhibiting the proliferation of cancer cells and the growth and metastasis of cancer. In addition, the fusion protein can be usable as an anti-cancer agent and exhibits an effective anti-cancer effect at a dose lower than that of a conventional angiogenesis inhibitor.

FIG. 1

Veep

VEGFR1

Nrp1

VEGFR1 lg2

Nrp1 b1

Fc

VEGF

Veep
Description

Technical Field

[0001] The present invention relates to a novel angiogenesis inhibiting fusion protein that inhibits cancer growth and metastasis, and a method for producing the same.

Background Art

[0002] Angiogenesis is an essential process for the proper growth and repair of organs and a highly elaborately regulated process. The regulatory imbalance of these processes results in inflammatory, cardiovascular, immune or malignant diseases. Vascular endothelial growth factor A (VEGFA) is a major inducer of angiogenesis and is known to be involved in cancer growth and progression. VEGFA genes comprise 8 exons and produce at least six major VEGFA isoforms, VEGFA-121, VEGFA-145, VEGFA-165, VEGFA-183, VEGFA-189, and VEGFA-206. Among them, three major homologous proteins, VEGFA-121, VEGFA-165, and VEGFA-189 are secreted in cells, and their characteristics, bioavailability, and distribution are different from one another. However, it is regarded that their angiogenic functions are generally regulated by VEGF_{165}.

[0003] VEGFAs bind to two kinds of tyrosine kinase receptors, the VEGF receptor (VEGFR) 1 and VEGFR2, not only to participate in development of neovascularization, but also to prevent apoptosis thereby achieving vascular maintenance. Angiogenesis signaling is mediated by the binding of VEGFA to VEGFR2 (KDR) and a co-receptor, neuropilin-1 (NRP1). Although VEGFR2 is a major receptor involved in angiogenesis and vasculogenesis, VEGFR1 has much higher binding affinity to VEGFA than VEGFR2 does.

[0004] The VEGFA expression level is increased by physiologically necessary conditions such as wound healing and hypoxic conditions, and it is also increased in pathological conditions such as proliferative retinopathy, arthritis, psoriasis, and cancer. Furthermore, VEGFA is an important mediator of tumor vasculogenesis, because it induces new blood vessel growth from peripheral blood vessels, allows cancer cells to obtain oxygen and nutrients, and facilitates cancer metastasis.

[0005] Many anti-VEGF drugs, such as antibody formulations, aptamers and tyrosine phosphorylation inhibitors, have been developed. Recently, aflibercept (VEGF-trap), which is a fusion protein having high affinity to VEGFA, has been highlighted as next-generation drug. This drug consists of the binding sites to VEGFR1 and VEGFR2.

[0006] As related prior arts, Korean Patent Laid-Open No. 1397088 entitled as "A fusion protein for inhibiting both angiogenesis and tumor cell proliferation, and composition comprising the same" discloses a fusion protein comprising an angiogenesis inhibitor and a cancer-specific antibody having treatment effect on gastric cancer or breast cancer.

[0007] In a related literature, Regeneron and Bayer HealthCare have reported the results of Phase III clinical trials of VEGF Trap-Eye (aflibercept; brand name, Eylea®) for macular degeneration, which shows that it can achieve treatment effect with lower doses compared with Ranibizumab.

[0008] Finely et al. developed a mouse model transplanted with human cancer cells for study of cancer characteristics including cancer growth rate and VEGF secretion, to predict VEGF secretion rate using VEGF-trap, which suggests that it can be used as in vivo cancer model at a preclinical stage.

[0009] The present inventors prepared an angiogenesis inhibitor (VEGF Deep Blocker: VEEP) fusion protein, which is a decoy receptor comprising an Ig2 domain of VEGFR1 and a b1 domain of NRP1 so as to bind to VEGFA with higher affinity than VEGF-trap and block VEGFA signal transduction, to complete the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Technical Problem

[0010] It is an object of the present invention to provide a novel angiogenesis-inhibiting fusion protein that inhibits cancer growth and metastasis and a method for preparing the same.

[0011] It is an object of the present invention to provide a recombinant DNA encoding a novel angiogenesis-inhibiting fusion protein that inhibits cancer growth and metastasis.

Technical solution

[0012] To this end, one aspect of the present invention provides a fusion protein characterized by comprising the Ig2 domain of vascular endothelial growth factor receptor 1 (VEGFR1) and the b1 domain of neuropilin 1 (NRP1). The composition comprising the protein is capable of inhibiting cancer growth and metastasis, and can be used for the treatment of cancer.
Effects of the Invention

[0013] The novel fusion protein according to the present invention is an angiogenesis inhibitor that blocks vascular endothelial growth factor from binding to its receptor on the cell membrane, and has an effect of inhibiting cancer cell proliferation, growth, and metastasis. The composition containing the fusion protein can be usefully employed as an anticancer agent. In addition, the composition exhibits a high anticancer effect with a lower dose compared with conventional angiogenesis inhibitors.

Brief Description of Drawings

[0014]

FIG. 1 is a schematic diagram showing a VEGFR1-NRP1-Fc fusion protein.
FIG. 2 shows expression of a VEEP fusion protein.
FIG. 3 show results obtained by comparing anticancer effect of VEEP with that of a control VEGF-trap in LLC mouse model.
FIG. 4 shows results of endpoint analysis for LLC mouse model at 30th day after VEEP treatment.

Mode for the Invention

[0015] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[0016] A first embodiment of the present invention provides a fusion protein characterized by comprising an Ig2 domain of vascular endothelial growth factor receptor 1 (VEGFR1) and a b1 domain of neuropilin 1 (NRP1). More specifically, the vascular endothelial growth factor receptor 1 may be represented by, but is not limited to, SEQ ID NO: 1. The Ig2 domain and the b1 domain of neuropilin 1 (NRP1) may be in various forms, and preferably, the Ig2 domain and the b1 domain of neuropilin 1 (NRP1) may be represented by SEQ ID NO: 2. The fusion protein may further comprise a Fc domain of an immunoglobulin, and various Fc domains can be used. Preferably, the Fc domain may be represented by SEQ ID NO: 3. The fusion protein may further comprise a leader sequence for expression, and various leader sequences can be used. Preferably, the leader sequence may be represented by SEQ ID NO: 4. More preferably, the fusion protein may have the amino acid sequence of SEQ ID NO: 5.

[0017] A second embodiment of the present invention provides a composition for cancer treatment, the composition comprising the above-described fusion protein. Those skilled in the art may add various forms of drug delivery materials, excipients, stabilizers, and the like, to the composition, and these various formulations also fall within the scope of the present invention.

[0018] A third embodiment of the present invention provides a DNA fragment encoding the protein. A person skilled in the art to which the present invention belongs can produce various DNA sequences encoding the fusion protein of the present invention according to the degeneration of the genetic code. Finally, any type of DNA sequence encoding the protein of the present invention may be within the scope of the invention.

[0019] A fourth embodiment of the present invention provides a transformant obtained by transformation/transfection using the above recombinant vector. For the transformant, various cells can be used; preferably human-derived cells and most preferably HEK293E cells can be used.

[0020] A fifth embodiment of the present invention provides a method of preparing a protein, comprising culturing the transformed cell according to the present invention; and separating the fusion protein from the cell culture media.

DETAILED DESCRIPTION OF THE INVENTION

Method

1. Preparation of VEGFR1-NRP1-Fc fusion protein

[0021] The DNA fragment encoding Ig2 domain of VEGFR1 was obtained by PCR of the synthesized DNA (Ezbio; EZbio), and the DNA fragment encoding b1 domain of NRP1 was obtained from the human gene bank of the Korea Research Institute of Bioscience and Biotechnology. The DNA fragment comprising the two fragments was linked to human Fc DNA to generate a fusion protein. The cloned DNA was transfected into HEK293E cells and the culture media was purified using Protein A resin. The concentration of the purified protein was calculated by measuring A280 absorbance.
2. In vitro Affinity Test (ELISA)

[0022] VEEP and VEGF-trap were added to a 96-well plate coated with VEGF. After washing several times, HRP-conjugated anti-human Fc was added and stabilized TMB is added thereto, and then, absorbance was measured at 450 nm.

3. VEEP Animal Test

Target disease and solid tumor animal model

[0023] As an animal model to verify anticancer effect of drugs, six-week-old male C57BL/6 mice were purchased from Koatech (Pyeongtaek City, Korea). The animals were subjected to a week of adaptation period according to the animal experiment ethics regulations. LLC (Lewis lung carcinoma) cell line was purchased from ATCC for the induction of solid tumor in mice.

In vivo Assay of anticancer effect of VEEP and end point

[0024] 1x10^6 LLC cells were subcutaneously injected into right flank region of 6-week-old C57BL/6 mice. 25 mg/kg of VEEP and 25 mg/kg of VEGF-trap was respectively administered subcutaneously every 3 days from 9th day after the LLC injection, and the administration was performed 8 times in total. The proliferation of solid tumors was measured every 3-4 days for 30 days and survival of the solid tumor was observed. The size of the solid tumor was measured using an electronic digital caliper to measure length (major axis) and width (minor axis), orthogonally. The volume of solid tumor was calculated by \( \pi/6 \times (\text{length})^2 \times \text{width} \). On day 30, mice were sacrificed and solid tumors were extracted and weighed using a microbalance.

<Example 1> Fusion protein

[0025] The purified VEGFR1-NRP1-Fc fusion protein was subjected to SDS-PAGE analysis under reducing condition and non-reducing condition, and it was observed that bands for monomer and dimer were shown at the positions corresponding to predicted size (Fig. 2).

<Example 2> Affinity Analysis

[0026] To determine the affinity of VEEP, a fusion protein, to VEGFA, the binding affinity of VEGF to receptor or blocker was analyzed by ELISA analysis. For affinity test, VEGF_{165} was selected among the VEGFA homologous proteins. For controls, comparisons and analyses were conducted using VEGFA receptors naturally expressed in a cell, such as VEGFR1, VEGFR2, NRP1, and NRP2, and commercially available blockers such as VEGF-trap, bevacizumab, and Ranibizumab. The analysis results showed that the binding affinity of VEGF-trap to VEGF_{165} was the highest among the control group. In contrast, VEEP, the fusion protein of the present invention, showed the highest binding affinity to VEGF_{165}, compared to all controls, and showed 10-times higher binding affinity than that of VEGF-trap (Table 1).

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<Example 3> Anticancer Effect Test

[0027] In order to measure the anti-cancer effect of the VEEP fusion protein prepared and purified according to the present invention, measurement of solid tumor growth and observation of survival were conducted using LLC mouse models. As a result of the measurement of solid tumor growth in LLC mice, the average tumor size in control group without blocker treatment was 13105 mm³. In VEGF-trap-treated group, the average tumor size was decreased to 9479 mm³, but the decrease effect showed large difference between individual mouse models. In contrast, the average size of solid tumors of VEEP-treated mice was measured as 5872 mm³, which shows significant increase in the anti-cancer effect compared to the control without the blocker (FIG. 3). In addition, the anti-cancer effect of VEEP was superior to that of VEGF-trap. The survival rate of VEEP fusion protein-treated mice was 80%, which is superior to that of VEGF-trap-treated control (80%) or blocker non-treated control (0%). Therefore, it is believed that the VEEP fusion protein prepared in the present invention can be usefully applied to patients who cannot be treated with VEGF-trap.

<Example 4> End point analysis

[0028] Tumor size and weight were measured at 30th day after treating LLC mice with VEEP. The solid tumor weight (5.15g) of the VEEP fusion protein-treated LLC mouse was significant decreased in compared with that (7.49 g) of VEGF-trap-treated mouse (Fig. 3). The VEGF-trap-treated mice showed a large variation in tumor size. In the VEEP-treated mice, the average tumor size was smaller than that of VEGF-trap-treated mice, and the tumor size decrease degrees of the VEEP-treated mice were similar to one another (Fig. 4). Thus, as in the results of the above anticancer effect analysis, the VEEP blocker of the present invention is considered to be effectively applied to patients who had no therapeutic effect with VEGF-trap.

References

[0029]


Sequence Listing 1: Sequence of VEGFR1 part

VSDTGRPFVEMYSEIEIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLPDGKRII

WDSRKGIISNATYKEIGLTLCEATVNGHLYKTNYLTHRQINT (VEGFR1)

Sequence Listing 2: Sequence of Nrpl part
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vygckit

Sequence Listing 3: Immunglobulin Fc domain

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Sequence Listing 4: Leader sequence
MYLGLNYVFIFLLNGVQS
Sequence Listing 5: Full-length sequence

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EP 3 486 254 A1

<110> IBENTRUS, Inc.

<120> Anticancer composition comprising VEGF Deep Blocker (Veep) suppressing tumor angiogenesis and methods of preparation thereof

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Claims

1. A fusion protein comprising an Ig2 domain of vascular endothelial growth factor receptor 1 (VEGFR1) and a b1 domain of neuropilin 1 (NRP1).

2. The fusion protein of claim 1, which comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3.

3. The fusion protein of claim 3, which further comprises SEQ ID NO: 4.

4. The fusion protein of claim 3, which comprises SEQ ID NO: 5.

5. A composition for cancer treatment, comprising the fusion protein of any one of claims 1 to 4.

6. A DNA fragment encoding the fusion protein of any one of claims 1 to 4.

7. A recombinant vector comprising the DNA fragment of claim 6.

8. A cell transformed with the recombinant vector of claim 7.

9. The cell of claim 6, wherein the cell is HEK293E.

10. A method of preparing a protein for cancer treatment, characterized by

    (a) culturing the transformed cells of claim 8; and

    (b) separating a fusion protein from the culture solution of said cells.
FIG. 1

Veep
FIG. 2

Expression of VEEP Fusion Protein

10% SDS-PAGE

- M
- -DTT
- +DTT

(KDa)
250
150
100
75
50
37
25

Dimer
Monomer
FIG. 3

Anticancer Effect

a  Tumor size

b  Survival

LLC Mouse Model

FIG. 4

End Point Analysis
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

*C07K 14/7551(2006.01)*, *C07K 14/71(2006.01)*, *A61K 38/17(2006.01)*, *C12P 21/00(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

*C07K 14/705; A61P 25/00; A61K 39/35; A61K 38/45; A61K 38/02; C07K 7/06; C07K 16/00; A61K 39/00; A61K 48/00; C12N 9/12; A61K 38/18; C07K 14/71; A61K 38/17; C12P 21/80*

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility models and applications for Utility models: IPC as above

Japanese Utility models and applications for Utility models: IPC as above

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

eKOMPASS (KIPO internal) & Keywords: fusion protein, VEGFR-1, Neuropillin-1, cancer treatment

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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**Date of the actual completion of the international search**

**16 MARCH 2017 (16.03.2017)**

**Date of mailing of the international search report**

**16 MARCH 2017 (16.03.2017)**

**Name and mailing address of the ISA/KR**

Korean Intellectual Property Office

Government Complex Daejeon, 109 Shamin-dong, Daejeon 340-701, Republic of Korea

**Facsimile No.** +82-42-891-8578

**Authority**

Authorized officer

**Telephone No.**

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