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COMPOSITIONS FOR GENE THERAPY OF RHEUMATOID ARTHRITIS INCLUDING A GENE ENCODING AN ANTI-ANGIOGENIC PROTEIN OR PARTS THEREOF

TECHNICAL FIELD

The present invention relates in general to compositions for gene therapy of rheumatoid arthritis including a gene encoding an anti-angiogenic protein or parts thereof, and more particularly to a gene therapy for treating rheumatoid arthritis by constructing a recombinant vector including a gene encoding an anti-angiogenic protein or parts thereof and transplanting the recombinant vector or the cells transfected or transduced with the recombinant vector into the affected area of a patient, and compositions for the gene therapy.

BACKGROUND ART

Rheumatoid arthritis is a chronic inflammatory disease involving multiple joints. The main pathology of the affected synovial tissue consists of the hyperplasia and the subintimal infiltration of T and B lymphocytes. Such inflammation of the synovial tissue is thought to be caused by T lymphocyte reactive to an unknown

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autoantigen. Nonetheless, the T lymphocyte infiltrated in the almost tissues does not show any indication of activation on the surface of cell and also does not almost express cytokines. In contrast to this, it is observed that both synovial tissue and fluid are enriched with the cytokines derived from macrophage. These cytokines may include interleukin-1 (IL-1) which can accelerate the growth of synovial fibroblast and tumor necrosis factors (TNFs). These experimental results suggest the hypothesis that T lymphocyte is importantly associated with the induction of inflammation to synovial tissues and the inflammation is maintained by the cytokines derived from the activated synovial cells.

One of the major intents of rheumatoid arthritis treatment is to prevent the synovial tissue hyperplasia, because it forms the pannus tissue that irreversibly destroys the cartilage and bone in the affected joint. Effective drugs for treating rheumatoid arthritis have not been developed until the present time and the developed drugs can exhibit limited efficacies. Once arthritis occurs, it causes economic loss as well as severe pain.

Medical treatments of rheumatoid arthritis being used presently are as follows. The drugs used often for initial treatment are non-steroidal anti-inflammatory drugs (NSAIDs). These NSAIDs limitedly improve a patient's condition, but cannot prevent the cartilage destruction of joint area or the progress of disease. Moreover, half the patients using this treatment should stop the treatment within one year because of

serious side effect. Next, gold drugs such as gold sodium thiomalate and gold sodium thiosulfate, or disease modifying anti-rheumatic drugs (DMARDs) such as penicillamine and anti-malarials are used. These drugs also decrease the progress of rheumatoid arthritis, but after 5 years of the treatment using DMARDs, only 5-15% of the patients adhere to use the drugs because the serious side effect can be accompanied. If the drugs mentioned above are not effective any more, the affected joint area with rheumatoid arthritis should be replaced by artificial joint by surgical operation.

In this manner, most of the treatments of rheumatoid arthritis used until now were not designed with a particular target molecule and had a limitation of showing slight effects in most cases. In the meantime, it has been reported that therapeutic effects appeared by taking notice of inflammation-inducing cytokines such as TNF as a target molecule for treating the rheumatoid arthritis and introducing an antibody specific to TNF or a soluble TNF-receptor into the affected area of a patient to result in inactivation of the TNF (Maini, R.N. *et al., Immunol. Rev.* 144:195, 1995; Moreland, L.W. *et al., N. Engl. J. Med.* 337:141, 1997). In company with this, various gene transfer experiments *in vivo* are progressing in an animal model of rheumatoid arthritis with continuously expressing the molecules having an immune inhibitory function. Consequently, most of the current treatments are directed to correction of the immune aberration that supposedly drives the synovial cell proliferation.

Angiogenesis, the formation of new blood vessels, is one of the earliest histopathologic findings in rheumatoid arthritis and appears to be required for pannus development. This neovascularization is thought not only to maintain the chronic architectural changes via delivery of required blood-borne elements to the pannus, but also to play an active role in inflammation as a source of both cytokine and protease activity. The expanded vascular-bed volume resulting from angiogenesis may provide increased access for inflammatory cells to infiltrate the synovium. Although the factors specifically promoting angiogenesis in rheumatoid arthritis have not yet been identified, both synovial tissue and fluid are enriched with angiogenesis-promoting molecules. These include cytokines, such as basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and soluble adhesion molecules, such as E-selectin. These data suggested a therapeutic potential for using an anti-angiogenic procedure for favorably changing the disease course of rheumatoid arthritis.

Until now a lot of factors that repress angiogenesis have been found. Most of them are created from the cleavage of protoprotein, and representatively angiostatin, endostatin and platelet factor-4 and the like have been known.

Angiostatin is composed of 98^{th} to 440^{th} amino acids of plasminogen. Angiostatin was initially isolated from mice bearing a Lewis lung carcinoma and was identified as a 38-kDa internal fragment of plasminogen that encompasses the first four

kringles of the molecule (O'Relly, M. *et al.*, *Cell* 79:715, 1994). It was reported that the growth of primary tumors was inhibited effectively by injecting purified angiostatin hypodermically in six cancer model experiments (O'Relly, M. *et al.*, *Nat. Med.* 2:689, 1996).

Endostatin consists of C-terminal 183 amino acids of collagen XVIII and has an anti-angiogenic activity. It was reported that the growth of primary tumors was inhibited effectively by injecting purified endostatin hypodermically in four cancer model experiments (O'Relly, M. *et al.*, *Cell* 88:277, 1997).

Platelet factor-4 belongs to CXC cytokine family, which consists of chemotactic polypeptides below 10 kDa, and has an anti-angiogenic activity. It was reported that the platelet factor-4 inhibited the growth of cancer, such as B-16 melanoma and HCT-116 colon carcinoma (Maion, T. E. *et al.*, *Cancer Research*, 51:2077, 1991).

Angiogenesis is known to be associated with various diseases, such as tumor formation and metastasis, retinitis, angioma, chronic inflammation, intestinal adhesions, atherosclerosis, rheumatoid arthritis and so on, but it has not yet been verified that anti-angiogenic factors were effective to all the diseases listed above actually. Only the tumor inhibitory effects of these factors associated with a particular disease have been reported (USP 5,856,315, USP 5,733,876, USP 5,792,845, USP 5,854,205, USP 6,024,688). The US patents disclose that the treatment effects for various kinds of

diseases, such as ovarian carcinoma (HTB161, A2780S), colon carcinoma (MIP, CACO2), Lewis Lung Carcinoma (LLC), fibrosarcoma (T241), prostate gland carcinoma (PC-3) and breast carcinoma (MDA-MB), were identified by injecting anti-angiogenic factors with the type of recombinant protein.

Meanwhile, unlike general treating method that applies a toxicity to cells directly, the treating method, which cures diseases by inhibiting angiogenesis by means of supplying anti-angiogenic factors as described above, is based on the principle of inhibiting the cell growth, so anti-angiogenic factors over certain concentration should be supplied continuously to exhibit effects *in vivo*. But, the method of supplying anti-angiogenic factors with the type of recombinant proteins costs too much for administering proteins continuously, is troublesome and has a problem in that it imparts toxicity to a patient. Therefore, it has been required to develop a method of supplying anti-angiogenic factors to the affected area continuously and locally.

Accordingly, the present inventors have attempted new approaches for the probability of the treatment of rheumatoid arthritis by anti-angiogenesis in order to replace the prior treatment of rheumatoid arthritis having focused on solving the immunological problems. More particularly, the present inventors obtained the cell lines for producing representative anti-angiogenic factors such as angiostatin, endostatin

and platelet factor-4 through inserting their genes into a viral vector and then transplanting them the affected area of mice induced with rheumatoid arthritis. We also performed the histological examinations for the level of hyperplasia in synovial cell and cartilage destruction and the immunological examinations for the concentrations of cytokines associated with the joint inflammation, as well as macroscopic examination for joint swelling to obtain the results for the progressive level of rheumatoid arthritis. The results showed that the incidence of rheumatoid arthritis in our treatment was remarkably reduced in comparison with the control group. This is to show that our gene therapy using an anti-angiogenic gene is effective to treatment of rheumatoid arthritis.

DISCLOSURE OF THE INVENTION

It is therefore an object of the present invention to provide compositions for gene therapy of rheumatoid arthritis including a DNA encoding an anti-angiogenic protein or parts thereof. More particularly, the present invention provides a recombinant vector including a DNA encoding an anti-angiogenic protein or parts thereof, a cell into which the recombinant vector is introduced and compositions for the gene therapy of rheumatoid arthritis including the recombinant vector or the recombinant cell as an active ingredient.

To accomplish this object, the present invention provides compositions for gene

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therapy including a DNA encoding an anti-angiogenic protein or parts thereof, which shows therapeutic effects on rheumatoid arthritis.

According to the compositions of the present invention, the DNA encoding an anti-angiogenic protein or parts thereof can be provided with an inserted form in retroviral vector, adenoviral vector, adeno-associated viral vector, herpes simplex viral vector or plasmid that can be expressed in an animal cell, or with the form of a recombinant cell that is collected by transfecting or transducing a cell with the recombinant vector including the DNA in order to supply anti-angiogenic factor to the affected area continuously and locally.

Furthermore, the present invention provides a gene therapy of treating rheumatoid arthritis by delivering the compositions to the affected area of a patient.

As used herein, the term "anti-angiogenic gene" means a DNA encoding an anti-angiogenic protein or parts thereof. It is not limited to a natural DNA, but may include any forms of proper modifications under maintenance of anti-angiogenic activity and additions of elements for expression regulation if it can be used suitably for the purpose of the present invention, regardless of whether or not it is obtained by genetic engineering method or chemical method.

Hereinafter, the present invention will be described in detail.

The present invention is characterized in that the composition for gene therapy

of treating arthritis comprises a DNA encoding anti-angiogenic protein or parts thereof as an active ingredient. The anti-angiogenic gene used in the gene therapy for treating arthritis according to the present invention is preferably a gene encoding at least one protein selected from the group consisting of angiostatin, endostatin, platelet factor-4, thrombospondin-1, thrombospondin-2, METH-1, METH-2 (anti-angiogenic proteins having metalloprotease domain and thrombospondin domain; Vanzquez F. *et al., J. Biol. Chem.* 274(33):23349-57, 1999) and hepatocyte growth factor.

Particularly, the anti-angiogenic gene included in the composition for gene therapy of treating arthritis according to the invention is preferably the gene encoding the angiostatin including 98^{th} to 440^{th} amino acids of human plasminogen and the four kringles, the gene encoding the endostatin including 1334^{th} to 1516^{th} amino acids of human collagen XVIII, or the gene encoding the mouse platelet factor-4 protein.

Although an entire amino acid sequence may be used as the anti-angiogenic factor, a fragment known as having anti-angiogenic activity may also be used. For example, angiostatin kringle 1-3 fragment may be used for this purpose.

The example of the anti-angiogenic gene included in the composition for gene therapy of treating arthritis according to the invention may preferably be the DNA derived from human, mouse, rhesus, pig or bovine plasminogen, and more preferably the cDNA of human angiostatin. The endostatin gene may also preferably be the DNA derived from human, mouse, rhesus, pig or bovine collagen XVIII, and more preferably

the cDNA of human endostatin. The preferable embodiments of the invention identified that the angiostatin and endostatin genes cloned from human foreskin fibroblast (HFF) are effective to gene therapy for treating arthritis.

The recombinant vector included in the composition for gene therapy for treating arthritis according to the invention also comprises the anti-angiogenic gene. It is preferable that the recombinant vector further comprises a nucleotide sequence encoding a signal peptide required for secretion in the upstream or downstream in order to secrete the protein expressed by the gene out of the cell.

The signal peptide may include any signal peptides known as associated to protein secretion in eukaryotic cell, and may preferably be 18 of amino-terminal amino acids of human plasminogen represented as the SEQ ID No. 2 or the signal peptide of mouse immunoglobulin κ chain encoded by the nucleotide sequence represented as SEQ ID No. 13.

For example, as described below in one preferable example according to the invention, the signal sequence can be functionally connected to the anti-angiogenic gene by synthesizing a nucleotide including the nucleotide sequence encoding 18 of the amino-terminal amino acids of human plasminogen and the sequences having restrictive enzyme cleaving sites, hybridizing it into double helical DNA, and treating it with restrictive enzyme to insert it into a certain site of target vector. However, the construction of vector having a signal sequence for producing the secretion type of anti-

angiogenic protein is not limited to as mentioned above, but may be accomplished by the method well known in the field of this art.

The vector used in the composition for gene therapy of treating arthritis according to the invention is a vector in which the anti-angiogenic gene can be inserted to supply the anti-angiogenic factor continuously to the affected area. The vector may include virus-derived vectors such as retroviral vector, adenoviral vector, adeno-associated viral vector, and herpes simplex viral vector, plasmids capable of being expressed in bodies of animals such as pCXN2 (Gene, 108:193-200, 1991) and PAGE207 (Japan Patent Laid-Open No. Sho6-4684), and their modified vectors.

In the preferable example according to the present invention, a viral vector was prepared, which was to produce angiostatin, endostatin or platelet factor-4 as a anti-angiogenic factor, using MT5 retroviral vector which had been filed before Korean Industrial Property Office (KIPO) by the present inventors (Korean Patent Appl. No. 97-48095; KCCM-10205), and the effect for treating arthritis was verified by experimental results as to mouse using the viral vector. The MT5 retroviral vector is a vector based on murine leukemia virus (MLV) including mutant noncoding sequence of human elongation factor 1 (EF 1), without coding sequence derived from virus and is good in both external gene expressing efficiency and viral titer. That is to say, the vector is to enhance stability by completely removing the *gag*, *pol* and *env* sequence of MLV, is to include at the upstream of external gene insertion site, a part of noncoding

sequence of EF 1a as a noncoding sequence derived from heterogeneous gene for providing splicing receptor, and is to control splicing efficiency appropriately to maintain gene expressing highly with also maintaining virus producing concentration highly.

Particularly, in one example of the present invention, angiostatin DNA fragment encoding 93rd to 368th amino acids of human plasminogen represented as SEQ ID No. 2 was obtained from human foreskin fibroblast (HFF) through PCR and was inserted into pGEM T easy vector to construct pGEM T easy-hASTi vector. The sequence encoding 18 of amino-terminal amino acids of plasminogen of SEQ ID No. 2 was synthesized as a signal sequence, and was inserted into pGEM T easy-hASTi vector to construct pGEM T easy-hAST vector. The obtained vector was cleaved with BamHI to prepare human angiostatin gene fragment connected with the signal sequence, which was inserted into MT5 vector. In the meantime, to prepare cell lines expressing human angiostatin, MT5-hAST vector DNA was transfected into 293T cell with the plasmid expressing *gag-pol* and *env* gene of murine leukemia virus and then non-cellular virus was obtained from the cell culture media.

The obtained virus was transduced into NIH3T3, and then the cell lines introduced with retrovirus were selected and cultivated to prepare NIH3T3 cells expressing human angiostatin protein. By means of the same method, NIH3T3 cells expressing human endostatin protein and mouse platelet factor-4 protein were prepared

respectively.

Another example of the present invention verified the effect of gene therapy for arthritis using the cell lines respectively expressing anti-angiogenic proteins such as human angiostatin protein, human endostatin protein and mouse platelet factor-4 protein.

Particularly, a gene therapy to mice without any macroscopic signs of inflammation in collagen-induced arthritis mouse model was performed. Namely, anti-angiogenic protein-producing NIH3T3 cell lines mixed with PBS were transplanted in the knee joint area of rear leg of the arthritis-mouse, and then the progressive level of arthritis was investigated by measuring joint swelling, synovial hyperplasia, destruction of cartilage, and joint inflammation-associated cytokine level.

The results showed that the swelling level in the knee joint area in case of transplanting angiostatin-producing NIH3T3 cell lines was remarkably decreased to 27% in comparison that the control group in case of injecting only PBS or transplanting the cell lines transferred with only MT5 vector was 47% or 67%. The frequency showing the significant level of IL-1 was also remarkably decreased. The synovial hyperplasia and cartilage destruction in the knee joint area were also remarkably decreased. Furthermore, similar results to the transplant of antiostatin producing NIH3T3 cell lines were obtained when endostatin producing NIH3T3 cell lines and platelet factor-4 producing NIH3T3 cell lines were transplanted. These results show that the gene therapy using anti-angiogenic gene according to the present invention is

effective to suppression and treatment of arthritis.

In another example of the present invention, duration of therapeutic effects was measured. The result showed that therapeutic effect of a single injection lasts for 14 days after treatment.

The composition of gene therapy for treating arthritis according to the present invention can be prepared by preparing viral particles including recombinant DNA encoding anti-angiogenic protein or cell lines transduced with the viral particles and mixing them with carriers used in gene therapy (Crystal RG *et al.*, *Nature Genet.* 8:42-51, 1994).

The carriers used in gene therapy may include any carriers generally used in injection liquid. For example, the carriers may include distilled water, sodium chloride solutions, the mixtures of sodium chloride and inorganic salts or their similar mixtures, the solutions of materials such as mannitol, lactose, dextran, and glucose, amino acid solutions such as glycine and arginine, the mixtures of organic acid solutions or salt solutions and glucose solutions, and their similar solutions. The injection liquid may be prepared in the form of solution, suspension or colloidal solution by adding osmotic modulator, pH controller, vegetable oil such as sesame oil or bean oil, lecithin or surfactant such as non-ionic surfactant to the carriers in the conventional manners. This injection may be prepared in the form of powder or lyophilization and then dissolved in the form of solution before being used.

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The composition of gene therapy for treating arthritis according to the present invention may be dissolved in a sterilized carrier, in case of solid phase, if needed, before treatment of gene therapy, or may be used as it is without further treatment in case of liquid phase.

The composition of gene therapy for treating arthritis according to the present invention can be absorbed in the synovial cell of blood vessel of target area through the injection manner that the total daily dose of effective ingredient locally administered to a patient may range from 1 ng to 1 g or it may be administered once through catheter after surgical operation.

The cell line including anti-angiogenic factor producing vector may be any cell which has the major histocompatibility complex (MHC) antigen identical or similar to patient's such that it does not generate histoincompatibility during transplantation. That the synovial cell of patient's are separated and used for gene transfer is preferable for excluding histoincompatibility completely.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram that shows the process of preparing the pGEM T-hASTi vector by cloning human angiostatin gene through PCR and introducing the cloned gene into pGEM T easy vector, the process of preparing the pGEM T-hAST

vector by linking the signal sequence obtained through PCR functionally to the upstream of the angiostatin gene introduced in pGEM T-hASTi vector, and the process of preparing the MT5-hAST vector by introducing the angiostatin gene linked to the signal peptide into the *Bam*HI site of retroviral MT5 vector.

Fig. 2 is a schematic diagram that shows the structure of a double stranded DNA produced by hybridizing two synthetic nucleotides, which were prepared to have the nucleotide sequences represented by SEQ. ID. NO: 5 and SEQ. ID. NO: 6, and the amino acid sequence of the signal peptide encoded therefrom, in order to obtain a gene that encodes the signal peptide consisting of N-terminal 18 amino acids of human plasminogen.

Fig. 3 shows photographs illustrating the level of hyperplasia by staining with hematoxylin/eosin, knee joint area in the collagen-induced arthritis (CIA) mice, into which NIH3T3 cell line expressing angiostatin (B) and NIH3T3 control cell line transduced with only MT5 vector not including an angiostatin gene (A) are injected, respectively.

Fig. 4 shows photographs illustrating the level of cartilage destruction by staining with Safranin O/fast green, knee joint area in the collagen-induced arthritis (CIA) mice, into which NIH3T3 cell line expressing angiostatin (B) and NIH3T3 control cell line transduced with only MT5 vector not including an angiostatin gene (A) are injected, respectively.

Fig. 5 shows photographs illustrating the neovascularization level investigated using an antibody specific to CD31, which is surface protein of vascular endothelial cell by immunohistochemically staining knee joint area in the collagen-induced arthritis (CIA) mice, into which NIH3T3 cell line expressing angiostatin (B) and NIH3T3 control cell line transduced with only MT5 vector not including an angiostatin gene (A) are injected, respectively.

Fig. 6 is a graph showing the measurement results on duration of therapeutic effects of angiostatin-expressing NIH3T3 cells.

■ : control group

• : group transplanted with 1×10^5 of angiostatin-expressing NIH3T3 cells

* : P<0.05 ** : P<0.06

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will hereinafter be described in further detail by examples. It should however be borne in mind that the present invention is not limited to or by examples.

Example 1 : Construction of retroviral vector introduced with a gene encoding an anti-angiogenic protein

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(1-1) Construction of retroviral vector introduced with human angiostatin gene

(1-1-1) Cloning of human angiostatin gene (Construction of pGEM T easy-hASTi vector)

Human angiostatin gene was cloned by extracting RNA from the human foreskin fibroblast (HFF), obtaining cDNA, and performing PCR. First, RNA was extracted from the HFF cells by using the Tryzol method (Gibco BRL, USA), and cDNA was obtained from the extracted RNA by using reverse transcriptase polymerization reaction. Then, a DNA fragment including human angiostatin gene was obtained through the PCR by using the cDNA as a PCR template and the synthetic nucleotides represented by SEQ. ID. NO: 3 and SEQ. ID. NO: 4 as primers. To perform the PCR, total 100 μl of a mixture solution was prepared by mixing 1 μl of template DNA, each of 1 μl of 10 pmol/μl primer, 10 μl of 10 mM dNTP, 3.5 units of Expand high fidelity enzyme (Gibco BRL, USA) and 10 μl of buffer for enzymes. The reaction condition for PCR was as follows: 94°C for 1 min, 50°C for 1 min, 72 °C for 1 min 30 sec. The PCR was performed 30 cycles. The amplified angiostatin gene includes a DNA encoding 93^{rd} ~ 368^{th} amino acids of human plasminogen represented by SEQ. ID. NO: 2. The amplified PCR product of about 800 bp was introduced into a pGEM T easy vector (Promega, WI, USA) to construct a pGEM T easy-hASTi vector (See Fig. 1).

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(1-1-2) Linking of signal sequence and construction of retroviral MT5-hAST vector

To make the protein secreted out of cells after expressed from the human angiostatin gene cloned in the above Example (1-1-1), a nucleotide sequence encoding a signal peptide was linked functionally to the upstream of the angiostatin gene. The signal sequence was introduced into the pGEM T easy-hASTi vector. Particularly, to prepare a nucleotide sequence encoding N-terminal 18 amino acids of human plasminogen as a signal peptide, nucleotides represented by SEQ. ID. NO: 5 and SEQ. ID. NO: 6 were synthesized. The synthetic nucleotides were mixed, incubated for 1 min at 94 °C, and consequently incubated for 1 min 30 sec at 50°C to form a double stranded DNA by pairing the two synthetic nucleotides each other. As shown in Fig. 2, the double stranded synthetic nucleotides have *Hind*III and *Bam*HI sites in one end, and *Nhe*I site in the other end. The nucleotides were introduced into *Hind*III and *Nhe*I sites of the pGEM T easy-hASTi vector to construct a pGEM T easy-hAST vector. And then, the pGEM T easy-hAST vector was digested with *Bam*HI to obtain human angiostatin gene linked to the signal sequence, and the obtained human angiostatin gene was introduced into the *Bam*HI site of the MT5 vector (KCCM-10205) to construct a MT5-hAST vector (Fig. 1).

(1-2) Construction of retroviral vector introduced with human endostatin gene

(1-2-1) Cloning of human endostatin gene (Construction of pGEM T easy-hESTi vector)

Human endostatin gene was cloned by extracting RNA from the human foreskin fibroblast (HFF), obtaining cDNA, and performing PCR. A DNA fragment including human endostatin gene was obtained by the same method and under the same PCR condition as the Example (1-1-1) above, except for using the synthetic nucleotides represented by SEQ. ID. NO: 9 and SEQ. ID. NO: 10 as primers. The amplified endostatin gene includes a DNA encoding 1334^{th} ~ 1516^{th} amino acids of human collagen XVIII represented by SEQ. ID. NO: 8. The amplified PCR product of about 550 bp was introduced into a pGEM T easy vector (Promega, WI, USA) to construct a pGEM T easy-hESTi vector.

(1-2-2) Linking of signal sequence and construction of retroviral MT5-hEST vector

To make the protein secreted out of cells after expressed from the human endostatin gene cloned in the above Example (1-2-1), a nucleotide sequence encoding a signal peptide was linked functionally to the upstream of the endostatin gene. The signal sequence was introduced into the pGEM T easy-hESTi vector. Particularly, to prepare a nucleotide sequence encoding N-terminal 25 amino acids of mouse immunoglobulin

kappa chain as a signal peptide, PCR was performed by using the pSecTag plasmid (Invitrogen) DNA as a template, and synthetic nucleotides represented by SEQ. ID. NO: 11 and SEQ. ID. NO: 12 as primers. To perform the PCR, total 100μl of a mixture solution was prepared by mixing 1μl of template DNA, each of 1μl of 10pmol/μl primer, 10μl of 10 mM dNTP, 3.5 units of Expand high fidelity enzyme (Gibco BRL, USA) and 10μl of buffer for enzymes. The reaction condition for PCR was as follows: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min 30 sec. The PCR was performed 30 cycles. The signal sequence of the amplified mouse immunoglobulin kappa chain is represented by SEQ. ID. NO: 13. The amplified PCR product of about 120 bp was introduced into a pGEM T easy vector (Promega, WI, USA) to construct a pGEM T easy-SigPep vector. And then, the pGEM T easy-hESTi vector was digested with *Nhe*I / *Bam*HI to obtain endostatin gene, and the obtained endostatin gene was introduced into the *Nhe*I / *Bam*HI sites of the pGEM T easy-SigPep plasmid to construct a pGEM T easy-hEST vector. Consequently, the pGEM T easy-hEST plasmid was digested with *Bgl*II / *Bam*HI to obtain human endostatin gene linked to the signal sequence, and the obtained human endostatin gene was introduced into the *Bam*HI site of the MT5 vector (KCCM-10205) to construct a MT5-hEST vector.

(1-3) Construction of retroviral vector introduced with mouse platelet factor-4 gene

(1-3-1) Cloning of mouse platelet factor-4 gene (Construction of pGEM T easy-mPF4 vector)

Mouse platelet factor-4 gene was cloned by extracting RNA from the spleen of the C57BL/6 mouse (Japan SLC, Hamamatsu, Japan), obtaining cDNA, and performing PCR. First, RNA was extracted from the spleen tissue of the mouse by using the Tryzol method (Gibco BRL, USA), and cDNA was obtained from the extracted RNA by using reverse transcriptase polymerization reaction. Then, a DNA fragment including mouse platelet factor-4 gene was obtained through the PCR by using the cDNA as a PCR template and the synthetic nucleotides represented by SEQ. ID. NO: 15 and SEQ. ID. NO: 16 as primers. To perform the PCR, total 100μl of a mixture solution was prepared by mixing 1μl of template DNA, each of 1μl of 10pmol/μl primer, 10μl of 10 mM dNTP, 3.5 units of Expand high fidelity enzyme (Gibco BRL, USA) and 10μl of buffer for enzymes. The reaction condition for PCR was as follows: 94°C for 1min, 50°C for 1min, 72°C for 1min 30sec. The PCR was performed 30 cycles. The amplified platelet factor-4 gene is represented by SEQ. ID. NO: 14. The amplified PCR product of about 380 bp was introduced into a pGEM T easy vector (Promega, WI, USA) to construct a pGEM T easy- mPF4 vector.

(1-3-2) Construction of retroviral MT5- mPF4 vector

The pGEM T easy-mPF4 vector prepared in the Example (1-3-1) above was

digested with *EcoR*V to obtain the platelet factor-4 gene. The obtained platelet factor-4 gene was digested with *Bam*HI, treated with Klenow fragment, and then was introduced into the blunt-ended MT5 vector (KCCM-10205) to construct a MT5- mPF4 vector.

Example 2: Preparation of cell lines expressing anti-angiogenic protein

(2-1) Preparation of cell lines expressing human angiostatin

In order to prepare cell lines expressing human angiostatine, the retrovirus including the MT5-hAST vector was produced. At first, the MT5-hAST vector DNA prepared in the example (1-1-2) described above was transfected into 293 T cells with the plasmid (retrovirus packaging kit ampho catalogue #6161; TAKARA SHUZO CO., LTD., SHIGA, Japan) expressing *gag-pol* and *env* gene of murine leukemia virus (MLV) and after 8 hrs, new culture medium was exchanged. After 48 hrs, the cell culture medium was filtered with 0.45 μm of filter paper to obtain non-cellular virus.

The produced MT5-hAST retrovirus was transduced into NIH3T3 cells (ATCC CRL1658), and then the cells were treated with G418 (1㎍/㎕, Gibco BRL, USA) for 14 days after 24 hrs to select and cultivate the cell lines introduced with the retrovirus. Collecting the group of cells selected and cultivated gave rise to preparation of NIH3T3 cell lines expressing human angiostatin protein.

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(2-2) Preparation of cell lines expressing human endostatin

NIH3T3 cell lines expressing human endostatin protein was prepared according to the same method as the example (2-1), except using the MT5-hEST vector prepared in the example (1-2-2) instead of the MT5-hAST vector.

(2-3) Preparation of cell lines expressing mousce platelet factor-4

NIH3T3 cell lines expressing platelet factor-4 protein was prepared according to the same method as the example (2-1), except using the MT5-mPF vector prepared in the example (1-3-2) instead of the MT5-hAST vector.

Example 3: Efficacy of gene therapy using anti-angiogenic factors in mouse collagen-induced arthritis model

(3-1) Preparation of mouse collagen-induced arthritis model

Mouse collagen-induced arthritis (CIA) model is an autoimmune type of arthritis model which shows many characteristics in common with human rheumatoid arthritis. The collagen-induced arthritis model was prepared by the following method. DBA/1 mice (Jackson Laboratory, Maine, USA), aged 9-10 weeks at the start of experiments, were immunized intradermally at the base of the tail with bovine type II collagen (100 ㎍; Chondrex, WA, USA) emulsified in Freund's complete adjuvant (Gibco BRL, NY, USA). On day 21, the animals were boosted with an intradermal

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injection of 100 ㎍ type II collagen. Gradual onset of arthritis normally starts approximately 4 weeks after initial immunization. In this example, mice that did not yet have any macroscopic signs of arthritis were chosen at 30 days after the initial immunization to perform gene therapy.

(3-2) Measurement of arthritis progress level

Measurement and detailed decision on indices for measuring arthritis progress level were performed by the following manner. First, histological analysis was performed for the knee joint cavity in which the cell lines expressing anti-angiogenic protein (human angiostatin, human endostatin, and mouse platelet factor-4) were transplanted. The main phathology of rheumatoid arthritis may be summarized to consist of the hyperplasia of joint synovial cells and irreversible destruction of cartilage tissues due to the hyperplasia. Histological analysis is the most effective to decide the progress level of the synovial cell hyperplasia and cartilage destruction. For this analysis, knee joints of mice were dissected, fixed in 10% phosphate-buffered formalin, decalcified in 10% EDTA, and then embedded in paraffin. Standard frontal sections of 7 μm were prepared and stained with either hematoxylin/eosin or Safranin O/fast green (Bakker A.C. *et al., Arthritis and Rheumatism* 40:893-900, 1997; Apparailly Florence *et al., J. Immunol.* 160:5213-5220, 1998).

The hyperplasia levels of synovial cells were scored based on the results of

hematoxylin/eosin stain according to the criterion suggested in the published paper (Apparailly Florence *et al., J. Immunol.* 160:5213-5220, 1998; Lubberts Erik *et al., J. Immunol.* 163:4546-4556, 1999). The scores are 0 in normal condition, 1 in slight inflammation and faint synovial hyperplasia, and 2 in the formation of pannus and synovial hyperplasia heavier than severe level. It was decided that arthritis occurred in the knee if the hyperplasia level of synovial cell was 2 or more.

The levels of cartilage destructions were scored based on the results of Safranin O/fast green stain according to the criterion suggested in the published paper (Lubberts Erik et al., *J. Immunol.* 163:4546-4556, 1999). The scores are 0 in normal condition, 1 in vacuolation of cartilage cell and slight loss of proteoglycan, 2 in the observation of cartilage erosion in addition to the previous, and 3 in cartilage erosions at two or more regions and destruction of subchondral layer. It was decided that arthritis occurred in the knee if the level of cartilage destruction was 2 or more.

Neovascularization level in the joint area was investigated by performing immunohistochemical stain using the antibody specific to CD31, a surface protein of vascular endothelial cell (Xu, M. *et al., Molecular Genetics and Metabolism* 63:103-109, 1998).

The progress level of arthritis in the ankle area was determined with macroscopic sign such as joint swelling and immunological sign such as the expression amount of inflammation-associated cytokine. The levels of joint swellings were scored

based on the results of macroscopic signs according to the criterion suggested in the published paper (Apparailly Florence *et al., J. Immunol.* 160:5213-5220, 1998). The scores are 0 in normal condition, 1 in swelling and slight erythema, 2 in distint swelling and erythema, and 3 in severe edema accompanied with wound. It was decided that arthritis occurred in the ankle if the level of joint swelling was 2 or more. It was also decided to be a serious case of arthritis if the level was 3 or more. The detection of inflammation-associated cytokine was performed by measuring the expression amount of mouse interleukine-1 (IL-1), which was known to play an important role during the progress of arthritis, in the ankle joint with ELISA (R&D systems, Minneapolis, USA) according to the instruction of manufacturer. It was decided that arthritis occurred if the expression amount of interleukine-1 was 10 ng or more per 1g of proteins in the joint tissue.

(3-3) Implementation of gene therapy and analysis of experimental results

(3-3-1) In case of transplanting NIH3T3 cell lines expressing human angiostatin protein

In one group were selected 15 of mice. The NIH3T3 cell lines expressing human angiostatin were transplanted by mixing 1 x 10^5 of the NIH3T3 cell lines with 25 $\mu\ell$ of PBS solution in aseptic condition and injecting the mixture into the knee joint cavity of mouse using a syringe of 30 gauge. Into one negative control group, NIH3T3

cells transferred with only MT5 vector were transplanted, and into another negative control group, only PBS was injected. After that time, the arthritis progresses were observed. On day 10 after the transplantation of the cell lines, the progressive levels of arthritis were measured by the investigation of macroscopic sign such as joint swelling, histological signs such as joint synovial cell hyperplasia and cartilage destruction, and immunological sign such as the level of joint inflammation-associated cytokine. The neovascularization in the joint area was also investigated using immunohistochemical stain. The results are shown in Table 1 and Figs. 3 through 5.

Table 1. The incidences of arthritis for each item

	Ankle joint swelling (%)	Synovial cell hyperplasia (%)	Joint cartilage destruction (%)	IL-1 detection (%)
PBS	47	65	53	41
MT5	67	93	67	67
MT5-hAST	27	47	33	27

As shown in Table 1, in the results of joint swelling in the ankle of mouse rear leg, the level of joint swelling in case of transplanting the cells expressing angiostatin was remarkably decreased as compared to those in cases of control groups. Namely, when the incidence of arthritis was investigated based on the joint swelling, the incidence of arthritis was seen in 27% in case of transplanting the cells expressing

angiostatin, while the incidences of arthritis was respectively seen in 67% and 47% in cases of transplanting the control cell lines and of injecting only PBS. These results verified that the incidence of arthritis in the cells expressing angiostatin was significantly decreased.

In the results obtained through the investigation of synovial cell hyperplasia in the knee joint of mouse rear leg as shown in Fig. 3, the growth of synovial cell in case of transplanting the cells expressing angiostatin was remarkably decreased as compared to those in cases of the control groups. Namely, when the incidence of arthritis was investigated based on the synovial cell hyperplasia, the incidence of arthritis was seen in 47% in case of transplanting the cells expressing angiostatin, while the incidences of arthritis was respectively seen in 93% and 65% in cases of transplanting the control cell lines and of injecting only PBS. These results verified that the incidence of arthritis in the cells expressing angiostatin was significantly decreased.

In the results obtained through the investigation of the desctruction of cartilage tissue in the knee joint of mouse rear leg as shown in Fig. 4, the destruction of cartilage tissue in case of transplanting the cells expressing angiostatin was remarkably decreased as compared to those in cases of the control groups. Namely, when the incidence of arthritis was investigated based on the destruction of cartilage tissue, the incidence of arthritis was seen in 33% in case of transplanting the cells expressing angiostatin, while the incidences of arthritis was respectively seen in 67% and 53% in cases of

transplanting the control cell lines and of injecting only PBS. These results verified that the incidence of arthritis in the cells expressing angiostatin was significantly decreased.

In the results of the neovascularization level in the knee joint of mouse rear leg obtained by performing immunohistochemical stain using antibody specific to CD31, a surface protein in vascular endothelial cell as shown in Fig. 3, the level of neovascularization in case of transplanting the cells expressing angiostatin was remarkably decreased as compared to that in case transplanted with the control cell lines.

The concentration of IL-1 in the ankle joint of mouse rear leg in case of transplanting the cells expressing angiostatin into the knee of the same leg was remarkably decreased as compared to those in cases of the control groups. Namely, in the results obtained through the investigation of the frequency in case that the concentration of IL-1 is 10 ng or more per 1g of the joint tissue, the frequency was 27% in case of transplanting the cells expressing angiostatin, while the frequency was 67% in case of transplanting the control cell lines and 41% in case of injecting only PBS. These results verified that the frequency showing the significant level of IL-1 was remarkably decreased in the cells expressing angiostatin.

The total results described as above showed that the transplantation of the cells expressing angiostatin into the knee with occurrence of arthritis remarkably decreased the incidence of arthritis not only in the knee joint but also in the ankle joint of the same leg. These results may suggest that when a gene therapy is performed in a large joint

such as knee joint, the treatment effect can be also showed in a small joint such as ankle joint of the same leg by movements of inflammation-inducible or inflammation-inhibitory factors along with the flow of fluid connected between the joints.

(3-3-2) In case of transplanting NIH3T3 cell lines expressing human endostatin protein

In one group were selected 15 of mice. The NIH3T3 cell lines expressing human endostatin were transplanted by mixing 1 x 10^5 of the NIH3T3 cell lines with 25 μl of PBS solution in aseptic condition and injecting the mixture into the knee joint cavity of mouse using a syringe of 30 gauge. Into one negative control group, NIH3T3 cells transferred with only MT5 vector were transplanted. After that time, the arthritis progresses were observed, and macroscopic sign such as joint swelling was investigated. The results are shown in Table 2.

Table 2. The incidences of arthritis

	Ankle joint swelling (%)
MT5	75
MT5-hEST	23

As shown in Table 2, in the results of joint swelling in the ankle of mouse rear leg, the level of joint swelling in case of transplanting the cells expressing endostatin

was remarkably decreased as compared to that in case of the control group. Namely, when the incidence of arthritis was investigated based on the joint swelling, the incidence of arthritis was seen in 23% in case of transplanting the cells expressing endostatin, while the incidence of arthritis was seen in 75% in case of transplanting the control cell lines. These results verified that the incidence of arthritis in the cells expressing endostatin was significantly decreased. These results may also suggest that the gene therapy using endostatin can effectively inhibit the occurrence of arthritis.

(3-3-3) In case of transplanting NIH3T3 cell lines expressing mouse platelet factor-4 protein

In one group were selected 15 of mice. The NIH3T3 cell lines expressing platelet factor-4 were transplanted by mixing 1×10^5 of the NIH3T3 cell lines with 25 $\mu\ell$ of PBS solution in aseptic condition and injecting the mixture into the knee joint cavity of mouse using a syringe of 30 gauge. Into one negative control group, NIH3T3 cells transferred with only MT5 vector were transplanted. After that time, the arthritis progressions were observed. The progressive levels of arthritis were measured by the investigation of macroscopic sign such as joint swelling, histological signs such as joint synovial cell hyperplasia and cartilage destruction, and immunological sign such as the level of joint inflammation-associated cytokine. The results are shown in Table 3.

Table 3. The incidences of arthritis for each item

	Ankle joint swelling (%)	Synovial cell hyperplasia (%)	Joint cartilage destruction (%)	IL-1 detection (%)
MT5	64	77	54	64
MT5-hAST	29	43	36	29

As shown in Table 3, in the results of joint swelling in the ankle of mouse rear leg, the level of joint swelling in case of transplanting the cells expressing platelet factor-4 was remarkably decreased as compared to that in case of the control group. Namely, when the incidence of arthritis was investigated based on the joint swelling, the incidence of arthritis was seen in 29% in case of transplanting the cells expressing platelet factor-4, while the incidence of arthritis was seen in 64% in case of transplanting the control cell lines. These results verified that the incidence of arthritis in the cells expressing platelet factor-4 was significantly decreased.

In the results obtained through the investigation of synovial cell hyperplasia in the knee joint of mouse rear leg, the growth of synovial cell in case of transplanting the cells expressing platelet factor-4 was remarkably decreased as compared to that in case of the control group. Namely, when the incidence of arthritis was investigated based on the synovial cell hyperplasia, the incidence of arthritis was seen in 43% in case of transplanting the cells expressing platelet factor-4, while the incidence of arthritis was seen in 77% in case of transplanting the control cell lines. These results verified that the

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incidence of arthritis in the cells expressing platelet factor-4 was significantly decreased.

In the results obtained through the investigation of the destruction of cartilage tissue in the knee joint of mouse rear leg, the destruction of cartilage tissue in case of transplanting the cells expressing platelet factor-4 was remarkably decreased as compared to that in case of the control group. Namely, when the incidence of arthritis was investigated based on the destruction of cartilage tissue, the incidence of arthritis was seen in 36% in case of transplanting the cells expressing platelet factor-4, while the incidence of arthritis was seen in 54% in case of transplanting the control cell lines. These results verified that the incidence of arthritis in the cells expressing platelet factor-4 was significantly decreased.

The concentration of IL-1 in the ankle joint of mouse rear leg in case of transplanting the cells expressing platelet factor-4 into the knee of the same leg was remarkably decreased as compared to that in case of the control group. Namely, in the results obtained through the investigation of the frequency in the case that the concentration of IL-1 is 10 ng or more per 1g of the joint tissue, the frequency was 29% in case of transplanting the cells expressing platelet factor-4, while the frequency was 64% in case of transplanting the control cell lines. These results verified that the frequency showing the significant level of IL-1 was remarkably decreased in the cells expressing platelet factor-4.

The total results described as above showed that the transplantation of the cells

expressing platelet factor-4 into the knee with occurrence of arthritis remarkably decreased the incidence of arthritis not only in the knee joint but also in the ankle joint of the same leg.

Example 4: Measurement for duration of therapeutic effects

To measure the duration of therapeutic effect for NIH3T3 cell lines expressing angiostatin, the NIH3T3 cell lines were transplanted by mixing 1 x 10^5 of the NIH3T3 cell lines with 25 $\mu\ell$ of PBS solution in aseptic condition and injecting the mixture into the knee joint cavity of mouse using a syringe of 30 gauge. Into one negative control group, NIH3T3 cells transferred with only MT5 vector were transplanted.

The incidence of serious-grade arthritis in feet (as the assessment criteria of ankle joint swelling is 3 or more) was measured on every third or fourth day from the transplant day to the 20^{th} day. As shown in Fig. 6, the result showed that the occurrence of serious-grade arthritis was inhibited up to the 14^{th} day after transplantation ($P<0.05$). This means that the therapeutic effect can be maintained for 14 days after treatment with one injection.

INDUSTRIAL APPLICABILITY

The gene therapy for treating rheumatoid arthritis using the genes encoding anti-angiogenic factors provided by the present invention can significantly inhibit the

progress of arthritis not only in the macroscopic sign such as joint swelling, but also immunological and histological signs such as inflammation-associated cytokine level, synovial cell hyperplasia, and destruction of cartilage tissue. Accordingly, the present invention can be used to treat arthritis effectively in the situation that no effective treatment has been suggested for arthritis up to now.

What is claimed is:

1. A use of a DNA encoding an anti-angiogenic protein or parts thereof, for the manufacture of the medicament for gene therapy of rheumatoid arthritis.

2. The use of claim 1, wherein the anti-angiogenic protein is angiostatin, endostatin, platelet factor-4, thrombospondin-1, thrombospondin-2, METH-1, METH-2 or hepatocyte growth factor.

3. The use of claim 1, wherein the DNA encodes 93^{rd} ~ 368^{th} amino acids of plasminogen protein (SEQ.ID.NO: 2).

4. The use of claim 1, wherein the DNA encodes 1334^{th} ~ 1516^{th} amino acids of collagen XVIII protein (SEQ.ID.NO: 8).

5. The use of claim 1, wherein the DNA encodes platelet factor-4 protein represented by SEQ.ID.NO: 14.

6. The use of claim 1, wherein the DNA encoding an anti-angiogenic protein or parts thereof links with a nucleotide sequence encoding a signal peptide.

7. The use of claim 6, wherein the signal peptide has N-terminal 18 amino acids of human plasminogen (SEQ.ID.NO: 2).

8. The use of claim 6, wherein the signal peptide is a signal peptide of mouse immunoglobulin κ chain encoded by a nucleotide sequence represented by SEQ.ID.NO: 13.

9. The use of claim 1, wherein the DNA encoding an anti-angiogenic protein or parts thereof is provided with an inserted form in retroviral vector, adenoviral vector, adeno-associated viral vector, herpes simplex viral vector or plasmid that can be expressed in an animal cell.

10. The use of claim 9, wherein the retroviral vector is MT5 vector (KCCM-10205).

11. The use of claim 1, wherein the DNA encoding an anti-angiogenic protein or parts thereof is provided with the form of a recombinant cell that is collected by transfecting or transducing a cell with a recombinant vector including the DNA.

12. The use of claim 11, wherein the recombinant cell has an MHC antigen that is identical or similar to that of a rheumatoid arthritis patient.

13. The use of claim 11, wherein the recombinant cell is a synovial cell isolated from a rheumatoid arthritis patient.