Title: THE PHARMACEUTICAL COMPOSITION COMPRISING ARGinine DEIMINASE FOR INHIBITING ANGIOGENESIS

Abstract: The present invention relates to a pharmaceutical composition for inhibiting angiogenesis which comprises arginine deiminase as an active ingredient, where the arginine deiminase, obtained from Mycoplasma arginini or prepared by a genetic recombination technique, may be conjugated to an activated polymer to lower its immunogenicity and increase its life time. The pharmaceutical composition of the present invention exhibits an excellent inhibitory activity against angiogenesis.
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THE PHARMACEUTICAL COMPOSITION COMPRISING ARGinine DEiminase FOR INHIBITING ANGIOGENESIS

Field of the Invention

The present invention relates to a pharmaceutical composition for inhibiting angiogenesis which comprises arginine deiminase as an active ingredient. More particularly, it pertains to a pharmaceutical composition comprising arginine deiminase or conjugates of the enzyme with activated polymers; said arginine deiminase can be obtained either by purification from Mycoplasma arginini or by a genetic recombination technique.

Background of the Invention

Angiogenesis, the process of forming new capillary blood vessels from microvessels, occurs during embryonic development, wound healing and female menstruation, but not under normal conditions. A failure in angiogenesis regulatory system may lead to: angiogenesis-related diseases such as angioma, angiofibroma and blood vessel malformation; cardiovascular diseases such as arteriosclerosis, intravascular coagulation and edematous scleroderma; and ophthalmological diseases such as corneal transplantation-related neovascularization, neovascular glaucoma, corneal disease, involutional macula, degeneration of macula, pterygium, retinal degeneration, retrolental fibroplasias and granular conjunctivitis. In addition, such failure may result in chronic diseases such as rheumatism and dermatological diseases such as psoriasis, telangiectasis, pyogenic granuloma, seborrheic dermatitis and acne. Solid tumor growth and metastasis, in particular, are angiogenesis-dependent (Ophthalmol., 102, 1261-1262, 1995; J. Am. acad. Derm., 34(3), 486-497, 1996; Circulation, 93(4), 632-682, 1996; Cell, 86, 353-364, 1996).

Angiogenesis is accompanied by the processes of: degradation of the vascular basal membrane; migration and growth of the vascular endothelial cell; formation of canal cavity by differentiation of vascular endothelial cell; and re-
formation of the blood vessels. Normal angiogenesis occurs during the luteinization and placentation, but abnormal angiogenesis leads to such diseases as mentioned above. Therefore, there have been many attempts to develop compounds for the prevention and treatment of these angiogenesis-dependent diseases.

Arthritis, a well known inflammatory disease, is caused as an autoimmune disease. But if the abnormality continues to proceed, angiogenesis is induced by the chronic inflammation appearing in the synovial cavity in the joints and synovial cell. Then, the growth of vascular endothelial cell in the synovial cavity is induced by cytokine, which leads to the formation of articular lamina leak to eventually destroy such normal tissues as the cartilage which act as a cushion in the articulation (Koch A.E. et al., Arthritis Rheum., 29, 471-479, 1986; Stupack D.G., Braz. J. Med. Biol. Res., 32, 578-581, 1999; Koch A.E., Arthritis Rheum., 41, 951-962, 1998).

Angiogenesis is one of major causes of ophthalmological diseas, millions of people over the world are suffering from the loss of their eye power (Jeffrey M.I. and Takayuki A., J. Clin. Invest., 103, 1231-1236, 1999). Such diseases as geriatric muscular degeneration, diabetic retinopathy, premature infant’s retinopathy, neovascular glaucoma and corneal neovascularization are caused, at least partially, by angiogenesis (Adamin A.P., et al., Angiogenesis, 3, 9-14, 1999). Diabetic retinopathy, in particular, is a diabetic complication which leads to blindness through capillary vessel penetration into the vitrina.

The ocular tissue contains the least amount of blood vessels among all tissues, and abnormal blood vessel growth in the ocular tissue leads to blindness. Because there is no suitable treatment available for ophthalmological diseases caused by angiogenesis, only steroid or antibiotic treatment is practiced, together with cauterity or photocoagulation at an advance stage of the disease. However, these treatments are temporarily effective and the symptom recurs because of the failure to prevent angiogenesis. Therefore, an effective therapy for such diseases must be based on the inhibition of angiogenesis.

Psoriasis, represented especially by flush macula and scaly skin, is one of the proliferative disorders in the skin, and if not cured, it can cause pain and
malformation. Normal horny cells usually divide once a month, but psoriatic skin cells, at least once a week. If the horny cell grows rapidly, angiogenesis takes place to supply blood (Folkman J., *J. Invest. Dermatol.*, 59, 40-48, 1972).

Angiogenesis is critical to the growth and metastasis of cancer cell. If angiogenesis is inhibited and the supply of blood is prevented, cancer cells grow to a size of about 1 - 2 mm in diameter and remain localized (Folkman and Tyler, Cancer Invasion and Metastasis; Biologic Mechanisms and Therapy[S.B. Day ed.], Raven press, New York, p94-103, 1997).

Hitherto, there have been reported such angiogenesis inhibitors as the fumagillin and its derivative called AGM-1470 which inhibit the vascular endothelial cell growth, platelet factor-4 and its synthetic peptide, herbinycin A and the collagen inhibitory tetracycline antibiotics.

Although arginine deiminase has been reported to inhibit the growth of cancerous cells in vivo and in vitro (Takaku et al., *Int. J. Cancer*, 51, 244-249, 1992; Komada et al., *Int. J. Hematol.*, 65, 129-141, 1997; Misawa et al., *J. Biotechnol.*, 36, 145-155, 1994; Miyazaki et al., *Cancer Res.*, 50, 4522-4527, 1990; Sugimura et al., *Melanoma Res.*, 2, 191-196, 1992), it has not yet been disclosed as an angiogenesis inhibitor.

The bioavailability of a protein drug is generally low because it is easily hydrolyzed and degraded by enzymes in vivo after it is administered, and if an immune response is induced by repeated administration thereof, life-threatening hypersensitivity may develop; furthermore, its clearance is enhanced by the reticuloendothelial system (RES).

U.S. Patent No. 4,179,337 discloses a peptide-polymer complex prepared by linking a peptide or polypeptide to a polyethylene glycol (hereinafter, PEG) having a molecular weight of 500-20,000 or a water-soluble polymer. The in vivo biological activity of this complex remains high while the immune response against the complex is suppressed.

Abuchowski et al. have shown that the in vivo half-lives of various PEG-conjugated proteins are prolonged and their immunogenicities were low in the plasma (Abuchowski et al., *Cancer Biochem. Biophys.*, 7, 175-186, 1984), and Davis et al. have demonstrated that polyethylene glycol-uricase complex has an
increased half-life and the side effect during the metabolism of uric acid is reduced (Davis et al., *Lancet.*, 2, 281-283, 1981). These results suggest that biologically active peptides or proteins, when conjugated to PEG, exhibit prolonged half-lives, increased solubilities and reduced immune responses.

Arginine deiminase has been known as an anti-cancer agent, but not as an inhibitor of angiogenesis. The present inventors have identified for the first time that arginine deiminase has inhibitory activity against angiogenesis. Arginine deiminase obtained from microorganisms or prepared by a genetic recombination technique, or said arginine deiminase conjugated to polymers like PEG, may have an extended *in vivo* half-life, reduced immunogenicity, and high activity of angiogenesis inhibition. The present inventors have thus found that arginine deiminase may be advantageously used in a pharmaceutical composition for inhibiting angiogenesis.

**Summary of the Invention**

The primary object of the present invention is to provide a novel use of arginine deiminase having inhibitory activity against angiogenesis for preventing and/or treating various diseases related to angiogenesis.

**Brief Description of the Drawings**

The above and other objects and features of the present invention will become apparent from the following description of the invention taken in conjunction with the following accompanying drawings; which respectively show:

Figs. 1a to 1d show the effect of arginine deiminase treatment on the tube formation of HUVEC in Matrigel, wherein Fig. 1a represents, untreated, and Fig. 1b to 1d, treated with 10 μg/ml, 1 μg/ml and 0.4 μg/ml of arginine deiminase, respectively;

Fig. 2 provides a graph showing the inhibiting effect of arginine deiminase
on tube formation of HUVEC;

Fig. 3 presents a photograph showing angiogenesis inhibition by arginine
deminase in the Chorioallantoic membrane (CAM) assay;

Fig. 4 shows the result of measuring angiogenesis inhibition by arginine
deminase in the mouse Matrigel model;

Fig. 5 describes the PCR-directed mutagenesis procedure of arginine
deminase from *Mycoplasma arginini*;

Fig. 6 illustrates the base sequence of arginine deminase obtained in Fig. 5;

Fig. 7 demonstrates the SDS-PAGE analysis to measure the molecular weight
of the overexpressed recombinant thioredoxin-linked arginine deminase
(67kDa) and the purified arginine deminase (45 kDa);

Fig. 8 shows the native-PAGE analysis to confirm the dimer (90 kDa) of
arginine deminase;

Fig. 9 displays the variation in activity of recombinant arginine deminase
with changing the pH;

Fig. 10 shows the variation in activity of recombinant arginine deminase
with changing temperature;

Fig. 11 shows the activity of recombinant arginine deminase at 41°C with
changing incubation time;

Figs. 12a and 12b show the effect of PEG-arginine deminase treatment on
the tube formation of HUVEC on Matrigel, wherein Fig. 12a represents
untreated, and 12b treated with 10 µg/ml of PEG-arginine deminase
respectively;

Figs. 13a and 13b show the inhibitory mechanism of PEG-arginine
deminase in the HUVEC tube formation. Fig. 13a shows the effect when treated
with 1 µg/ml of PEG-arginine deminase and Fig. 13b shows the effect when
treated with PEG-arginine deminase 1 µg/ml and arginine;

Fig. 14 is the result of the CAM assay showing the inhibitory effect of PEG-
arginine deminase on angiogenesis;

Fig. 15 is a graph showing the inhibitory effect of PEG-arginine deminase
on angiogenesis in the mouse Matrigel model.
Detailed Description of the Invention

In accordance with the above object, the present invention provides a pharmaceutical composition comprising arginine deiminase as an active ingredient for inhibiting angiogenesis.

In the above-mentioned pharmaceutical composition, available arginine deiminase may be obtained from microorganisms such as Mycoplasma sp. or prepared by a genetic recombination method.

Further, pharmaceutical composition of the present invention may comprise complexes wherein arginine deiminase is conjugated with activated polymers such as PEG; the enzyme is either obtained from a microorganism or prepared by a genetic recombination method.

In order to provide arginine deiminase from a microorganism such as Mycoplasma sp., the present inventors isolated arginine deiminase from Mycoplasma arginini by conducting ion-exchange chromatography and affinity chromatography, and measured its activity.

Also, the present inventors isolate the genomic DNA from Mycoplasma arginini, conduct PCR, and clone the arginine deiminase gene to an expression vector plasmid. The tryptophan codon (TGA) specific to Mycoplasma sp. is replaced with TGG to overexpress arginine deiminase in E. coli. The arginine deiminase gene thus obtained is cloned to an E. coli overexpression vector, and an E. coli cell is transformed with this vector. The transformants are isolated, cultured in a large scale, and then, arginine deiminase is overexpressed for purification. Accordingly, the present invention provides the recombinant arginine deiminase protein encoded in the polynucleotide of SEQ ID No.: 9, or its equivalents and a derivative thereof.

In addition, arginine deiminase, purified or prepared by a genetic recombination method, is mixed with various activated polymers and the mixture is stirred to produce various polymer-ADI complexes.

In the present invention, one or more polymers selected from such watersoluble polymers as polyethylene glycol (PEG), polypropylene glycol (PPG), polyoxyethylene (POE), polytrimethylene glycol, polylactic acid and its
derivative, polyacrylic acid and its derivative, polyphosphazenes, poly[L-lysine],
polyalkylene oxide (PAO) and polysaccaride, and such nonimmune polymers as
dextran, polyvinyl pyrrolidone, polyvinyl alcohol(PVA) and polyacryl amide
could be used to offer a complex that has angiogenesis inhibitory activity, by
conjugating thereto arginine deiminase, purified or prepared by genetic
recombination method.

In the present invention, polymers having a molecular weight in the range
of 200 to 100,000, preferably 1,000 to 45,000, may be used in the reaction.
The molar ratio of enzyme to activated polymer in the synthesis of the
arginine deiminase-polymer complex of the present invention may range from
1:1 to 1:100, preferably from 1:1 to 1:50. The complexes linked one to 30
polymers to one enzyme molecule may be produced.
The conjugation reaction between enzyme and activated polymer may be
carried out at a temperature ranging from 0 to 25°C in 0.1 M phosphate buffer
with pH 6 to 9 for a period of several minutes to 12 hours.
The activation of a polymer is conducted as follows; an unactivated
polymer is converted into a polyalkylene oxide( PAO) form as monomethoxy-
poly[ethylene glycol] (mPEG) and the other end-group of this PAO is converted
into a reactive functional group to obtain an activated form of the polymer. The
activated polymer is reacted with the α-amine group in the lysine residue of the
enzyme to form the enzyme-polymer complex. In addition to the amine group of
the lysine, the carboxyl group, activated carbonyl group, oxidized sugar or
mercapto group of the enzyme may be used as the conjugation site of the
polymer.

The extent of the tube formation in the human vascular endothelial cells
is measured to evaluate the inhibitory effect of arginine deiminase, purified or
prepared by the recombination method as mentioned above, and the PEG-
conjugated arginine deiminase. Also, the inhibitory effect of arginine deiminase
on angiogenesis is determined by using the CAM assay and the mouse Matrigel
model, two in vivo methods to measure the angiogenesis inhibitory effect.

Namely, human umbilical vein endothelial cells (HUVEC) are cultured
on the gelified matrigel and then vascular formation, which is a process of
angiogenesis, is induced. It is shown that the tube formation is strongly inhibited and the tube can not be formed when 10 μg/ml, 1 μg/ml or 0.4 μg/ml of arginine deiminase is added.

It is also confirmed that arginine deiminase inhibits angiogenesis in the CAM assay, an in vivo method to measure angiogenesis.

Therefore, the pharmaceutical composition comprising arginine deiminase as an active ingredient may be used as a drug for preventing and treating angiogenesis-dependent diseases.

The above-mentioned pharmaceutical composition is effectively used for treating not only arthritis, but also many angiogenesis-dependent ophthalmologic diseases, such as diabetic retinopathy, premature infant's retinopathy, neovascular glaucoma, involutional macula, degeneration of macula, pterygium, retinal degeneration, retrolental fibroplasias, granular conjunctivitis and corneal disease. This composition is also useful for preventing and/or treating angioma, angiofibroma, and some dermatologic diseases such as psoriasis, telangiectasis, pyogenic granuloma, seborrheic dermatitis and acne, and cancer metastasis.

As mentioned above, arginine deiminase, isolated from a microorganism or obtained by genetic recombination method, and a complex thereof conjugated to an activated polymer may be mixed with general carriers. In order to prepare oral formulations such as tablets, capsules, pills, granules, suspensions and solutions, the formulations for injection such as solutions or suspensions, or dried powders that may be mixed with distilled water before injection, the locally-applicable formulations such as ointments, creams and lotions, and other formulations in accordance with any of the conventional procedures in the pharmaceutical field.

Carriers generally used in the pharmaceutical field may be employed in the composition of the present invention. For example, orally-administered formulations may include binders, emulsifiers, disintegrating agents, excipients, solubilizing agents, dispersing agents, stabilizing agents, suspending agents, coloring agents or spicery. Injection formulations may comprise preservatives, unagonizing agents, solubilizing agents or stabilizing agents. Preparation for local administration may contain bases, excipients, lubricants or preservatives.
Any of the suitable formulations known in the art (Remington's Pharmaceutical Science [the new edition], Mack Publishing Company, Eaton PA) may be used in the present invention.

The inventive pharmaceutical composition can be clinically administered as various oral and parenteral formulations. A suitable formulation may be prepared using such excipients as additives, enhancers, binders, wetting agents, disintegrating agents and surfactants, or diluents. Solid formulations for oral administration include pills, tablets, dusting powder, granules and capsules. Those solid formulations may be prepared by mixing one or more excipients, e.g., starch, calcium carbonate, sucrose, lactose and gelatin with dibenzylbuthylacton lignan derivatives. Also, lubricants such as magnesium stearate and talc may be included in the present formulation. Liquid formulations for oral administration include suspension, solution, emulsion and syrup. Those formulations may contain wetting agents, sweeteners, aromatics and preservatives, in addition to general simple diluents such as water and liquid paraffin. Formulations for parenteral administration include sterilized aqueous solution, suspension, emulsion, freeze-dried alternative treatment and suppositories. Water-insoluble excipients and suspending agents comprise vegetable fats such as propylene glycol, polyethylene glycol and olive oil, and injectable esters such as ethyl oleate. Witepsol®, Macrogel, Tween® 61, cacao fats, laurin fats and glycerogelatin may be used as bases of suppositories.

The pharmaceutical composition may be administered orally or via parenteral routes such as intravenous, intramuscular, subcutaneous, intraabdominal, sternal and arterial injection or infusion, or topically through rectal, intranasal, inhalational or intraocular administration.

The typical daily dose of arginine deiminase of the present invention may range from 0.05 to 200 mg/kg body weight, preferably from 0.1 to 100 mg/kg body weight and can be administrated in a single dose or in divided dose. However, it should be understood that the amount of the active ingredient actually administrated ought to be determined in light of various relevant factors including the conditions to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's
symptom. Therefore, the above dose should not be construed as a limitation to the scope of the invention in any way.

The following examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Purification of Arginine deiminase

*Mycoplasma arginini* was inoculated and cultured in PPLO broth containing 20% horse serum, 2.5% yeast extract and 1% L-arginine to obtain arginine deiminase thererfrom.

The culture was centrifuged at 15,000 g for 20 minutes and the precipitated cells were washed twice with 30 ml of the 10 mM potassium phosphate, pH 7.0. The resulting cell precipitates were suspended in 10 ml of 10 mM potassium phosphate buffer, pH 7.0, sonicated at 20 kHz in an ice bath for 15 minutes, and centrifuged to collect the supernatant. The resulting supernatant was subjected to the following purification steps carried out in a cold chamber maintained at 4°C.

First, the cell lysate was passed through a DEAE anion exchange column (Amersham Pharmacia, Cat. No.: 17-0709) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was then washed with a sufficient amount of buffer (three times column volume) to minimize the non-specific binding, and fractionated under a 0 - 1 M NaCl salt gradient. The arginine deiminase activity of each fraction was measured to identify active fractions.

The active fractions were pooled and precipitated with 0 - 80% ammonium sulfate to obtain pellets. The pellets were dissolved in 1 M ammonium sulfate, passed through a phenyl sepharose column (Amersham Pharmacia, Cat. No.: 17-0965) pre-equilibrated with 1 M ammonium sulfate, and fractionated under a reverse-phase salt gradient of 1 - 0 M ammonium sulfate. The activity of each fraction was measured, active fractions were combined and dialysed for 14 hours in 10 mM potassium phosphate buffer.

The resulting dialysate was passed through an arginine-sepharose affinity
column (Amersham Pharmacia, Cat. No.: 17-0524) pre-equilibrated with 10 mM potassium phosphate buffer, washed with the same buffer, and subjected to a salt gradient of 0 - 1.5 M NaCl. The activity of each fraction was measured and the fractions containing pure arginine deiminase were combined, concentrated and desalted by using an ultrafiltration unit (YM10).

Example 2: Measurement of Arginine Deiminase Activity

Arginine deiminase obtained from *Mycoplasma arginini* is an enzyme having activity to remove the imine group from arginine to produce citrulline. The activity of arginine deiminase may thus be estimated by measuring the citrulline level produced in the reaction mixture.

The level of the citrulline produced from arginine deiminase was measured by the colorimetric determination described by Boyde and Rahmatulalah (Boyde T.R. and Rahmatulalah M., *Anal. Biochem.*, 107, 424-431, 1980). An aliquot (0.1 ml) of the enzyme solution and 10 mM L-arginine were added to 1 ml of 0.1 M potassium phosphate buffer, pH 7.0 and the resulting solution was incubated at 37°C for 5 minutes. After the reaction was complete, the reaction mixture was deproteinized with 5% TCA solution and centrifuged.

A 0.1 ml portion of the supernatant was transferred to a new tube, 2 ml of a ferric acid solution (550 ml of deionized water, 200 ml of concentrated phosphoric acid, 250 ml of sulfuric acid and 150 ml of ferric chloride) and 1 ml of a diacetyl monoxime solution (5 mg of thiosemicarbazide, 50 ml of deionized water and 250 ml of diacetyl monoxime) were added thereto. The mixture was heated in a boiling water bath for 5 minutes, cooled to room temperature, and the optical density at 530 nm was measured. 1 mM citrulline was diluted and used as a standard solution.

A total 7 mg of protein was obtained from 600 mg of *Mycoplasma arginini* and 250 µg of pure arginine deiminase was recovered therefrom by way of conducting chromatography using DEAE-sepharose, phenyl-sepharose and arginine-sepharose columns. Defining one unit as the amount of the enzyme needed to convert 1 µmole of arginine into citrulline at 37°C in 1 minute, the
specific activity of the purified arginine deiminase was found to be 31.36 units/mg of the protein. The conversion reaction was carried out in 0.1 M potassium phosphate buffer, pH 7.4 with 10 mM L-arginine.

Example 3: Production of Recombinant Arginine Deiminase

(3-1) Substitution of the Codon Usage of the Arginine Deiminase gene

The genomic DNA was isolated from *Mycoplasma arginini*, ATCC 23243 and subjected to a polymerase chain reaction (PCR) using primers specific to arginine deiminase gene, and the PCR product was cloned to pBluescript KS(+).

In *Mycoplasma arginini*, the TGA stop codon is specifically recognized as tryptophan (Misawa et al., *J. Biotechnol.*, 36, 145-155, 1994), and this TGA codon was replaced with TGG tryptophan codon in order to overexpress the arginine deiminase gene in *E. coli*.

The arginine deiminase gene has a total of 5 TGA codons. The last TGA codon was previously mutated by inserting TGG codon in the 3'-primer during the first PCR. The remaining four TGA codons were substituted by site-directed mutagenesis during the process of overlap extension by PCR with the antisense and sense oligonucleotides of SEQ ID No.'s.: 1 to 8, respectively, described by Steffan et al. (*Vet. Parasitol.*, 31, 269-273, 1989). The resulting product was cloned to the pBluescrip KS(+) and the DNA sequencing was carried out to confirm the sequence of the mutated sites (Fig. 6, SEQ ID No.: 9).

(3-2) Overexpression and Purification in *E. coli*

To overexpress the above recombinant arginine deiminase (designated ADI) gene in *E. coli*, the ADI gene was cloned to the BamH I and Xho I sites of pET-32a expression vector(Novagen, USA) to construct pET32a/ADI (Fig. 5). Plasmid pET32a/ADI was transformed into *E. coli* BL-21, and the transformed *E. coli* was isolated and then cultured in a large scale. The recombinant ADI was overexpressed by induction with 1 mM of IPTG, and analyzed by SDS-PAGE showing that the molecular weight of ADI was about 63kDa (Lane (a) of Fig. 7).
Almost all of the recombinant ADI were overexpressed in the form of an inclusion body. The inclusion bodies were collected, denatured in a guanidine-HCl solution and neutralized for 48 hours.

The neutralized ADI solution was subjected to ion-exchange and affinity chromatographies according to the method by Kang et al. (Kang et al., *Mol. Cells*, 10, 343-347, 2000), and active fractions were collected. When the collected fractions were analyzed by SDS-PAGE, it was found that the combined active fractions contained pure ADI protein of about 45 kDa (Lane (b) in Fig. 7), which was somewhat different from the molecular weight of the initially overexpressed ADI in *E. coli*.

The analysis of the N-terminal amino acid sequence of the purified ADI showed that a portion of the amino acid sequence in the S-Tag from the pET-32a was self-processed. The native-PAGE and sephacryl S-100 column (Amersham Pharmacia, Cat. No. 17-0612-01) chromatographic analyses also confirmed that ADI exists as a dimer having a molecular weight of 90 kDa (Fig. 8).

(3-3) Activity and Stability of the Recombinant ADI

The method of determining the amount of citrulline produced from arginine was applied to measure the activity of recombinant ADI (Boyde et al., *Anal. Biochem.*, 107, 424-431, 1980). The recombinant ADI had similar activity as the ADI purified from *Mycoplasma agrinini*, and optimal temperature was 41 °C in 20 mM potassium phosphate buffer solution, pH 7.4 (Fig. 9) and optimal pH was 6.4 (Fig. 10). Also, it remains stable with 70% of its original activity for 48 hours at 41 °C and pH 7.4 (Fig. 11).

Example 4: Effect of ADI on the Tube Formation of the HUVEC Cell on the Matrigel

To examine the effect of ADI on angiogenesis in an *in vitro* experiment, capillary vessel formation in the human vascular endothelial cells was studied. First, human umbilical vein endothelial cell (HUVEC) was isolated to carry out the tube formation experiment.
Endothelial cells of the vein were isolated from fresh human cords obtained during Caesarean operation and cultured on Matrigel. It was confirmed by immunocytochemical staining with the antibody against VIII factor that HUVEC had been successfully isolated. The resulting vascular endothelial cells were cultured on Matrigel (Matrigel, BD Bioscience, USA, Cat. No.354234). The endothelial cells formed two-dimensional tubes on gelified Matrigel. The reticular tube structure were shown when the endothelial cells were cultured at 37°C for 16 - 18 hours, and it may be considered as one of steps in angiogenesis. The effects of ADI on angiogenesis were observed when treated with 10 µg/ml, 1 µg/ml and 0.4 µg/ml of ADI. ADI of this experiment was purified from Mycoplasma arginini, and the results are shown in Fig. 2 and Table I.

Table I

<table>
<thead>
<tr>
<th>Concentration of ADI</th>
<th>Area of Tube (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>0.4 µg/ml</td>
<td>75.7</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>28.1</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>15.8</td>
</tr>
</tbody>
</table>

As the result shows, the tube formation was strongly inhibited by 10 µg/ml of ADI and the tube-forming cells were not properly shaped (Fig. 1b). When treated with 1 µg/ml or 0.4 µg/ml of ADI, the tube formation was suppressed as compared with the control (Fig. 1a), and exhibited disconnections in the tube (Fig. 1c and Fig. 1d). The tube formation was inhibited, relative to the control group, in a concentration-dependent manner. The area of tube formation in Table I was determined by using image analysis program Image-Pro Plus (Media Cybernetica, USA).

Example 5: CAM assay to measure angiogenesis

Fertilized chicken eggs were incubated for three days in an incubator
maintained at a relative humidity of over 70% at 37°C. From each egg, 2 – 3 ml of albumin was extracted using a 26 gauge syringe and the egg was sealed with a transparent adhesive tape to prevent drying, and then, a window of 1 x 1 cm size was made in the central region of the fertilized egg by drilling. 1 µg of recombinant ADI obtained in Example 3 was solubilized in 10 µg of distilled water, the resulting solution was dried on Thermanox disc (Miles Scientific co.), and laid on the chorioallantoic membrane of the egg exposed through the window. The egg was then sealed with a transparent adhesive tape and incubated for 3 days in the incubator.

To distinguish the blood vessels in the allantoic cavity from those distributed in the chorioallantoic membrane, Intralipid was injected into the allantoic cavity with a 26 gauge syringe to shield the blood vessels in the allantoic cavity, and the change of the blood vessels in the chorioallantoic membrane was observed.

As shown in Fig. 3, angiogenesis in the chorioallantoic membrane of the fertilized control egg was not affected but the formation of the capillary vessels was significantly inhibited by 1 µg of ADI and angiogenesis was inhibited to the extent of 88% when the fertilized egg was treated with 1 µg of ADI.

Example 6: Animal Experiment to Measure Angiogenesis (a Mouse Matrigel Model)

The inhibitory effect of ADI on angiogenesis was measured using in vivo mouse Matrigel model. In order to induce angiogenesis, a mixture of 0.4 ml of Matrigel (Collaborative Biomedical Products), 50 µg/ml of basic fibroblast growth factor (FGF) and 50 units/ml of heparin were injected subcutaneously to each 6 - 8 week-old C57BL/6 mouse (control group). For each mouse in the ADI-treated group, 8.9 µg/head of recombinant ADI obtained in Example 3 was included in the Matrigel.

After 3 - 5 days, the epidermis was removed, Matrigel was carefully recovered and the hemoglobin content was measured with the Drabkin reagent (Sigma). As shown in Table II, angiogenesis was almost completely inhibited
in the ADI-treated experimental group, as compared with the control group.

Table II

<table>
<thead>
<tr>
<th></th>
<th>Content of Hemoglobin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>66.4 ± 27.6</td>
</tr>
<tr>
<td>ADI-treated group</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

10 **Example 7: Production of PEG5000-ADI**

Monomethoxy-poly (ethylene glycol) was made from PEG 5000 (Shearwater Corp., Huntsville, Alabama, USA) according to the method described by Abuchowski et al. (*Cancer Biochem. Biophys.*, 7, 175-186, 1984), to protect one terminal hydroxyl group of PEG, and treated with phosgene and N-hydroxysuccinimide to obtain ester of succinyl-N-hydroxysuccinimide monomethoxy-poly (ethylene glycol) (designated SS-PEG). Activated SS-PEG (2 mg) solubilized in 0.1 M phosphate buffer, pH 8.0 was added to 0.2 mL of 5 mg/mL ADI purified from *Mycoplasma arginini* or prepared by the genetic recombination method in 0.1 M phosphate buffer, pH 8.0. The mixture was shaken for 30 minutes at room temperature, and 0.1 M glycine was included in the reaction mixture to stop the reaction. PEG5000-ADI was recovered after removal of unreacted ADI and PEG by dialysis in phosphate buffered saline (PBS), pH 7.4.

25 **Example 8: Production of branched PEG25000-ADI**

Branched PEG2-COOH (MW 5,000) (Shearwater Corp., Huntsville, Alabama, USA) was activated with succinimidyl succinate, and 5 mg of the activated branched SS-PEG25000 was used to make branched PEG25000-ADI, according to the procedure of Example 7.
Example 9: Production of PEG220000-ADI

Branched PEG2-COOH (MW 20,000) (Shearwater Corp., Huntsville, Alabama, USA) was activated with succinimidyl succinate, and 10 mg of the activated branched PEG220000 was used to make branched PEG220000-ADI, according to the procedure of Example 7.

Example 10: Production of PEG-ADI at various pH

6.8 µl of ADI (10 mg/ml) and 10 µl of SS-PEG5000 (10 mg/ml) were mixed in 0.1 M phosphate buffer having a pH in the range of 5 to 9, and then the mixture was shaken at room temperature for 30 minutes. The reaction was stopped with 5 µl of 1 M glycine. Unreacted ADI and PEG were removed by dialysis in PBS at pH 7.4 to obtain PEG-ADI.

Example 11: Production of PEG-ADI using various Molar Ratio of SS-PEG/ADI

Using 10 µl of SS-PEG solution and 6.8 µl of ADI(10 mg/ml), 4 mixtures having ADI : SS-PEG molar ratio of 1 : 10, 1 : 20, 1 : 50 and 1 : 100 were prepared and subjected to the procedure of Example 7.

Example 12: Measurement of PEG-ADI Enzyme Activity

The enzyme activities of PEG-ADIs produced in Examples 7 to 11 were measured by the method of Example 3. The result showed that all the PEG-ADI conjugates had an activity of over 80% based on that of unconjugated ADI.

Example 13: Effect of PEG-ADI on the Tube Formation in HUVEC

To examine the anti-angiogenic activity of PEG-ADI, an experiment was conducted using PEG5000-ADI as a representative PEG-ADI. Human HUVEC was treated with 10 µg/ml or 1 µg/ml of PEG-ADI and cultured on gelified
Matrigel at 37°C for 16 - 18 hours to observe its effect on the reticular tube formation of the endothelial cells. The result showed that the tube formation was strongly inhibited by 10 µg/ml of PEG-ADI and the tube-forming cells were deformed as compared with the control (Figs. 12a and 12b), and the tube formation was also strongly suppressed by adding 1 µg/ml of PEG-ADI. In order to see whether the inhibition of tube formation by PEG-ADI is due to the depletion of the arginine as a NO donor, 2 mM of arginine was added in a repeat run with 1 µg/ml of PEG-ADI (Figs. 13a and 13b). The inhibitory effect of PEG-ADI on tube formation in this run was reversed in the presence of arginine (Fig 13b): the extent of the formed tube was similar to that of the control group. Accordingly, it was confirmed that PEG-ADI inhibited the tube formation by depleting the arginine in the cell.

Example 14: the CAM Assay of PEG-ADI

The chorioallantoic membrane (CAM) assay of Example 5 was repeated to measure the in vivo inhibitory effect of PEG-ADI on angiogenesis.

The result showed that there was no change in the capillary blood vessels in saline-treated fertilized eggs, while angiogenesis inhibition was observed in 81% (n=16) of PEG-ADI-treated fertilized eggs (Fig. 14).

Example 15: the Inhibitory Effect of PEG-ADI on Angiogenesis in Animal Experiment (a Mouse Matrigel Model)

The inhibitory effect of PEG-ADI on angiogenesis was quantitatively measured as in Example 6 using the mouse Matrigel model. 2 µg of PEG-ADI was included in the Matrigel and compared with the control group. After 3 – 5 days, the epidermis was removed and the content of hemoglobin was measured in excised Matrigel. As shown in Table III, experimental group exhibited angiogenesis inhibition to the extent of 50% as compared with the control group (Fig. 15).
Table III

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As the above results demonstrated, both ADI, either purified from *Mycoplasma arginini* or prepared by the genetic recombination method, and PEG-ADI, inhibit angiogenesis in human HUVEC tube formation assay and mouse Matrigel model.

As shown in the above specific embodiments, the pharmaceutical composition of the present invention for inhibiting angiogenesis which comprises ADI as an active ingredient is remarkably effective in inhibiting the tube formation of human vascular endothelial cells and angiogenesis in *in vivo* tests such as the chorioallantoic membrane assay and the mouse Matrigel model. Accordingly, ADI may be advantageously used as a drug for preventing and treating angiogenesis-dependent diseases and it may be conjugated with an activated polymer such as PEG to be used as a pharmaceutical product.
What is claimed is:

1. A pharmaceutical composition for inhibiting angiogenesis which comprises arginine deiminase as an active ingredient.

2. The pharmaceutical composition of claim 1, which is used for treating or inhibiting a disease selected from the group consisting of: angioma, angiofibroma, arthritis, diabetic retinopathy, premature infant's retinopathy, neovascular glaucoma, corneal disease, involutional macula, degeneration of macula, pterygium, retinal degeneration, retroental fibroplasias, granular conjunctivitis, psoriasis, telangiectasis, pyogenic granuloma, seborrheic dermatitis, acne, cancer and metastasis.

3. The pharmaceutical composition of claim 1 or claim 2, wherein arginine deiminase is obtained from *Mycoplasma arginini*.

4. The pharmaceutical composition of claim 1 or claim 2, wherein arginine deiminase is a recombinant protein.

5. The pharmaceutical composition of claim 4, wherein the arginine deiminase is the protein encoded by the polynucleotide of SEQ ID No.: 9, its equivalent or a derivative thereof.

6. The pharmaceutical composition of claim 1 wherein the arginine deiminase is conjugated to an activated polymer.

7. The pharmaceutical composition of claim 6, wherein the polymer is selected from the group consisting of: polyethylene glycol, polypropylene glycol, polyoxymethylene, polytrimethylene glycol, polylactic acid and its derivative, polyacrylic acid and its derivative, polyamine acids, polyurethane, polyphosphazenes, poly[L-lysine], polyalkylene oxide, polysaccharide, dextran, polyvinyl pyrrolidone, polyvinyl alcohol and polyacryl amide.
FIG. 1c

FIG. 1d
FIG. 2

% Area of tube formation

control  0.4  1  10

Conc. of Arginine deiminase (ug/ml)
FIG. 5

PCR (ADI-5', ADI-3')

5' 3' ADI

pT7-Blue(R) T

Site directed mutagenesis (*) : TGA(Stop) → TGG(Trp)

B X

*p *** *
pBluescript KS(+)

Trx.Tag 6xHis S.Tag 6xHis

pET32a(+)

pT7-Blue(R) T

5' 3' ADI
FIG. 6

```
AGT GCC GGG ATT TTT AAA GCA ATT CAC GTT TAT TCA GAA ATT 51
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G E L E S V L V H E P G R E I D Y 34
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I T P A R L D E L L F S A I L E S 51
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H D A R K E H K Q F V A E L K A N 68
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D I N V V E L I D L V A E T Y D L 85
GCA TCA CAA GAA GCT AAA GA(1) AAA TTA ATC GAA GAA TTT TTA GAA GAC TCA 306
A S Q E A K D K L I E E F L E D S 102
GAA CCA GTT CTA TCA GAA GAA CAC AAA GTA GTT GTA AG(2) AAC TTC TTA AAA 357
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R F V F S N H P K L I N T P W Y Y 204
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FIG. 6 (continue)

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G M G N A R C M S M P L S R K D V
AAA ©C ©AG
K W

* nucleotide in circle: mutations in original clone,

* nucleotide in box: site-directed mutation for codon usage in E.coli

1233
420
(a) Recombinant ADI overexpressed in BL21
(b) Finally purified recombinant ADI
FIG. 11

Relative activity (100%)

Incubation time (hr)

- ⋄ 37°C
- ⋄ 41°C
- ▼ 55°C
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Artificial Sequence

site-directed mutagenesis product from polynucleotide coding
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DNA

Artificial Sequence

recombinant polynucleotide coding Mycoplasma Arginin Arginine Deiminase

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85  90  95

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## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 A61K 38/46**

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)

IPC 7 : A61K

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

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

CA On-Line

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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\[ Further documents are listed in the continuation of Box C. \]

\[ See patent family annex. \]

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

16 NOVEMBER 2001 (16.11.2001)

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

19 NOVEMBER 2001 (19.11.2001)

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

Korean Intellectual Property Office
Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon
Metropolitan City 302-701, Republic of Korea

Faximile No. 82-42-472-7140

**Authorized officer**

HAN, Hyung-Mee

**Telephone No.** 82-42-481-5601

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