



(11) **EP 2 239 325 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
13.10.2010 Bulletin 2010/41

(21) Application number: **09702346.9**

(22) Date of filing: **13.01.2009**

(51) Int Cl.:
C12N 15/09 (2006.01) **A61K 35/76** (2006.01)
A61P 9/10 (2006.01) **A61P 11/00** (2006.01)
A61P 25/00 (2006.01) **A61P 35/00** (2006.01)
C07K 14/03 (2006.01) **C07K 14/47** (2006.01)
C07K 19/00 (2006.01) **C12N 7/00** (2006.01)

(86) International application number:
PCT/JP2009/050299

(87) International publication number:
WO 2009/090933 (23.07.2009 Gazette 2009/30)

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR
Designated Extension States:
AL BA RS

(30) Priority: **16.01.2008 JP 2008007179**

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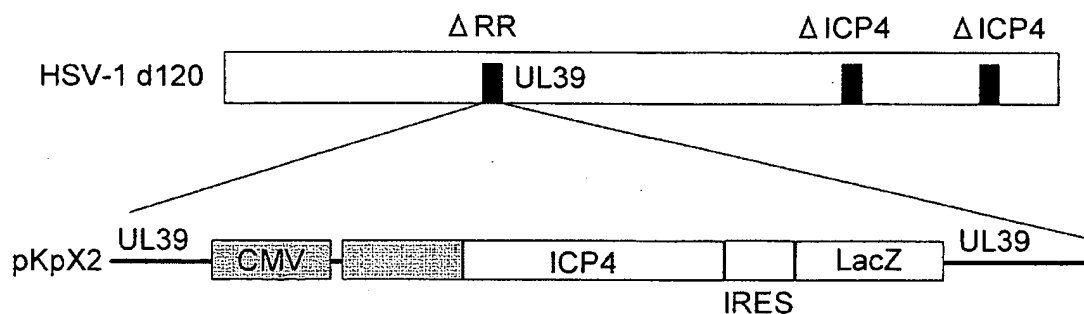
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(54) **VIRUS GROWING IN HYPOXIC CELL OR VIRUS VECTOR EXPRESSING GENE THEREIN**

(57) The present invention provides a virus or a viral vector capable of expressing a gene specifically in a cell having replication ability in a hypoxic state such as a cancer stem cell and injuring the cell, and a pharmaceutical

composition comprising the same. Specifically, the present invention provides a virus or a viral vector which comprises a gene encoding a fusion protein of an ODD and a protein essentially required for viral proliferation, and a pharmaceutical composition comprising the same.

FIG. 3



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Description

Technical Field

5 **[0001]** The present invention relates to a virus that comprises a gene encoding a fusion protein of an ODD and a protein essentially required for viral proliferation, a method for producing a virus that uses the virus, a virus or a viral vector that is obtainable by the method, and a pharmaceutical composition comprising the virus or viral vector for treating or preventing a disease characterized by a cell in a hypoxic state. Further, the present application claims the benefit of priority from Japanese Patent Application No. 2008-7179, filed on January 6, 2008, and the entire content of this Japanese Patent Application No. 2008-7179 is incorporated herein by reference.

Background Art

15 **[0002]** During the course of previous research, the inventors of the present invention modified herpes simplex virus type I (HSV-1) gene to enable the virus to only proliferate in cells in the case of having infected cells that express calponin gene, namely smooth muscle cells growing in leiomyoma, mesothelioma or sites of vascular stenosis, and as a result thereof, developed a method for expressing genes within infected cells and disrupting infected cells (Patent Document 1).

20 **[0003]** Cancer cells present in a hypoxic environment within tumors are unresponsive to radiotherapy or chemotherapy, and have the ability to metastasize to distant organs. In addition, cancer stem cells have been reported to be present in cell populations having self-replicating ability in hypoxic regions of bone marrow in acute myelogenous leukemia (Non-Patent Document 1). Moreover, the presence of cancer stem cells has also been recently reported in solid cancers such as breast cancer, brain tumors and colorectal cancer (Non-Patent Document 2).

In general, cancer stem cells are considered to be cancer cells that are resistant to therapy, are highly metastatic, and are able to self-replicate even in hypoxic environments (Non-Patent Document 3). Thus, the development of a treatment

25 method is sought that enables cancer cells present in hypoxic regions such as cancer stem cells to be selectively disrupted.

[0004] Research has been conducted on a method for stably expressing arbitrary proteins under hypoxic conditions by using an oxygen-dependent degradation domain (ODD) that serves as a marker for protein degradation at normal oxygen partial pressure present in the amino acid sequence of transcription factor HIF1 α , which stabilizes only in cells in a hypoxic state. For example, a method has been developed for inducing apoptosis in cancer cells by producing a fusion gene in which an ODD sequence has been added to the amino terminal of the apoptosis stimulating factor, caspase 3, and activating the caspase 3 only in cells in a hypoxic state, and a method has been developed for expressing diphtheria toxin protein (Non-Patent Documents 4 and 5). However, these methods not only target abnormal cells such as cancer cells, but target normal cells as well. In addition, since these methods involve direct administration of protein whose expression has been inhibited by an ODD sequence, there is the disadvantage of weak therapeutic effects since expression is unable to be sustained due to degradation and the like. Thus, there is a need to develop a method that allows obtaining specific and potent therapeutic effects in target cells. In addition, although adenovirus has previously been produced that is capable of replicating only in cells under hypoxic conditions by coupling E1A gene downstream to the promoter sequence, Hypoxia-Responsive Element (HRE), which responds to HIF1 α , there is concern over toxic activity against normal cells due to insufficient on-off control of oxygen partial pressure-dependent promoter activity (Non-Patent Document 6).

Patent Document 1: Japanese Patent Application No. 2006-205006

Non-Patent Document 1: Sipkins D.A. et al. Nature 435, 969-973, 2005

Non-Patent Document 2: Ailles L.E. and Weissman I.L. Curr. Opin. Biotech. 18, 460-466, 2007

45 Non-Patent Document 3: De Toni F. et al. Oncogene 25, 3113-3122, 2006

Non-Patent Document 4: Harada H. et al. Cancer Res. 62, 2013-2018, 2002

Non-Patent Document 5: Koshikawa N. and Takenaga K. Cancer Res. 65, 11622-11630, 2005

Non-Patent Document 6: Cuevas Y. et al. Cancer Res. 61, 6877-6884, 2003

50 Disclosure of the Invention

Problems to be Solved by the Invention

55 **[0005]** An object of the present invention is to provide a virus for specifically injuring cells such as cancer stem cells in a hypoxic state, a viral vector that expresses more genes in cells in a hypoxic state, and preferably in cells having the ability to replicate in a hypoxic state, and a pharmaceutical composition comprising the virus and the viral vector.

Means for Solving the Problems

[0006] As a result of conducting extensive studies with the foregoing in view, the inventors of the present invention fused an oxygen-dependent degradation domain (ODD) with a protein essentially required for viral proliferation, and produced a virus that comprises a gene encoding the fusion protein. Although the fusion protein is degraded following recognition by the ubiquitin-proteasome system as a result of the fusion protein being modified by hydroxylation of a proline residue in the ODD sequence by proline hydroxylase in the case the virus has infected a cell at normal oxygen partial pressure, the fusion protein is not degraded under hypoxic conditions such as at an oxygen partial pressure of 10 mmHg or less. Thus, it was found that the virus does not proliferate in cells at normal oxygen partial pressure, but rather proliferates specifically in the case of having infected cells growing in a hypoxic state, that the gene carried by the virus is expressed, and that specific cytolytic action can be made to occur, thereby leading to completion of the present invention.

[0007] Namely, the present invention provides the inventions indicated in (1) to (16) below:

(1) a virus or viral vector, comprising: a gene encoding a fusion protein of an oxygen-dependent degradation domain (ODD) and a protein essentially required for viral proliferation;

(2) the virus or viral vector described in (1), wherein the protein essentially required for viral proliferation is selected from the group consisting of herpes virus ICP4, γ 34.5, adenovirus E1A, E1B, retrovirus LTR R and U5;

(3) the virus or viral vector described in (2), wherein the protein essentially required for viral proliferation is ICP4;

(4) the virus or viral vector described in any of (1) to (3) which has a deficiency in ribonucleotide reductase (RR);

(5) a method for producing a virus or viral vector, comprising the steps of:

(a) infecting a cell with the virus described in any of (1) to (4);

(b) culturing the cell in a hypoxic state; and

(c) recovering the virus that has proliferated;

(6) a virus or viral vector, which is obtainable by the method described in (5);

(7) the virus or viral vector described in (6) which is of the strain d12.ODD Δ RR;

(8) a pharmaceutical composition for treating or preventing a disease characterized by a cell having replicating ability in a hypoxic state, which comprises the virus or viral vector described in any of (1) to (4) or the virus or viral vector described in (6) or (7);

(9) the pharmaceutical composition described in (8), wherein the disease is selected from the group consisting of cancer, pulmonary fibrosis, pulmonary hypertension, and vascular stenosis in ischemic heart disease or ischemic brain disease;

(10) the pharmaceutical composition described in (9), wherein the disease is cancer;

(11) the pharmaceutical composition described in (10) which targets a cancer stem cell present in cancer;

(12) the pharmaceutical composition described in any of (8) to (11), wherein the hypoxic state is a state in which oxygen partial pressure is 10 mmHg or less;

(13) a method for treating or preventing a disease comprising: administering to a subject the virus or viral vector described in any of (1) to (4) or the virus or viral vector described in (6) or (7), wherein the disease is characterized by a cell in the subject having replicating ability in a hypoxic state;

(14) a use of the virus or viral vector described in any of (1) to (4) or the virus or viral vector described in (6) or (7) in manufacturing a medicament for treating or preventing a disease in a subject, wherein the disease is characterized by a cell in the subject having replicating ability in a hypoxic state;

(15) a fusion protein of an ODD and a protein essentially required for viral proliferation; and,

(16) a polynucleotide encoding the protein described in (15).

Effect of the Invention

[0008] Thus, according to the present invention, cells such as cancer stem cells in a hypoxic state, and particularly cells having the ability to replicate in a hypoxic state, can be specifically injured, thereby making it possible to effectively treat or prevent diseases such as cancer, pulmonary fibrosis, pulmonary hypertension or vascular stenosis in ischemic heart disease or ischemic brain disease.

Brief Description of the Drawings

[0009]

FIG. 1 indicates expressions of EGFP, HIF1 α and ICP4 after culturing human gastric cancer cell line AZP7a, transfected with a CMV-NLS-ODD-ICP4-IRES-EGFP-polyA DNA fragment, in RPMI medium containing 10% FBS at normal oxygen partial pressure (O₂: 20%) or low oxygen partial pressure (O₂: 1%) as observed with a fluorescence microscope;

FIG. 2 indicates plaque formation by HSV-1 virus in human gastric cancer cell line AZP7a, co-transfected with DNA of ICP4-deficient HSV-1 mutant strain d120 and a CMV-NLS-ODD-ICP4-IRES-EGFP-polyA DNA fragment, at normal oxygen partial pressure (O₂: 20%) or low oxygen partial pressure (O₂: 1%);

FIG. 3 indicates construction of d12.ODD Δ RR;

FIG. 4 represents the proliferation ability of HSV-1 virus as total plaque area when Vero cells are infected with purified d12.ODD Δ RR and cultured for 24 hours at normal oxygen partial pressure (O₂: 20%) or low oxygen partial pressure (O₂: 1%), and indicates that d12.ODD Δ RR proliferated well under conditions of low oxygen partial pressure (O₂: 1%);

FIG. 5 is a Western blot indicating expression of ICP4 protein when human gastric cancer cell line AZP7a was infected with purified d12.ODD Δ RR at a multiplicity of infection (MOI) of 0.1 and cultured for 48 hours at normal oxygen partial pressure (O₂: 20%) or low oxygen partial pressure (O₂: 1%), and indicates that ICP4 was only expressed at low oxygen partial pressure (O₂: 1%);

FIG. 6 shows the results of viral replication analysis indicating ganciclovir sensitivity when Vero cells were infected with a purified d12.ODD Δ RR at an MOI of 0.01 and cultured for 26 hours at normal oxygen partial pressure (O₂: 20%) or low oxygen partial pressure (O₂: 1%);

FIG. 7 indicates immunohistochemical results showing expression of ICP4 protein and the presence of HSV-1 envelope antigen indicating proliferation of d12.ODD Δ RR in a hypoxic region labeled with pimonidazole (injection of d12.ODD Δ RR at 1×10^7 pfu) located in a tumor in which human gastric cancer cell line AZP7a was transplanted into the abdominal cavities of SCID mice;

FIG. 8 indicates antitumor effects of d12.ODD Δ RR against a tumor in which cultured human mesothelioma cell line MSTO was transplanted subcutaneously into the backs of SCID mice (intratumoral injection of d12.ODD Δ RR at 1 to 2×10^3 pfu/injection), and tumor volume was determined by quantifying activity of luciferase transfected into the tumor as a photon count by real-time in vivo imaging;

FIG. 9 indicates antitumor effects of d12.ODD Δ RR against tumors in which primary-cultured human leiomyoma cells established from surgical specimens (n = 8) were transplanted subcutaneously into the backs of SCID mice (d12.ODD Δ RR initially injected intratumorally at 1 to 2×10^3 pfu/injection, and subsequently injected 17 times at 3 to 4 day intervals) as compared with d12.CALP Δ RR (d12.CALP Δ RR initially injected intratumorally at 1 to 2×10^4 pfu/injection, and subsequently injected 17 times at 3 to 4 day intervals), with the left side of the drawing indicating changes in tumor volume, and the right side of the drawing indicating residual tumor cells present in the tumor mass on day 82 following the start of treatment as observed with calponin immunostaining; and

FIG. 10 shows the results of a viral replication analysis in which cultured GIST cells were divided into fractions positive and negative for the cancer stem cell surface marker, CD133, using AutoMACS Pro (Miltenyi), followed by culturing in Petri dishes and infecting with purified d12.ODD Δ RR at MOI of 0.01 to 0.0001.

Best Mode for Carrying Out the Invention

[0010] The present invention in one embodiment relates to a virus or viral vector comprising a gene encoding a fusion protein of an ODD and a protein essentially required for viral proliferation. As was previously described, an ODD (oxygen-dependent degradation domain) refers to a domain that serves as a marker for protein degradation by the ubiquitin-proteasome system at normal oxygen partial pressure present in the amino acid sequence of transcription factor HIF1 α , which stabilizes only in a hypoxic state. The amino acid sequence of the ODD is shown in SEQ ID NO: 1, while the nucleic acid sequence thereof is shown in SEQ ID NO: 2. The ODD used in the present invention is composed of an amino acid sequence in which one or several amino acids have been deleted, substituted or added in the amino acid sequence indicated in SEQ ID NO: 1, and contains a nuclear localization signal (NLS) composed of 23 amino acids and the amino acid proline, which functions as a marker of protein degradation, on the amino terminal thereof. That amino acid sequence is shown in SEQ ID NO: 3. In addition, the nucleic acid sequence encoding the NLS-ODD sequence used in the present invention contains a Kozak consensus sequence on the 5' terminal thereof. That nucleic acid sequence is shown in SEQ ID NO: 4.

[0011] The protein essentially required for viral proliferation includes protein not only essentially required for viral proliferation, but also for replication of viral vector and/or viral nucleic acids. Various proteins are known as such proteins, and examples include, but are not limited to HSV-1 ICP4 and γ 34.5, adenovirus E1A and E1B, retrovirus LTR R and U5, and herpes simplex virus ribonucleotide reductase (RR). The amino acid sequences of these proteins are known. The protein essentially required for viral proliferation used in the present invention comprises a protein that is composed of an amino acid sequence in which one or several, such as 9, 8, 7, 6, 5, 4, 3 or 2, amino acids have been deleted,

substituted or added in the natural amino acid sequence, and that retains the function of the protein. Since the virus or viral vector of the present invention is used to injure infected cells by being replicated within the infected cells, the virus is preferably a replicative virus. In addition, the virus may be a DNA virus or an RNA virus. The mechanism by which infected cells are injured includes, for example, direct cytolysis due to viral proliferation, induction of apoptosis of virus-infected cells, activation of the immune system and the like.

5 **[0012]** The fusion protein of an ODD and a protein essentially required for viral proliferation is a protein in which these two proteins exist as a single polypeptide and are fused so as to retain the functions of each protein. A person with ordinary skill in the art is able to produce such fusion protein based on the sequences of the ODD and the protein essentially required for viral proliferation. Since such fusion protein is able to be degraded incidental to degradation by the ubiquitin-proteasome system of ODD, the presence and/or amount of the fusion protein can be controlled dependent on oxygen partial pressure of infected cells by using the virus or viral vector of the present invention. Preferably, the virus or viral vector of the present invention can be used as a vector that allows the expression of an arbitrary exogenous gene by coupling that gene through an internal ribosomal entry site (IRES) (U.S. Patent No. 4937190) downstream of a gene encoding the sequence of the protein essentially required for proliferation, and allows expression of a greater number of carried genes in cells in a hypoxic state, preferably in cells that have the ability to replicate in a hypoxic state, and for example, cancer stem cells.

10 **[0013]** Preferably, the virus or viral vector of the present invention has a deficiency in ribonucleotide reductase (RR). This deficiency refers to a state in which the function of RR has been lost, and is caused by, for example, deletion, insertion or mutation of a nucleotide in a gene encoding RR. RR is an enzyme that functions during the course of DNA synthesis, and is produced in large amounts in actively growing cells such as cancer cells. Thus, a deficiency in RR causes replication of the virus of the present invention to be carried out more frequently in actively growing cells such as cancer cells than in normal cells. In addition, as was previously described, since cells resistant to existing treatment methods, such as cancer stem cells, exist in a hypoxic state and have the ability to self-replicate, the virus or viral vector of the present invention is able to specifically injure growing cells and/or self-replicating cells in a hypoxic state, including cancer stem cells, as a result of having an ODD and being deficient in RR.

20 **[0014]** Preferably, the virus or viral vector of the present invention does not contain a gene encoding a protein essentially required for viral proliferation, such as a gene encoding RR, other than a gene encoding the protein essentially required for viral proliferation contained in the fusion protein. Virus replication can be controlled according to oxygen partial pressure more favorably by not containing such a gene.

25 **[0015]** In another embodiment, the present invention relates to a method for producing a virus or viral vector that comprises:

- 30 (1) infecting a cell with the above-mentioned virus of the present invention;
- (2) culturing the cell in a hypoxic state; and,
- 35 (3) recovering the virus that has proliferated. Introduction of viral genes of the present invention into cells is carried out by ordinary virus infection. Cells infected by the virus may be any cells provided that the virus is able to replicate within the cells.

40 **[0016]** A hypoxic state refers to a state in which oxygen partial pressure is lower than the oxygen partial pressure of the normal proliferation environment of the virus or lower than the normal oxygen partial pressure within a cell, and for example refers to a state in which oxygen partial pressure is about 20 mmHg or less (or about 2% or less as oxygen partial pressure), and preferably a state in which oxygen partial pressure is about 10 mmHg or less (or about 1% or less as oxygen partial pressure). The fusion protein of ODD and the protein essentially required for viral proliferation is stabilized by culturing virus-infected cells in such a hypoxic state, and as a result, viral proliferation is begun, increased or accelerated. Culturing conditions other than a hypoxic state are suitably selected dependent on, for example, the cells infected by the virus.

45 **[0017]** The virus that has proliferated is recovered from the culture supernatant according to means or methods known among persons with ordinary skill in the art. The resulting virus can be infected into target cells in a hypoxic state, and preferably target cells that have the ability to replicate in a hypoxic state, and be allowed to proliferate for a fixed period of time enabling it to specifically injure the cells, or the virus or viral vector of the present invention can be obtained in a culture supernatant or in a supernatant obtained by lysing the virus-infected cells and centrifuging.

50 **[0018]** In another embodiment, the present invention relates to a virus or viral vector that is obtainable by a method for producing the above-mentioned virus or viral vector of the present invention. The virus or viral vector of the present invention efficiently proliferates in cells in a hypoxic state, and preferably in cells that have the ability to replicate in a hypoxic state. In addition, in the case the virus used in the method for producing the virus or viral vector of the present invention is HSV-1 deficient in RR, the virus or viral vector of the present invention is able to proliferate more favorably in actively growing cells such as cancer cells. Preferably, the virus or viral vector of the present invention is of strain d12.ODD Δ RR. This strain d12.ODD Δ RR will be described in detail herein-below. In addition, the virus or viral vector of

the present invention is obtainable by infecting cells with the virus or viral vector of the present invention and causing viral nucleic acids to be replicated within the cells.

5 [0019] In a further embodiment, the present invention relates to cells that have been infected or introduced with the virus or viral vector of the present invention as previously described. Infection or introduction of the virus or viral vector of the present invention into cells can be carried out using any known methods. Cells that have been infected or introduced with the virus or viral vector of the present invention can be used to induce an immune response to the cells by being injected into the body.

10 [0020] In a still further embodiment, the present invention relates to a pharmaceutical composition for treating or preventing a disease characterized by cells being in a hypoxic state, and preferably cells having a replicating ability in a hypoxic state, comprising: the above-mentioned virus or viral vector of the present invention. Although previously defined, a hypoxic state preferably refers to a state in which oxygen partial pressure is about 10 mmHg or less (or about 1% or less as oxygen partial pressure). Examples of diseases characterized by cells having the ability to replicate in a hypoxic state include, but are not limited to, all forms of cancer, pulmonary fibrosis, pulmonary hypertension and vascular stenosis in ischemic heart disease or ischemic brain disease. In the case of cancer, for example, cells such as cancer stem cells have the ability to replicate in a hypoxic state. Since only these cells are considered to have the ability to allow cancer to recur or metastasize and be resistant to radiotherapy and chemotherapy, use of the pharmaceutical composition of the present invention enables diseases such as cancer that are resistant to existing treatment methods to be treated, and enables prevention of metastasis thereof.

20 [0021] The virus or viral vector contained in the pharmaceutical composition of the present invention is characterized by replicating or proliferating in cells in a hypoxic state, and preferably in cells that have the ability to replicate in a hypoxic state. Thus, the pharmaceutical composition comprising the virus or viral vector of the present invention is able to target only cells in a hypoxic state. More specifically, in the case a virus is contained in the pharmaceutical composition of the present invention, the pharmaceutical composition is able to injure only cells in a hypoxic state as a result of the virus proliferating in those cells. In the case a viral vector is contained in the pharmaceutical composition of the present invention, a specific gene can be expressed only in cells in a hypoxic state. Since the viral vector is preferably replicated, the amount of gene expressed in the cells in a hypoxic state is sustained at a high level.

25 [0022] Moreover, in the case the virus or viral vector contained in the pharmaceutical composition of the present invention has a deficiency in RR, the pharmaceutical composition of the present invention is able to more specifically injure actively growing cancer cells, and preferably cells such as cancer stem cells having the ability to self-replicate.

30 [0023] The pharmaceutical composition of the present invention may also contain various components such as a carrier, excipient or additive in addition to containing the virus or viral vector of the present invention. The components preferably increase infection efficiency of the virus or viral vector, and are suitably selected depending on various factors such as the disease, state of the target cells to be treated, or the administration mode. The amount and number of administrations of the virus or viral vector contained in the pharmaceutical composition of the present invention are suitably selected depending on the disease, target cells, subject status, administration mode and the like. In addition, known therapeutic agents of a disease to be treated or prevented may also be used in the pharmaceutical composition of the present invention or concomitantly with the pharmaceutical composition of the present invention.

35 [0024] In a yet further embodiment, the present invention relates to a method for treating and/or preventing a disease characterized by a cell of a subject being in a hypoxic state, comprising: administering the pharmaceutical composition of the present invention to the subject. A known treatment method of a disease to be treated or prevented may be used concomitantly in the therapeutic and/or preventive method of the present invention.

40 [0025] In a further embodiment, the present invention relates to a use of the virus or viral vector of the present invention in manufacturing a medicament for treating and/or preventing a disease characterized by a cell being in a hypoxic state.

45 [0026] In another embodiment, the present invention relates to a fusion protein of an ODD and a protein essentially required for viral proliferation. The amino acid sequence of a fusion protein in which the protein essentially required for viral proliferation is HSV-1 ICP4 is shown in SEQ ID NO: 5. The fusion protein encompasses a protein which is composed of an amino acid sequence in which one or several, such as 9, 8, 7, 6, 5, 4, 3 or 2, of amino acids have been deleted, substituted or added in the amino acid sequence shown in SEQ ID NO: 5, and which has the function of the fusion protein, namely the function of the fusion protein of an ODD and a protein essentially required for viral proliferation. The fusion protein of the present invention can be produced according to a known method such as genetic recombination. The fusion protein of the present invention can be used to label a hypoxic region in cells in vivo, by introducing into the cells in advance.

50 [0027] In still another embodiment, the present invention relates to a polynucleotide that encodes the above-mentioned fusion protein of an ODD and a protein essentially required for viral proliferation. The nucleic acid sequence that encodes a fusion protein in which the protein essentially required for viral proliferation is HSV-1 ICP4 is shown in SEQ ID NO: 6.

55 [0028] Although the following provides a specific and detailed explanation of the present invention by indicating the following examples thereof, the examples should not be understood to limit the present invention.

Example 1

Production of Virus

5 **[0029]** A polypeptide composed of 57 proteins containing the 564th proline residue, which is the ubiquitin-proteasome recognition site within the ODD (oxygen-dependent degradation domain) of HIF1 α , which is an amino acid sequence serving as a marker of protein degradation at normal oxygen atmospheric pressure, was added to the amino terminal of ICP4, which is a transcription factor essentially required for the initiation of HSV-1 replication. Moreover, a nuclear localization signal composed of 23 amino acids was added to the amino terminal thereof.

10 **[0030]** ICP4 gene was PCR-amplified using a 4085-bp blunt end Sall-MseI fragment (provided by Dr. Hayward of John Hopkins School of Medicine) derived from pGH108 (J. Virol., 56, 558-570, 1985) containing the coding region thereof, from the initiation codon to a PvuII site, and a 261-bp DNA encoding a Kozak sequence (aattcccagcttgac), a sequence of 23 amino acids serving as a nuclear localization signal, and 57 ODD sequences, was coupled to the 5' terminal thereof. A PvuII-MseI fragment of ICP4 was coupled to the 3' side thereof to construct an ODD-fused ICP4 gene (4221 bp). Moreover, an approximately 9-kb CMV-NLS-ODD-ICP4-IRES-LacZ-polyA fragment was constructed in which a CMV promoter (588 bp) was coupled to the upstream side thereof, while a LacZ-polyA sequence (3.3 kb) derived from *E. coli* was coupled to the downstream side thereof through IRES (585 bp). Moreover, a CMV-NLS-ODD-ICP4-IRES-EGFP-polyA fragment was also constructed in which LacZ-polyA was substituted to EGFP-polyA.

20 **[0031]** First, the CMV-NLS-ODD-ICP4-IRES-EGFP-polyA fragment was co-transfected with a drug resistance gene expression vector, pSV2neo, into a sub-confluent single-layer culture of human gastric cancer cell line AZP7a (provided by Dr. Nishimori of the Sapporo Medical University) in a 6-well tissue culture plate (2.5×10^5 /well) using Lipofectamine[®] (Gibco/BRL) in accordance with the manufacturer's protocol. G418-resistant clones of the gastric cancer cells that constitutively expressed EGFP were then selected using a method well known among persons with ordinary skill in the art. Whether or not expression of ICP4 protein changes depending on oxygen partial pressure was then examined using a fluorescence microscope under conditions of normal oxygen partial pressure (O_2 : 20%) and low oxygen partial pressure (O_2 : 1%) using the gastric cancer cell clones. Both EGFP and ICP4 were confirmed to be expressed within the same cells only under hypoxic conditions (FIG. 1). Although EGFP was expressed under both conditions of normal oxygen partial pressure (O_2 : 20%) and low oxygen partial pressure (O_2 : 1%), HIF1 α and ICP4 were expressed only in cells cultured under conditions of low oxygen partial pressure (O_2 : 1%). Expression of ODD-ICP4 fusion protein was confirmed to be controlled according to the oxygen partial pressure of the cells (FIG. 1). Moreover, the CMV-NLS-ODD-ICP4-IRES-EGFP-polyA fragment and HSV-1 variant d120 viral DNA deficient in ICP4 were co-transfected into AZP7a cells in accordance with the manufacturer's protocol under conditions of normal oxygen partial pressure (O_2 : 20%) and low oxygen partial pressure (O_2 : 1%), and the number of plaques was counted after 48 hours. Plaques were confirmed to only have been formed under conditions of low oxygen partial pressure (O_2 : 1%) (FIG. 2). Plaque formation, which indicates viral proliferation, was observed to a much greater degree in the case of culturing at low oxygen partial pressure (O_2 : 1%). Not only the expression of ODD-ICP4 fusion protein (FIG. 1 and Western blot), but also the function thereof were confirmed to be controlled according to the oxygen partial pressure of the cells.

30 **[0032]** The CMV-NLS-ODD-ICP4-IRES-LacZ-polyA fragment was inserted into an StuI site of pKpX2 (J. Virol., 62, 196-205, 1998) (provided by Dr. Weller of Connecticut University), which contains a 2.3 kb sequence of the 5' side of a gene (UL39) that encodes RR, which is an enzyme essentially required for replication of viral DNA, followed by linearization by digesting with XhoI (in which the XbaI site further towards the 5' side of the RR sequence on the 5' side of pKpX2 and the HindIII site further towards the 3' side of the RR sequence on the 3' were substituted with XhoI) to construct an 11.3-kb UL39-CMV-NLS-ODD-ICP4-IRES-LacZ-polyA-UL39 homologous recombination vector from which pUC 19 sequence is removed. This was then co-transfected with HSV-1 variant d120 viral DNA deficient in ICP4 into a sub-confluent, single-layer culture of VeroE5 cells constitutively expressing ICP4 in a 6-well tissue culture plate (2.5×10^5 /well) using Lipofectamine[®] (Gibco/BRL) in accordance with the manufacturer's protocol. 1 ml of 20% DMEM culture medium was added 3 hours after transfection followed by culturing in the above-mentioned culture medium (10% FBS/DMEM) containing 0.5 mg/ml of 4-hydroxymethyl benzoic acid (HMBA) until 96 hours after transfection. Following confirmation of plaque formation, the cells were cultured for 24 hours in 10% FBS/DMEM not containing HMBA. The cells were then suspended in cold virus buffer (20 mM Tris-HCl containing 150 mM NaCl, pH: 7.5) at 500 μ l/well and placed in frozen storage.

45 **[0033]** Freezing and thawing treatment combined with ultrasonic treatment (3 times for 30 seconds each) were carried out three times to dissolve the above-mentioned suspension. The dissolved suspension was then stepwise-diluted and infected into sub-confluent, single-layer cultured VeroE5 cells in a 96-well tissue culture plate. Following infection, the cells were cultured for 96 hours in 1% FBS/DMEM containing 100 μ l of 11.3 μ g/ml human IgG (Jackson ImmunoResearch Laboratories) per well. VeroE5 single-layer cultured cells from those wells in which plaque formation was able to be confirmed were suspended in 100 μ l of the above-mentioned culture medium, and 6 μ l of the resulting suspension was used to measure β -galactosidase enzyme activity using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) as

the substrate and using a β galactosidase enzyme assay system (Promega). Suspensions of VeroE5 cells from wells positive for β -galactosidase enzyme activity were centrifuged for 5 minutes at 5000 rpm, and the resulting pellets were suspended in cold virus buffer at 100 μ l/well. Similar measurement of limited dilution infection/ β -galactosidase enzyme activity using a 96-well tissue culture plate was repeated two more times using VeroE5 cells to purify the recombinant virus d12.ODD Δ RR as a single plaque (FIG. 3). The recombinant virus d12.ODD Δ RR is maintained and managed by the inventors of the present invention.

[0034] Vero cells were infected in 10 to 20 150 cm² tissue culture flasks (T-150, Iwaki Glass), and after culturing for 48 hours in VP-SMF serum-free medium (Invitrogen), separated cells were centrifuged for 45 minutes at 42000 g and recovered in the sediment fraction to prepare the virus. The cells were then suspended in 10 ml of cold virus buffer (20 mM Tris-HCl containing 150 mM NaCl, pH: 7.5). Freezing and thawing treatment combined with ultrasonic treatment (3 times for 30 seconds each) were carried out three times to dissolve the above-mentioned cells, followed by centrifuging for 5 minutes at 4°C and 500 \times g, and further centrifuging the supernatant for 45 minutes at 4°C and 26100 \times g. The resulting pellet was suspended in cold virus buffer, a plaque assay was carried out using Vero cells under conditions of normal oxygen partial pressure (O₂: 20%) and low oxygen partial pressure (O₂: 1%), and proliferating activity of the purified d12.ODD Δ RR, sensitivity of its cell-injuring action to oxygen partial pressure, and its titer were determined. d12.ODD Δ RR proliferated more actively and demonstrated cell-injuring activity under conditions of low oxygen partial pressure (O₂: 1%) (FIG. 4).

Immunoblot Analysis of ICP4 Expression

[0035] AZP7a cells were respectively infected with d12.ODD Δ RR to a multiplicity of infection (MOI) of 0.1 or only virus buffer under conditions of normal oxygen partial pressure (O₂: 20%) and low oxygen partial pressure (O₂: 1%), followed by recovery of the cells after culturing for 48 hours. An equal amount of protein was applied to SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). After blocking the membrane for 2 hours at room temperature using 5% skim milk (Difco Laboratories), the protein was incubated overnight at 4°C with anti-ICP4 antibody (Goodwin Institute for Cancer Research, dilution factor: 1:500). A larger amount of ICP4 protein was expressed under conditions of low oxygen partial pressure (O₂: 1%) (FIG. 5). Expression of ICP4 was not observed with virus buffer only even after addition of AZP7a.

Virus Replication Analysis Indicating Ganciclovir Sensitivity of d12.ODD Δ RR

[0036] Vero cell line was cultured in a 24-well culture plate at 5 \times 10⁴ cells/well, and after infecting with d12.ODD Δ RR virus at an MOI of 0.01, various concentrations (0 to 1 μ g/ml) of ganciclovir (Wako Pure Chemical Industries) in 1% FBS/DMEM were added followed by culturing for 26 hours. After fixing the cells with 10% formalin PBS, the resulting plaques were stained with X-Gal and counted. Proliferation of d12.ODD Δ RR was inhibited concentration-dependently by addition of ganciclovir under both conditions of normal oxygen partial pressure (O₂: 20%) and low oxygen partial pressure (O₂: 1%). The results are shown in FIG. 6.

(In Vivo Treatment and Histological Analysis)

[0037] AZP7a cells were injected into the peritoneal cavities of 6-week-old, female severe combined immunodeficiency (SCID) mice (Nippon Clea), and MSTO human malignant mesothelioma cells (ATCC, CRL-2081), primary-cultured human leiomyoma cells and MCF7 human breast cancer cells (ATCC, HTB-22) were injected subcutaneously into the trunk to establish tumors in the mice. The MSTO cells were transfected with luciferase gene pGL4.13 (Promega), and clones demonstrating the highest chemiluminescence intensity and growth rate were selected. Among the cloned cells, tumor masses of the mesothelioma measuring 4 to 5 mm square were subcutaneously transplanted from those that had become established beneath the skin on the backs of the SCID mice to backs of 6-week-old, female SCID mice. The tumors grew to about 6 to 7 mm in diameter (50 to 70 mm³) at 30 days after being transplanted to the SCID mice. 50 μ l (per 100 mm³ of tumor volume) of virus suspension containing 1 \times 10⁷ pfu of d12.ODD Δ RR, or an equal volume of virus buffer, were injected intraperitoneally or intratumorally using a 30 gauge needle. The procedure was then repeated in exactly the same manner. Tumor diameter was measured at prescribed times after injection, and tumor volume was calculated using the following formula:

$$(\text{tumor volume}) = 0.53 \times (\text{length}) \times (\text{width})^2$$

In the case of MSTO, luciferin (Sigma Chemicals) was injected intraperitoneally, and in use of a high-sensitivity CCD

camera the intensity of chemiluminescence from tumor cells beneath the skin of the back was measured employing a real-time in vivo imaging system (Berthold). d12.ODD Δ RR demonstrated remarkable antitumor effects against each of the subcutaneously transplanted human malignant mesothelioma, leiomyoma and breast cancer cells by direct injection. The immunohistochemical results for human gastric cancer cells are shown in FIG. 7, while the treatment results for human malignant mesothelioma and leiomyoma are shown in FIGS. 8 and 9. Antitumor effects in a treatment group injected with d12.ODD Δ RR a total of seven times on the prescribed days indicated with arrows were clearly evident in comparison with a control group injected with virus buffer only (FIG. 8). In contrast to residual tumor cells not being observed in tumors treated with d12.ODD Δ RR, residual tumor cells were observed in tumors treated with d12.CALP Δ RR (FIG. 9).

[0038] Mice in which tumors were present were sacrificed on prescribed numbers of days following completion of administration of d12.ODD Δ RR at 1×10^7 pfu/100 mm³ of tumor volume for the purpose of histological research. The subcutaneous tumors were excised and fixed overnight at 4°C in PBS containing 1 mM MgCl₂ using 2% paraformaldehyde and 0.5% glutaraldehyde. Continuing, the tumors were immersed for 3 hours at 37°C in a substrate solution containing X-gal (1 mg/ml), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 1 mM MgCl₂ in PBS, followed by washing with PBS containing 3% DMSO. Immunohistochemistry consisted of fixing the specimens in Bouin's solution (15% (v/v) saturated picric acid solution, 1.65% (v/v) formalin and 1% (v/v) acetic acid/PBS) followed by embedding in paraffin. A section having a thickness of 4 μ m was placed on a microslide coated with poly-L-lysine followed by treatment in xylene and stepwise dehydration with alcohol, and in order to block intrinsic peroxidase, the section was immersed in a solution of 70% methanol and H₂O₂. Subsequently, antigen was recovered in 10 mM citrate buffer (pH 7.0) using an autoclave for 10 minutes at 121°C. The section was then incubated for 1 hour at room temperature using 1% (v/v) goat serum/PBS, followed by washing with PBS and incubating overnight at 4°C in 2% (w/v) BSA/PBS using the previously described anti-ICP4 antibody or anti-envelope antibody (Quartett). The section was then washed five times with 0.005% (v/v) Tween20/PBS followed by incubating for 1 hour at room temperature in 2% (w/v) BSA/PBS using biotinized goat anti-rabbit IgG (Tago Immunologicals), and further incubating for 30 minutes at room temperature using avidin-biotin-horse-radish peroxidase conjugate (Vector Laboratories). After washing with 0.005% (v/v) Tween20/PBS, the final reaction product was visualized with diaminobentidine (Wako Chemicals), and the section was counter-stained with hematoxylin. A tissue specimen treated with goat serum was used as a control to observe non-specific staining. Detection of hypoxic regions of tissue was carried out using the Hypoxyprobe-1 Kit (Natural Pharmacia International) in accordance with the manufacturer's protocol. In an SCID mouse intraperitoneal transplant model of human gastric cancer cell line AZP7a, proliferation of intraperitoneally injected d12.ODD Δ RR in coordination with hypoxic regions within tumors labeled with pimonidazole and expression of ICP4 protein were confirmed (FIG. 7).

[0039] After dividing cultured GIST cells into fractions positive and negative for CD133, which is a cell surface marker of cancer stem cells, using AutoMACS Pro (Miltenyi), the cells were cultured in Petri dishes and infected with purified d12.ODD Δ RR at MOI of 0.01 to 0.0001 followed by analyzing virus replication, the results of which are shown in FIG. 10. d12.ODD Δ RR proliferated more in CD133-positive cells and demonstrated potent cell-injuring action.

Industrial Applicability

[0040] Since a virus or viral vector comprising a gene encoding a fusion protein of an ODD and a protein essentially required for viral proliferation, and a pharmaceutical composition comprising the same, are provided by the present invention, the present invention can be used in the development and production of therapeutic agents in the field of pharmaceuticals and the like, such as those for treatment of cancer under hypoxic conditions and resistant to radiotherapy and chemotherapy, and preferably cancer stem cells.

Sequence Listing Free Text

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YAMAMURA, Hisako

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Claims

- 30
1. A virus or viral vector, comprising: a gene encoding a fusion protein of an oxygen-dependent degradation domain (ODD) and a protein essentially required for viral proliferation.
 - 35 2. The virus or viral vector according to claim 1, wherein the protein essentially required for viral proliferation is selected from the group consisting of herpes virus ICP4, γ 34.5, adenovirus E1A, E1B, retrovirus LTR R and U5.
 3. The virus or viral vector according to claim 2, wherein the protein essentially required for viral proliferation is ICP4.
 4. The virus or viral vector according to any one of claims 1 to 3, which has a deficiency in ribonucleotide reductase (RR).
 - 40 5. A method for producing a virus or viral vector, comprising the steps of:
 - (a) infecting a cell with the virus according to any one of claims 1 to 4;
 - (b) culturing the cell in a hypoxic state; and
 - 45 (c) recovering the virus that has proliferated.
 6. A virus or viral vector, which is obtainable by the method according to claim 5.
 7. The virus or viral vector according to claim 6, which is of the strain d12.ODD Δ RR.
 - 50 8. A pharmaceutical composition for treating or preventing a disease **characterized by** a cell having replicating ability in a hypoxic state, which comprises the virus or viral vector according to any one of claims 1 to 4 or the virus or viral vector according to claim 6 or 7.
 - 55 9. The pharmaceutical composition according to claim 8, wherein the disease is selected from the group consisting of cancer, pulmonary fibrosis, pulmonary hypertension, and vascular stenosis in ischemic heart disease or ischemic brain disease.

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10. The pharmaceutical composition according to claim 9, wherein the disease is cancer.

11. The pharmaceutical composition according to claim 10, which targets a cancer stem cell present in cancer.

5 12. The pharmaceutical composition according to any one of claims 8 to 11, wherein the hypoxic state is a state in which oxygen partial pressure is 10 mmHg or less.

10 13. A method for treating or preventing a disease comprising: administering to a subject the virus or viral vector according to any one of claims 1 to 4 or the virus or viral vector according to claim 6 or 7, wherein the disease is **characterized by** a cell in the subject having replicating ability in a hypoxic state.

15 14. A use of the virus or viral vector according to any one of claims 1 to 4 or the virus or viral vector according to claim 6 or 7 in manufacturing a medicament for treating or preventing a disease in a subject, wherein the disease is **characterized by** a cell in the subject having replicating ability in a hypoxic state.

15. A fusion protein of an ODD and a protein essentially required for viral proliferation.

20 16. A polynucleotide encoding the protein according to claim 15.

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FIG. 1

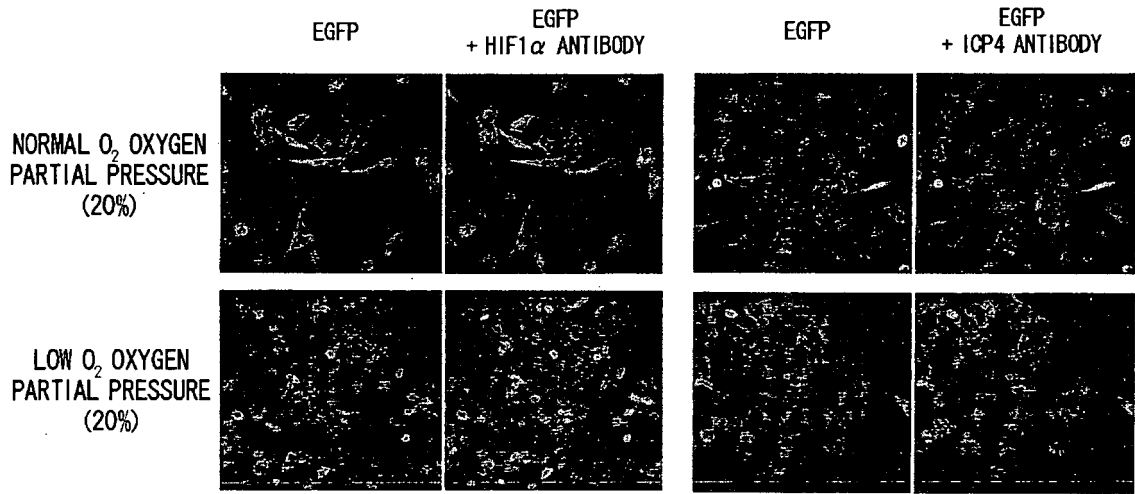


Fig. 2

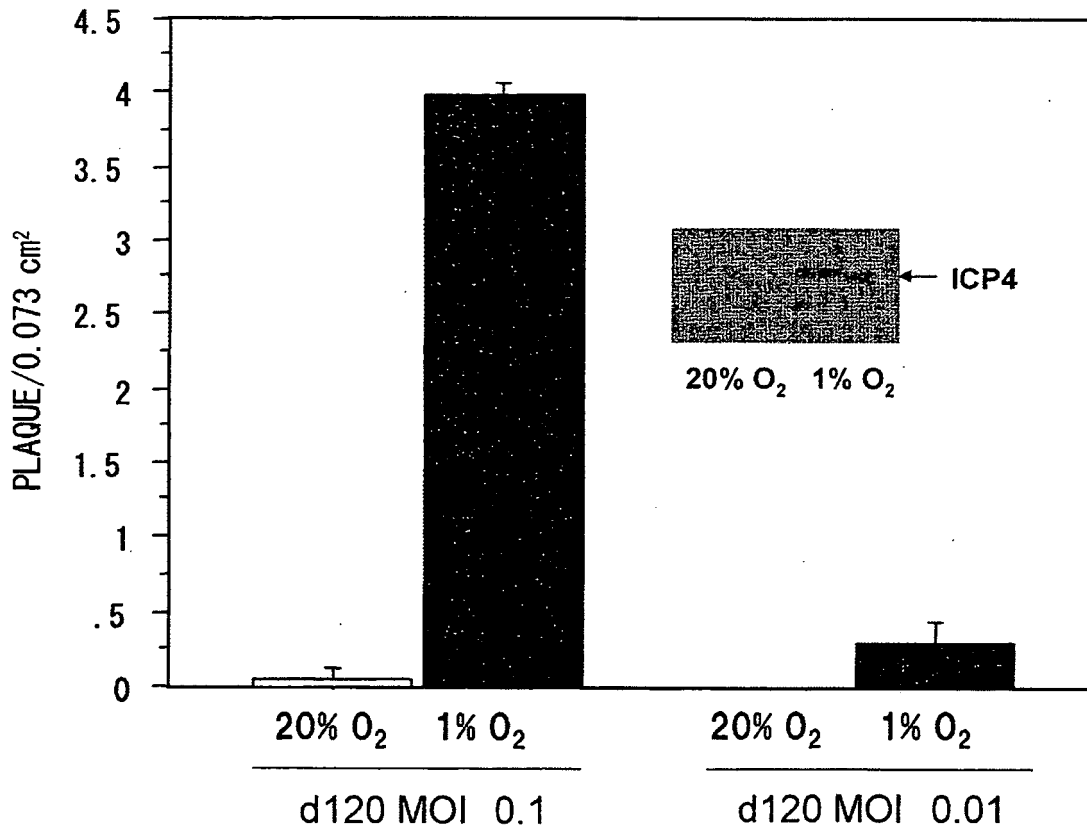


FIG. 3

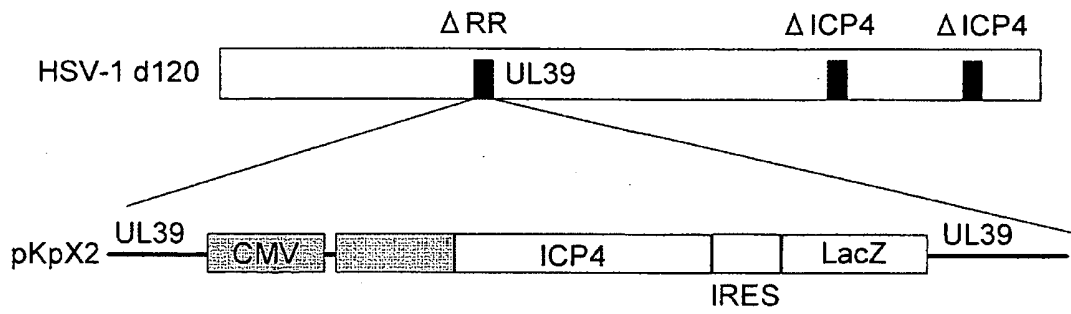


FIG. 4

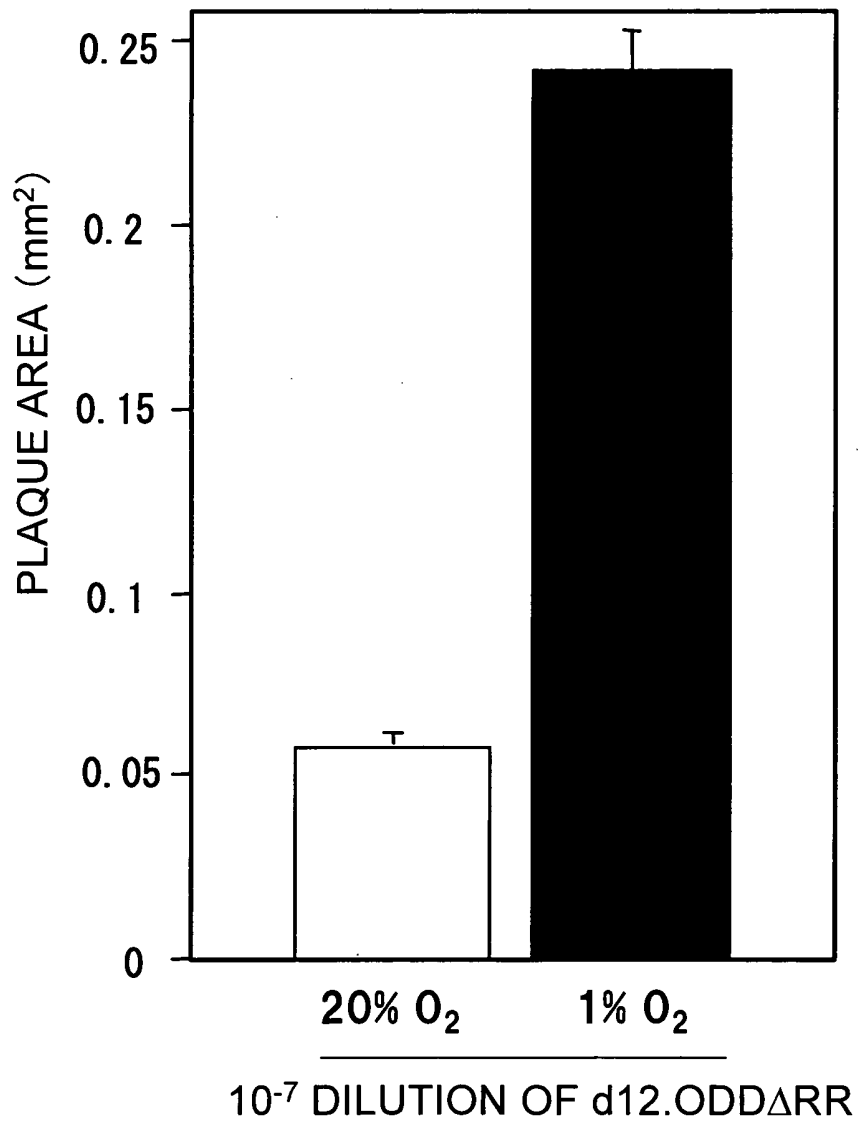


FIG. 5

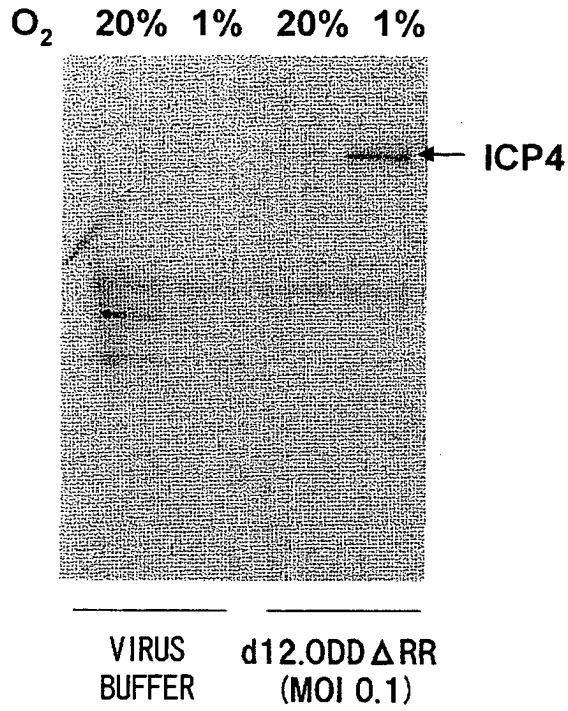


FIG. 6

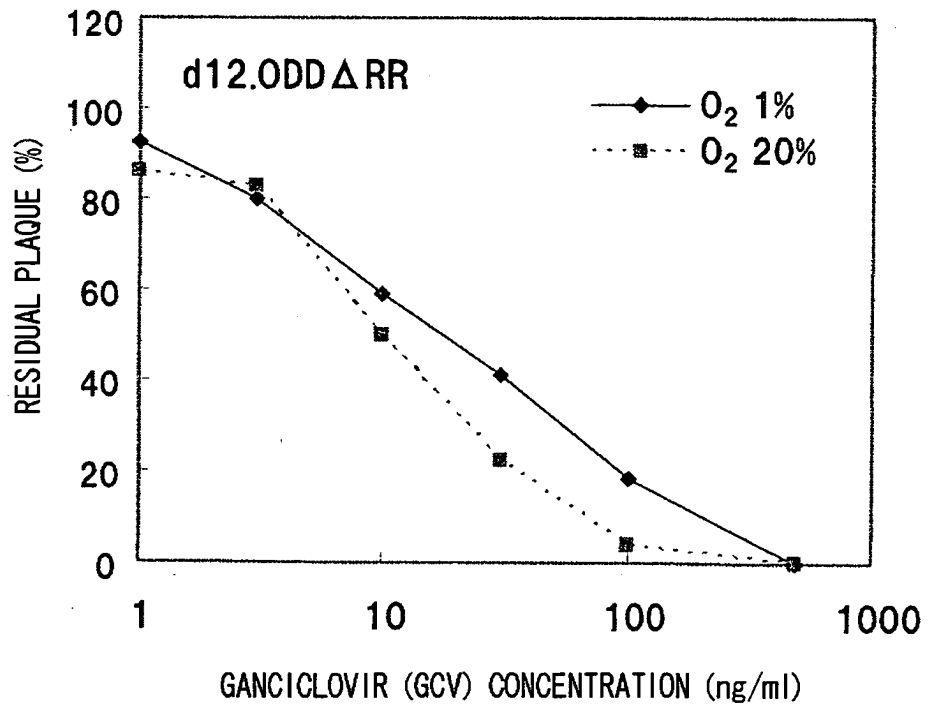


FIG. 7

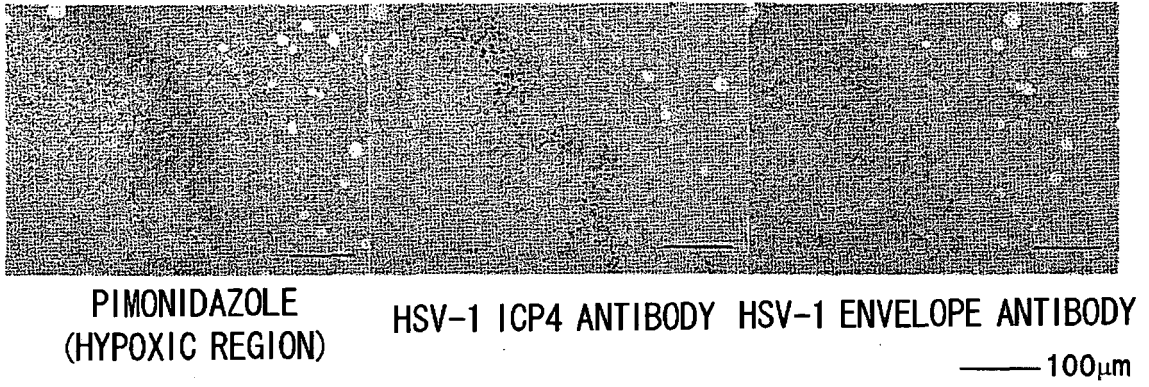


FIG. 8

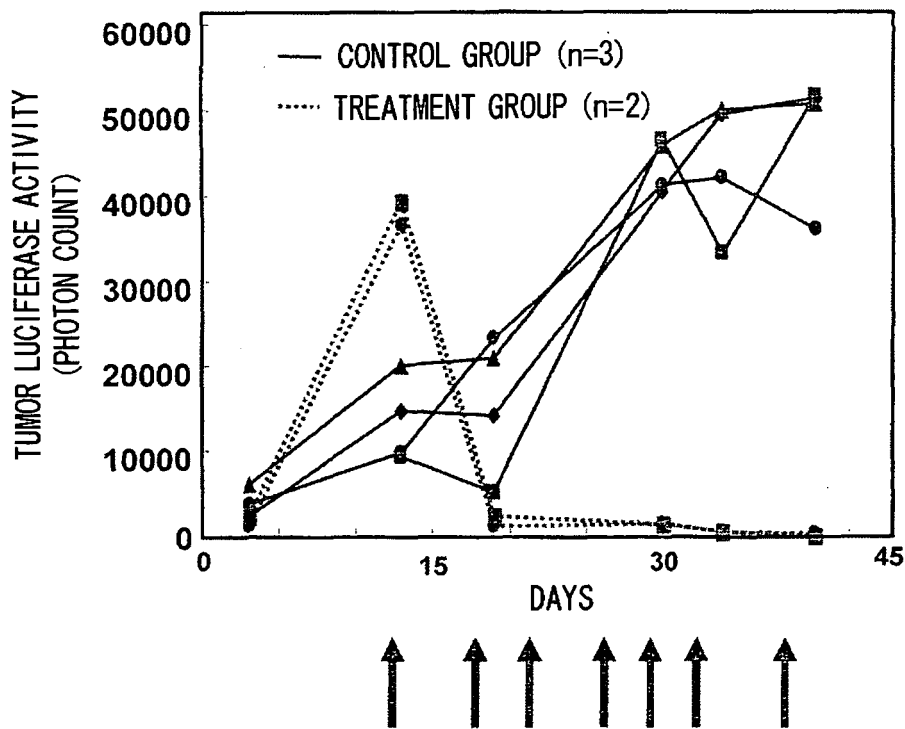


FIG. 9

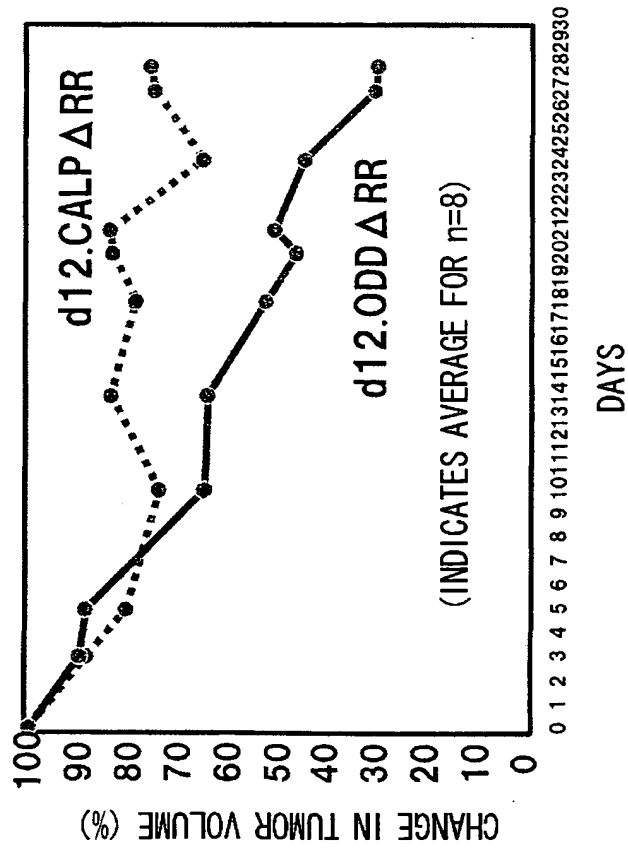
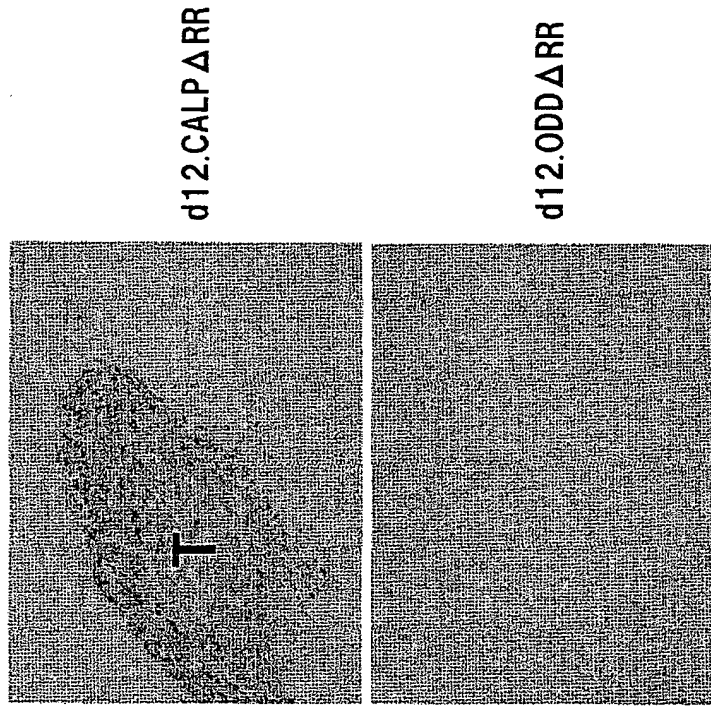
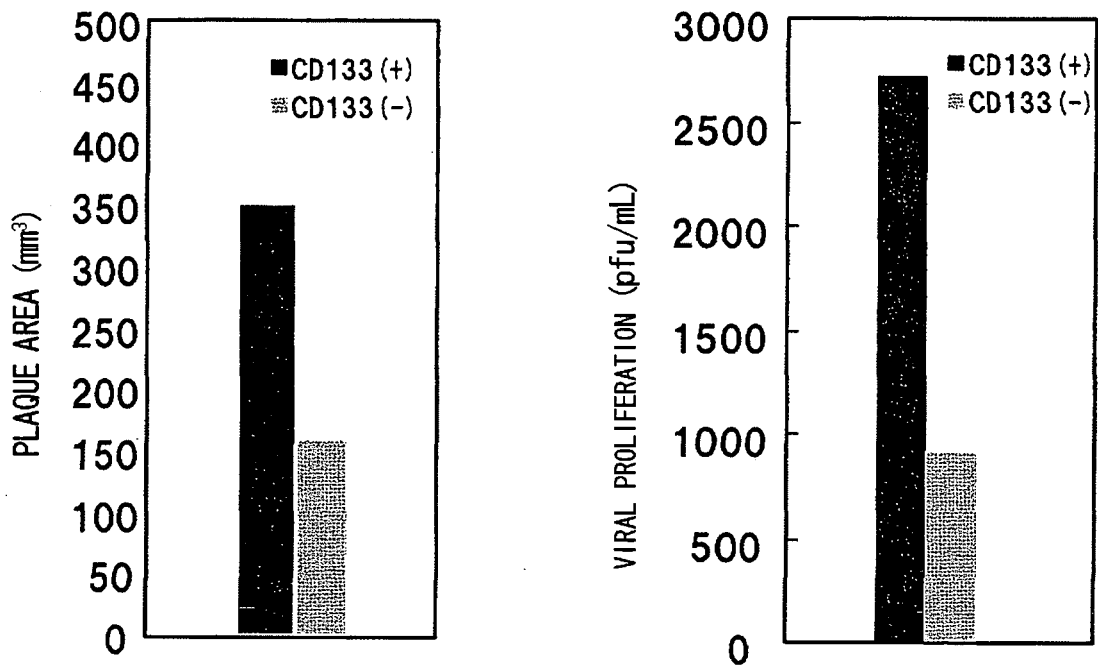


FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2009/050299

A. CLASSIFICATION OF SUBJECT MATTER C12N15/09(2006.01)i, A61K35/76(2006.01)i, A61P9/10(2006.01)i, A61P11/00(2006.01)i, A61P25/00(2006.01)i, A61P35/00(2006.01)i, C07K14/03(2006.01)i, C07K14/47(2006.01)i, C07K19/00(2006.01)i, C12N7/00(2006.01)i 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) C12N15/09, A61K35/76, A61P9/10, A61P11/00, A61P25/00, A61P35/00, C07K14/03, C07K14/47, C07K19/00, C12N7/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2009 Kokai Jitsuyo Shinan Koho 1971-2009 Toroku Jitsuyo Shinan Koho 1994-2009		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS/MEDLINE/WPIDS (STN), Cplus (STN), JSTPlus (JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, PubMed		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y/A	WO 2003/057888 A1 (JAPAN SCIENCE AND TECHNOLOGY CORP.), 17 July, 2003 (17.07.03), Full text & JP 2003-250579 A & US 2005/0032214 A1 & EP 1469077 A1	1-6,8-16/7
Y/A	HARADA, H., et al., Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells., Cancer Research, 2002, Vol.62, p.2013-2018, full text	1-6,8-16/7
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 26 February, 2009 (26.02.09)	Date of mailing of the international search report 10 March, 2009 (10.03.09)	
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer	
Facsimile No.	Telephone No.	

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2009/050299

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y/A	INOUE, M., et al., Targeting hypoxic cancer cells with a protein prodrug is effective in experimental malignant ascites., International Journal of Oncology, 2004, Vol.25, p.713-720, full text	1-6,8-16/7
Y/A	KOSHIKAWA, N., et al., Hypoxia-regulated expression of attenuated diphtheria toxin A fused with hypoxia-inducible factor-1alpha oxygen-dependent degradation domain preferentially induces apoptosis of hypoxic cells in solid tumor., Cancer Research, 2005, Vol.65, No.24, p.11622-30, full text	1-6,8-16/7

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REFERENCES CITED IN THE DESCRIPTION

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