Description

The present invention relates to the use of a human granulocyte colony stimulating factor (hereinafter abbreviated as "human G-CSF") for preparing a thrombus control agent.

The present invention aims at treating arterial and venous thrombi by utilizing human G-CSF which is one of the humoral factors that enhance the hematopoietic capability of the human body, in particular, the fission, proliferation and maturation of neutrophiles. None of the scholarly reports published to date have disclosures that have direct bearing to the use of human G-CSF as a thrombus control agent.

Human G-CSF has been known as a humoral hematopoietic factor that acts on precursor cells of granulocytes in in vitro experimental systems to promote their growth and differentiation into granulocytes [see, for example, Metcalf et al., Exp. Hematol., 1, 185 (1973)]. However, because of the extreme difficulty so far encountered in obtaining it, studies on the utility or effectiveness of human G-CSF as a medicine have not been fully conducted and it has been entirely unknown that human G-CSF has the potential to be used in the treatment of thrombi, which is the principal object of the present invention.

Heparin and sigmarol in various dosage forms have conventionally been used as thrombus control drugs that delay coagulation of blood but a need has also existed for the development of drugs that lyse fibrin clots. Recently, large-scale production of urokinase and a plasminogen activator (PA) which are capable of activating the fibrinolytic system has been established and these substances have come to be used as thrombus control agents.

At the same time, the recent advances in technology, in particular, genetic engineering have enabled the development of a process by which a pure and homogeneous human G-CSF can be produced in large quantities (see Ep-A-0 169 566 and Japanese Patent Public Disclosure Nos. 62-236497 and 62-236488). EP-A 0 169 566 discloses a colony stimulating factor (CSF) which can be used for promoting the differentiation and proliferation of human bone marrow cells to neutrophiles.

Based on this state of the art, the present inventors undertook studies on the possible effects of human G-CSF on vascular endothelial cells which produce PA capable of activating the fibrinolytic system. As a result, the present inventors have found that human G-CSF enhances the production of PA which is capable of activating the fibrinolytic system and that therefore it can be used as a thrombus control agent that has high efficacy and which yet causes limited side effects.

With a view to attaining the objective described above, the present inventors conducted intensive studies using mammalian endothelial cells. In the studies conducted on bovine carotid arterial endothelial cells and Rhesus monkey, the following facts were established:

(1) Bovine carotid arterial endothelial cells (hereinafter referred to simply as "endothelial cells") pre-incubated in the presence of human G-CSF synthesized and secreted about five times as much PA as when they were preincubated in the absence of human G-CSF;

(2) The PA production peaked when G-CSF was added in a concentration of about 50 ng/ml, which is substantially equal to an optimum concentration for the target cells in the hematopoietic system;

(3) As regards the promotion of PA synthesis and secretion by endothelial cells that were preincubated in the presence of human G-CSF, a significant correlation exists between the concentration of G-CSF addition and the period of preincubation;

(4) When fibrin gel was formed in a Petri dish and vascular endothelial cells were cultivated on the gel, PA was secreted in proportion as the cells grew in the presence of G-CSF and the plasminogen in the liquid culture was converted to plasmin, which lysed the fibrin gel; and

(5) When human G-CSF was administered continuously to Rhesus monkey which is akin to human and an analysis made on a thromboelastogram of the fresh blood taken after 2 weeks, the occurrence of fibrin lysis was observed immediately after blood coagulation, and this would probably be due to the activation of the fibrinolytic system.

These results suggest the possibility that human G-CSF acts on arterial and venous vascular endothelial cells and promotes their PA synthesis and secretion in such a way that the plasminogen present in large quantities in blood are converted to plasmin, with the resulting plasmin selectively lysing the fibrin clots in the blood. The present invention has been accomplished on the basis of this mechanism and provides a thrombus control agent containing human G-CSF as an active ingredient.

Fig. 1 is a graph showing the effect of human G-CSF on PA production by bovine vascular endothelial cells;

Fig. 2 is a graph showing the correlation between the time of preincubation in the presence of human G-CSF and PA production by bovine endothelial cells;

Fig. 3 shows the effect of human G-CSF on the promotion of the fibrinolytic activity of endothelial cells; and

Fig. 4 shows the time-dependent changes in TEG pattern upon continued administration of human G-CSF to Rhesus monkeys for 13 weeks.

The human G-CSF which is used as the active ingredient of the thrombus control agent of the present invention may derive from any origin as long as it has high purity. For example, a human G-CSF may be prepared by extracting, separating and purifying from a human viable sample. A human G-CSF may be isolated from the supernatant of the culture of human G-CSF producing cells. Alternatively, a human G-CSF may be recovered from human G-CSF producing hybridomas produced by cell fusion. The recombinant gene technology may be utilized in such a way that a host such as E. coli or animal cells is subjected to transformation and a human G-CSF is produced in the transformant and isolated and purified therefrom. If desired, the amino acid sequence of native human G-CSF may be chemically modified to produce a desired human G-CSF.

Particularly preferred examples of the human G-CSF that may be used in the present invention are the following two that have high purity and that can be produced in large volume:

(1) human G-CSF having the following physicochemical properties:

i) molecular weight: about 19,000 ± 1,000 as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;

ii) isoelectric point: having at least one of the three isoelectric points, pI = 5.5 ± 0.1, pI = 5.8 ± 0.1, and pI = 6.1 ± 0.1;

iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;

iv) amino acid sequence of the 21 residues from N-terminus:

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-
Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-

(2) human G-CSF containing either a polypeptide having the human granulocyte stimulating factor activity which is represented by all or part of the amino acid sequence shown below, or a glycoprotein having both said polypeptide and a sugar chain portion:

$(Met)_n$Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro
Gln	Ser	Phe	Leu	Leu	Lys	Cys	Leu	Glu	Gln	Val
Arg	Lys	Ile	Gln	Gly	Asp	Gly	Ala	Ala	Leu	Gln
Glu	Lys	Leu	(Val	Ser	Glu)$_m$	Cys	Ala	Thr	Tyr	Lys
Leu	Cys	His	Pro	Glu	Glu	Leu	Val	Leu	Leu	Gly
His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	Leu	Ser
Ser	Cys	Pro	Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly
Cys	Leu	Ser	Gln	Leu	His	Ser	Gly	Leu	Phe	Leu
Tyr	Gln	Gly	Leu	Leu	Gln	Ala	Leu	Glu	Gly	Ile
Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu	Asp	Thr	Leu
Gln	Leu	Asp	Val	Ala	Asp	Phe	Ala	Thr	Thr	Ile
Trp	Gln	Gln	Met	Glu	Glu	Leu	Gly	Met	Ala	Pro
Ala	Leu	Gln	Pro	Thr	Gln	Gly	Ala	Met	Pro	Ala
Phe	Ala	Ser	Ala	Phe	Gln	Arg	Arg	Ala	Gly	Gly
Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser	Phe	Leu

Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala
Gln Pro

(provided that m is 0 or 1; and n is 0 or 1).

The human G-CSFs described above may be produced by the methods shown in the Referential Examples to be given below. Stated specifically, the human G-CSF under (1) can be produced by the method described in Referential Example 1, whereas the human G-CSF under (2) can be produced by the method described in Referential Example 2 (when m = 0, the human G-CSF is conveniently designated -VSE, and when m = 1, it is designated +VSE).

For details of the respective process conditions to be employed in these methods, see the specifications of commonly assigned Japanese Patent Public Disclosure Nos. 61-22752, 62-236497 and 62-236488.

Another method that can be employed consists of performing fusion of a G-CSF producing cell with a self-proliferating malignant tumor cell and cultivating the resulting hybridoma in the presence or absence of mytogen.

All of the human G-CSFs that are obtained by the methods described above are included within the scope of the present invention.

The human G-CSF containing solution obtained may be stored frozen after aseptic filtration with a suitable filter medium such as a Millipore filter following further purification and concentration that may be performed as required by any known techniques. Alternatively, the solution may be stored after being dehydrated by such means as freeze-drying or vacuum drying.

If desired, the human G-CSF may be used as an injection after being dissolved in distilled water or an appropriate buffer solution.

For the purpose of formulating the thrombus control agent of the present invention in a dosage form adapted for administration to humans or animals, one or more of the following additives may be incorporated: a pharmaceutical carrier and excipient, as well as a stabilizer and an anti-adsorption agent.

The dose of the human G-CSF incorporated in the thrombus control agent of the present invention and the frequency of its administration may be determined in consideration of the severity of the disease to be treated. As guide figures, a formulation containing human G-CSF in an amount of 0.1 - 1,000 μg, preferably 1 - 500 μg per adult may be administered 1 - 7 times a week.

The present invention is hereunder described in greater detail with reference to referential examples (for the preparation of human G-CSF), experimental examples (for showing the pharmaceutical efficacy of human G-CSF) and examples (of pharmaceutical dosage forms).

Referential Example 1

Preparation of human G-CSF by cultivation of G-CSF producing cells:

G-CSF producing cell strain CHU-1 derived from human oral cancer cells was established by the method described in Example 1 provided in the specification of Japanese Patent Public Disclosure No. 61-227526. Similar cell strain CHU-2 (CNCM Accession Number I-483) was established by the same method. Each of these cell strains was suspended in a liquid culture RPMI 1640 containing bovine fetal serum, transferred into a roller bottle and whirl-cultured. When the cells grew to form a confluent layer on the inner surface of the roller bottle, the culture solution was replaced by a serum-free RPMI 1640. After 4-day culture, the supernatant of the culture was recovered and cultivation was continued with a serum-containing RPMI 1640 being added. After 3-day culture, the culture solution was again replaced by a serum-free RPMI 1640 and the supernatant of the culture was recovered 4 days later. By repeating these procedures, the supernatant of the culture was recovered. The so obtained serum-free supernatant of the culture was concentrated about 1,000 times by ultrafiltration and subsequently subjected to purification and assay by the same procedures as described in the examples provided in the specification of Japanese Public Disclosure No. 61-227526.

Referential Example 2

Preparation of human G-CSF by the recombinant gene technology:

The following description applies to the preparation of +VSE or -VSE depending upon whether m = 1 or 0 in the amino acid sequence described above.

A cDNA fragment harboring a human G-CSF gene that had been cut from Escherichia coli (E. coli) strain 1766 deposited by the assignee with the Fermentation Research Institute, the Agency of Science and Technology (FERM BP-955 for -VSE and FERM BP-954 for +VSE) was incorporated into a vector pdKCR to construct a plasmid pHGV2 (in the case of -VSE) or pHGG4 (in the case of +VSE). The plasmids were treated with SalI and subsequently reacted with a DNA polymerase Klenow fragment.

After attaching an EcoRI linker, this DNA was partially digested with EcoRI again and a fragment of ca. 2.7 kb was recovered by electrophoresis through an agarose gel.

In a separate step, a plasmid pAdD26SVpA [Kaufman, R.G. & Sharp, P.A. (1982) Mol. Cell. Biol., 2, 1304-1319] was treated with EcoRI and dephosphorylated by treatment with BAP. Subsequently, an EcoRI fragment of pAdD26SVpA was recovered by electrophoresis following treatment with phenol.

The 2.7 kb fragment and the pAdD26SVpA fragment thus obtained were annealed and used in transforming E. coli strain DHI by the rubidium chloride procedure to construct a plasmid pHGV2-dhfr (in the case of -VSE) or pHGG4-dhfr (in the case of +VSE).

CHO cells (dhfr$^-$ strain; courtesy of Dr. L. Chasin of Columbia University) were cultivated for growth in alpha-minimal essential medium containing 10% calf serum (α-MEN supplemented with adenosine, deoxyadenosine and thymidine) in plates (9 cm$^\phi$, Nunc). The cultured cells were transformed by the calcium phosphate procedure [Wigler et al., Cell, 14, 725 (1978)] in the following manner.

A carrier DNA (calf thymus DNA) was added in an appropriated amount to 1 μg of the previously prepared plasmid pHGV2-dhfr (in the case of -VSE) or pHGG4-dhfr (+VSE) and the mixture was dissolved in 375 μl of a TE solution, followed by addition of 125 μl of 1 M $CaCl_2$. After the solution was cooled on ice for 3 - 5 minutes, 500 μl of 2 x HBS (50 mM Hepes, 280 mM NaCl, and 1.5 mM phosphate buffer) was added to the solution. After re-cooling on ice, the solution was mixed with 1 ml of the culture of CHO cells, transferred onto plates, and incubated for 9 hours in a CO_2 incubator.

Following washing with TBS (Tris-buffered saline), addition of 20% glycerol-containing TBS, and re-washing, a non-selective medium (the α-MEN medium described above except that it was supplemented with nucleotides) was added. After 2-day incubation, a 10-fold dilution of the culture was transferred onto a selective medium (not supplemented with nucleotides). The cultivation was continued, with the medium being replaced by a fresh selective medium every 2 days, and the resulting colonies were selected and transferred onto fresh plates, where the cells grew in the presence of 0.02 μM methotrexate (MTX), followed by cloning through growth in the presence of 0.1 μM MTX.

As a result of further cloning procedures, it was confirmed that human G-CSF had been produced in an amount of at least 10 mg/ℓ (in the case of -VSE) or at least 1 mg/ℓ (in the case of +VSE).

Purification of human G-CSF and assaying of its activity were conducted by the procedures described in the examples provided in the specification of Japanese Patent Public Disclosure No. 62-236488.

Experimental Example 1

G-CSF triggered enhancement of plasminogen activator (PA) production by vovine carotid arterial endothelial cells:

Vascular endothelial cells collected from bovine carotid artery were dispersed in Eagle's minimum essential medium containing 20% bovine fetal serum, 50 unit/ml of penicillin and 50 μg/ml of streptomycin and the first-generation cultivation was conducted in a Petri dish at 37°C in a 95% air 5% CO_2 atmosphere. After a confluent layer formed on the bottom of the Petri dish, the cells were dispersed in a 0.05% trypsin solution and subcultured. In the next place, serial cultivation was conducted under the same conditions as in the first-generation cultivation except that the concentration of bovine fetal serum was changed to 10%. The resulting large volume of cells were cultivated for 2 days in a Petri dish using the already described Eagle's minimum essential medium containing 10% bovine fetal serum in the presence of the G-CSF prepared in Referential Example 2, with its concentration being varied from 0 to 300 ng/ml. Thereafter, the cells were washed with a medium containing neither G-CSF nor serum and further cultivated in a similar medium for 8 hours to prepare a conditioned medium. The activity of PA secreted in this conditioned medium was assayed by a coupled fluorometric method. In the first step, plasminogen was activated to plasmin by PA, and in the second step, the activity of the resulting plasmin was measured using t-butyloxycarbonyl-L-valyl-L-leucyl-lysine-4-methylcoumaryl-7-amide (BOC-Val-Leu-Lys-MCA) as a synthetic substrate. PA activity was expressed in terms of urokinase international unit (I.U.).

As will be clear from Fig. 1, the PA activity increased dramatically with the increasing addition of G-CSF and reached a plateau at a concentration of 50 ng/ml. As Fig. 2 shows, PA production increased with the lapse of pre-incubation time and substantially reached a maximum in 12 hours. The activity increase was

five times as great as that of the control group. These results show that G-CSF acts on vascular endothelial cells to promote their PA production by a marked degree.

In order to confirm that PA production increased not only extracellularly but also intracellularly, the following experiment was also conducted. After 2-day cultivation in the presence of G-CSF, the cells were washed and one half of them was homogenized with 0.5% Triton X-100 and the cellular extract obtained was assayed for its intracellular PA activity. The other half of the cells was cultivated for 8 hours in the manner already described and the resulting culture was assayed for the activity of extracellular PA secretion. The results are shown in Table 1 below, from which one can see that the intracellular activity of PA increased as much as the extracellular activity. When a protein synthesis inhibitor cycloheximide was added to the medium during the 2-day cultivation, both intracellular and extracellular PA production was completely inhibited in the subsequent period of cultivation for 8 hours.

These facts show that the PA production by vascular endothelial cells was appreciably enhanced by adding G-CSF to the medium.

Table 1

Intracellular and Extracellular PA Activities of Endothelial Cells Treated with Human G-CSF		
Treatment	PA Activity (urokinase I.U./10^8 cells)	
	Extracellular supernatant	Intracellular
Control	1.85 ± 0.45	2.59 ± 1.29
G-CSF (50 ng/ml)	9.09 ± 1.08	9.40 ± 3.30

Experimental Example 2

Promotion of the fibrinolytic activity of vascular endothelial cells by G-CSF:

A hundred microliters of thrombin (5 µ/ml) was added to 2.4 ml of a fibrinogen solution (13.5 mg/ml) in a culture dish and incubation was performed at 37°C for 3 hours to form a fibrin gel. On the gel, bovine carotid arterial endothelial cells were grown in Eagle's minimum essential medium containing 10% bovine fetal serum, penicillin (50 µ/ml) and streptomycin (50 µg/ml) in the presence of 500 µg/ml of the G-CSF obtained in Referential Example 2. An untreated control group was also cultivated under the same conditions. After 43 hours of the cultivation, a small lysis zone was observed in the control group as shown in Fig. 3 and this reflects nominal secretion of PA from the cells in the control group. A larger and more distinct lysis zone was observed in the cells of the treated group. G-CSF stimulated these cells to synthesize and secrete more PA, with consequent activation of the plasminogen in the culture to produce plasmin which lysed the fibrin gel.

These results show that human G-CSF acts on vascular endothelial cells to promote PA synthesis and secretion, with the secreted PA activating the surrounding plasminogen to produce plasmin, which then lyses fibrin which is the major component of thrombus.

The foregoing data establishes that human G-CSF stimulates vascular endothelial cells to promote PA release, is thereby enhancing the fibrinolytic activity of these cells.

Experimental Example 3

Enhancement of the fibrinolytic activity of Rhesus monkey by continued administration of human G-CSF:

After human G-CSF was administered continuously to Rhesus monkey, a peripheral blood sample was taken and its fibrinolytic activity was assayed with a thromboelastgraph (TEG) using a clot tracer Model TE-400 (Elmer). The result is shown in Fig. 4a, from which one can see that in the control group, the amplitude of a TEG pattern increased when coagulation started and the maximum amplitude (MA) of the pattern decreased gradually as fibrinolytic activity developed at a later time. In contrast, with the animals given subcutaneous injection of human G-CSF in an amount of 1 µg/kg, a marked enhancement of fibrinolytic activity occurred in the TEG pattern as early as 2 weeks after the injection (see Fig. 4b; also see Reference 1, "JISSEN SHIKETSU GYOKOGAKU", ed. by M. Fujimaki et al., pp. 189-191), revealing the decrease in

the elasticity of coagulation clot and fibrin clot in the process of blood coagulation. As Fig. 4c shows, the enhancement of fibrinolytic activity was more marked in the animals given continued administration of human G-CSF in an amount of 10 μg/kg [see Reference 2, "Analysis of Clot Tracer Patterns" in KISO TO RINSHO vol. 12, No. 6, June (1978), pp. 76-78]. As Reference 2 shows, when urokinase is added to a coagulated sample of normal human blood, lysis occurs to produce a single-lined pattern. A tendency which was entirely identical to this thromboelastogram was observed in almost all cases of human G-CSF administration.

The above results-show that human G-CSF was capable of achieving not only in vitro but also in vivo enhancement of fibrinolytic activity. In summary:

(1) vascular endothelial cells preincubated in the presence of human G-CSF synthesized and secreted about 5 times as much PA as when they were preincubated in the absence of human G-CSF;

(2) when human G-CSF and vascular endothelial cells were cultured on a fibrin gel, the synthesis and secretion of PA by the endothelial cells were promoted, with subsequent activation of the concomitant plasminogen to plasmin, which lysed the gel of fibrin which is the major component of thrombus (i.e., human G-CSF stimulated the vascular endothelial cells to liberate PA, thereby producing enhanced fibrinolytic activity); and

(3) analysis of a thromboelastogram of a peripheral blood sample taken from Rhesus monkey administered continuously with human G-CSF revealed enhancement of the fibrinolytic activity of the animal.

It was therefore established that human G-CSF is capable of achieving a marked enhancement of fibrinolytic activity both in vitro and in vivo.

Example 1

Polysorbate 20 (Tween 20®: polyoxyethylenesorbitan monolaurate) which was a nonionic surfactant was added to a 50 μg/ml solution of the human G-CSF prepared and purified in Referential Example 1 in 10 mM phosphate buffer (pH 7) so that the concentration of Polysorbate 20 became 0.1 mg/ml. After adjusting the osmotic pressure to be the same as that of the physiological saline, the solution was filtered through a membrane filter having a pore size of 0.22 μm to remove microorganisms, and charged into vials which had been sterilized. The vials were put on with sterilized rubber closures and then sealed with aluminum caps to give a liquid preparation for injection. The vials were stored in the dark at a temperature of lower than 10 °C.

Example 2

Polysorbate 80 (Tween 80®: polyoxyethylenesorbitan monooleate) which was a nonionic surfactant was added to a 100 μg/ml solution of the human G-CSF prepared and purified in Referential Example 2 in 10 mM phosphate buffer (pH 7) so that the concentration of Polysorbate 80 became 0.1 mg/ml. After adjusting the osmotic pressure to be the same as that of the physiological saline, the solution was filtered through a membrane filter having a pore size of 0.22 μm to remove microorganisms, and charged into vials which had been sterilized. The vials were put on with sterilized rubber closures and then sealed with aluminum caps to give a preparation for injection. The vials were stored in the dark at a temperature of lower than 10 °C.

Example 3

Polysorbate 20 (Tween 20®: polyoxyethylenesorbitan monolaurate) which was a nonionic surfactant, HSA, and mannitol was added to a 50 μg/ml solution of the human G-CSF prepared and purified in Referential Example 1 in 10 mM phosphate buffer (pH 7) so that the concentrations of Polysorbate 20, HSA and mannitol became at 0.1 mg/ml, 10 mg/ml and 50 mg/ml, respectively. The solution was filtered through a membrane filter having a pore size of 0.22 μm to remove microorganisms, and charged into vials which had been sterilized. The rubber closures are loosely fitted by the rubber insertion machine so that two gaps of the rubber closrue are exposed to allow for the freeze-drying process, and the charged solution was freeze dried. The vials were then sealed with the rubber closures and then with aluminum caps to give a freeze-dried preparation for injection. The preparation in the vials which can be stored at room temperature is used after reconstituting it with distilled water for injection.

Example 4

Polysorbate 80 (Tween 80®: polyoxyethylenesorbitan monooleate) which was a nonionic surfactant, gelatin, and mannitol was added to a 100 μg/ml solution of the human G-CSF prepared and purified in

Referential Example 2 in 10 mM phosphate buffer (pH 7) so that the concentrations of Polysorbate 80, gelation and mannitol became 0.1 mg/ml, 10 mg/ml and 50 mg/ml, respectively. The solution was filtered through a membrane filter having a pore size of 0.22 μm to remove microorganisms, and charged into vials which had been sterilized. The rubber closures are loosely fitted by the rubber insertion machine so that two gaps of the rubber closrue are exposed to allow for the freeze-drying process, and the charged solution was freeze dried. The vials were then sealed with the rubber closures and then with aluminum caps to give a freeze-dried preparation for injection. The preparation in the vials which can be stored at room temperature is used after reconstituting with distilled water for injection.

The thrombus control agent of the present invention which contains human G-CSF as an effective ingredient potentiates the ability of human vascular endothelial cells to produce PA, which ability is inherent in the human body, thereby converting the plasminogen in blood to its activated form (plasmin), which gives rise to fibrinolytic activity, with consequent dissolution of thrombus. Further, the number of neutrophiles increases as a result of G-CSF administration and they work in such a way as to promote the processing of lysed thrombus. These tow actions combine to effectively dissolve and eliminate thrombi. Therefore, the present invention provides a useful thrombus control agent with reduced side effects.

Claims

1. Use of a human granulocyte colony stimulating factor for preparing a thrombus control agent.

2. Use according to Claim 1 whereby the thrombus control agent is applicable to all thrombi that form in the arterial and venous vessels of a living body.

3. Use according to Claim 1 or 2, whereby said human granulocyte colony stimulating factor promotes the synthesis and secretion of plasminogen activator by arterial and venous vascular endothelial cells, said plasminogen activator forming plasmin to enhance the fibrinolytic activity of said endothelial cells.

4. Use according to any one of Claims 1 to 3, whereby said human granulocyte colony stimulating factor is obtained from the supernatant of a culture of human granulocyte colony stimulating factor producing cells.

5. Use according to any one of Claims 1 to 4, whereby said human granulocyte colony stimulating factor has the following physicochemical properties:
   i) molecular weight: about 19,000 ± 1,000 as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;
   ii) isoelectric point: having at least one of the three isoelectric points, pI = 5.5 ± 0.1, pI = 5.8 ± 0.1, and pI = 6.1 ± 0.1;
   iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;
   iv) amino acid sequence of the 21 residues from N-terminus:

$$H_2N\text{-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-.}$$

6. Use according to any one of Claims 1 to 5, whereby said human granulocyte colony stimulating factor is a polypeptide or glycoprotein having a human granulocyte colony stimulating factor activity produced from a transformant containing a recombinant vector that harbors a gene coding for a polypeptide having a human granulocyte colony stimulating factor activity.

7. Use according to Claim 6 whereby said polypeptide having a human granulocyte colony stimulating factor activity is represented by all or part of the amino acid sequence shown below:

* C L M S *

Referential Example 2 in 10 mM phosphate buffer (pH 7) so that the concentrations of Polysorbate 80, gelation and mannitol became 0.1 mg/ml, 10 mg/ml and 50 mg/ml, respectively. The solution was filtered through a membrane filter having a pore size of 0.22 μm to remove microorganisms, and charged into vials which had been sterilized. The rubber closures are loosely fitted by the rubber insertion machine so that two gaps of the rubber closrue are exposed to allow for the freeze-drying process, and the charged solution was freeze dried. The vials were then sealed with the rubber closures and then with aluminum caps to give a freeze-dried preparation for injection. The preparation in the vials which can be stored at room temperature is used after reconstituting with distilled water for injection.

The thrombus control agent of the present invention which contains human G-CSF as an effective ingredient potentiates the ability of human vascular endothelial cells to produce PA, which ability is inherent in the human body, thereby converting the plasminogen in blood to its activated form (plasmin), which gives rise to fibrinolytic activity, with consequent dissolution of thrombus. Further, the number of neutrophiles increases as a result of G-CSF administration and they work in such a way as to promote the processing of lysed thrombus. These tow actions combine to effectively dissolve and eliminate thrombi. Therefore, the present invention provides a useful thrombus control agent with reduced side effects.

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1. Use of a human granulocyte colony stimulating factor for preparing a thrombus control agent.

2. Use according to Claim 1 whereby the thrombus control agent is applicable to all thrombi that form in the arterial and venous vessels of a living body.

3. Use according to Claim 1 or 2, whereby said human granulocyte colony stimulating factor promotes the synthesis and secretion of plasminogen activator by arterial and venous vascular endothelial cells, said plasminogen activator forming plasmin to enhance the fibrinolytic activity of said endothelial cells.

4. Use according to any one of Claims 1 to 3, whereby said human granulocyte colony stimulating factor is obtained from the supernatant of a culture of human granulocyte colony stimulating factor producing cells.

5. Use according to any one of Claims 1 to 4, whereby said human granulocyte colony stimulating factor has the following physicochemical properties:
   i) molecular weight: about 19,000 ± 1,000 as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;
   ii) isoelectric point: having at least one of the three isoelectric points, pI = 5.5 ± 0.1, pI = 5.8 ± 0.1, and pI = 6.1 ± 0.1;
   iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;
   iv) amino acid sequence of the 21 residues from N-terminus:

$$H_2N\text{-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-}$$
$$\text{Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-.}$$

6. Use according to any one of Claims 1 to 5, whereby said human granulocyte colony stimulating factor is a polypeptide or glycoprotein having a human granulocyte colony stimulating factor activity produced from a transformant containing a recombinant vector that harbors a gene coding for a polypeptide having a human granulocyte colony stimulating factor activity.

7. Use according to Claim 6 whereby said polypeptide having a human granulocyte colony stimulating factor activity is represented by all or part of the amino acid sequence shown below:

* C L M S *

(Met)$_n$Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro
Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val
Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
Glu Lys Leu (Val Ser Glu)$_m$Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala
Gln Pro

(provided that m is 0 or 1; and n is 0 or 1).

8. Use according to any one of Claims 1 to 5, whereby said protein having a human granulocyte colony stimulating factor activity is a glycoprotein containing a polypeptide represented by all or part of the amino acid sequence shown below, and a sugar chain portion:

(Met)$_n$Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro
Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val
Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
Glu Lys Leu (Val Ser Glu)$_m$Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile

* C L M S *

Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala
Gln Pro

(provided that m is 0 or 1; and n is 0 or 1).

Patentansprüche

1. Verwendung eines menschliche Granulocyten koloniestimulierenden Faktors zur Herstellung eines Thrombus-Kontrollmittels.

2. Verwendung nach Anspruch 1, wobei das Thrombus-Kontrollmittel auf alle Thromben anwendbar ist, die sich in den arteriellen und venösen Blutgefäßen eines lebenden Körpers bilden.

3. Verwendung nach Anspruch 1 oder 2, wobei der menschliche Granulocyten koloniestimulierende Faktor die Synthese und Sekretion von Plasminogenaktivator durch arterielle und venöse vaskuläre Endothelialzellen fördert, wobei der Plasminogenaktivator Plasmin zur Erhöhung der fibrinolytischen Aktivität der Endothelialzellen bildet.

4. Verwendung nach einem der Ansprüche 1 bis 3, wobei der menschliche Granulocyten koloniestimulierende Faktor vom Überstand einer Kultur von Zellen, die menschliche Granulocyten koloniestimulierenden Faktor produzieren, erhalten wird.

5. Verwendung nach einem der Ansprüche 1 bis 4, wobei der menschliche Granulocyten koloniestimulierende Faktor die folgenden physikalisch-chemischen Eigenschaften aufweist:
   i) Molekulargewicht: etwa 19 000 ± 1000, gemessen durch Elektrophorese in einem Natriumdodecylsulfat-Polyacrylamidgel;
   ii) isoelektrischer Punkt: weist mindestens einen der drei isoelektrischen Punkte pI = 5,5 ± 0,1, pI = 5,8 ± 0,1 und pI = 6,1 ± 0,1 auf;
   iii) UV-Absorption: weist ein Absorptionsmaximum bei 280 nm und ein Absorptionsminimum bei 250 nm auf;
   iv) Aminosäuresequenz der 21 Reste am N-Terminus:

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-.

6. Verwendung nach einem der Ansprüche 1 bis 5, wobei der menschliche Granulocyten koloniestimulierende Faktor ein Polypeptid oder Glycoprotein mit einer Aktivität des menschliche Granulocyten koloniestimulierenden Faktors ist, das von einem Transformanten produziert wird, der einen rekombinanten Vektor mit einem Gen enthält, das ein Polypeptid mit einer Aktivität des menschliche Granulocyten koloniestimulierenden Faktors codiert

7. Verwendung nach Anspruch 6, wobei das Polypeptid mit einer Aktivität des menschliche Granulocyten koloniestimulierenden Faktors die gesamte oder einen Teil der nachstehenden Aminosäuresequenz aufweist:

* C L M S *

(Met)$_n$Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro
Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val
Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
Glu Lys Leu (Val Ser Glu)$_m$Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly

Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala
Gln Pro

mit der Maßgabe, daß m 0 oder 1 und n 0 oder 1 ist.

8. Verwendung nach einem der Ansprüche 1 bis 5, wobei das Protein mit einer Aktivität des menschliche Granulocyten koloniestimulierenden Faktors ein Glycoprotein ist, das ein Polypeptid enthält, das durch die gesamte oder einen Teil der nachstehend gezeigten Aminosäuresequenz und einen Zuckeranteil charakterisiert ist:

* C L M S *

(Met)$_n$Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro
Gln	Ser	Phe	Leu	Leu	Lys	Cys	Leu	Glu	Gln	Val
Arg	Lys	Ile	Gln	Gly	Asp	Gly	Ala	Ala	Leu	Gln
Glu	Lys	Leu	(Val	Ser	Glu)$_m$	Cys	Ala	Thr	Tyr	Lys
Leu	Cys	His	Pro	Glu	Glu	Leu	Val	Leu	Leu	Gly
His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	Leu	Ser
Ser	Cys	Pro	Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly
Cys	Leu	Ser	Gln	Leu	His	Ser	Gly	Leu	Phe	Leu
Tyr	Gln	Gly	Leu	Leu	Gln	Ala	Leu	Glu	Gly	Ile
Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu	Asp	Thr	Leu
Gln	Leu	Asp	Val	Ala	Asp	Phe	Ala	Thr	Thr	Ile
Trp	Gln	Gln	Met	Glu	Glu	Leu	Gly	Met	Ala	Pro
Ala	Leu	Gln	Pro	Thr	Gln	Gly	Ala	Met	Pro	Ala
Phe	Ala	Ser	Ala	Phe	Gln	Arg	Arg	Ala	Gly	Gly
Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser	Phe	Leu
Glu	Val	Ser	Tyr	Arg	Val	Leu	Arg	His	Leu	Ala
Gln	Pro								

mit der Maßgabe, daß m 0 oder 1 und n 0 oder 1 ist.

Revendications

1. Utilisation d'un facteur de stimulation des colonies de granulocytes humain pour la préparation d'un agent de contrôle de la formation des thrombus.

2. Utilisation suivant la revendication 1, dans laquelle l'agent de contrôle de la formation des thrombus est applicable à tous les thrombus qui se forment dans les vaisseaux artériels et veineux d'un corps vivant.

3. Utilisation suivant la revendication 1 ou 2, dans laquelle le facteur de stimulation des colonies de granulocytes humain favorise la synthèse et la sécrétion de l'activateur du plasminogène par les cellules endothéliales des vaisseaux artériels et veineux, l'activateur du plasminogène formant de la plasmine pour augmenter l'activité fibrinolytique de ces cellules endothéliales.

4. Utilisation suivant l'une quelconque des revendications 1 à 3, dans laquelle le facteur de stimulation des colonies de granulocytes humain est obtenu à partir du surnageant d'une culture de cellules produisant le facteur de stimulation des colonies de granulocytes humain.

5. Utilisation suivant l'une quelconque des revendications 1 à 4, dans laquelle le facteur de stimulation des colonies de granulocytes humain a les propriétés physico-chimiques suivantes :
   i) poids moléculaire : environ 19 000 ± 1000 tel que mesuré par électrophorèse sur un gel de polyacrylamide et de dodécylsulfate de sodium;
   ii) point isoélectrique : ayant au moins l'un des trois points isoélectriques, pI = 5,5 ± 0,1, pI = 5,8 ± 0,1, et pI = 6,1 ± 0,1;
   iii) absorption dans l'ultraviolet : ayant un maximum d'absorption à 280 nm et un minimum d'absorption à 250 nm;

* C L M S *

iv) séquence d'acides aminés des 21 résidus à partir de l'extrémité N :

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-
Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-.

6. Utilisation suivant l'une quelconque des revendications 1 à 5, dans laquelle le facteur de stimulation des colonies de granulocytes humain est un polypeptide ou une glycoprotéine ayant une activité de facteur de stimulation des colonies de granulocytes humain produit par un transformant contenant un vecteur recombinant qui éberge un gène codant pour un polypeptide ayant une activité de facteur de stimulation des colonies de granulocytes humain.

7. Utilisation suivant la revendication 6, dans laquelle le polypeptide ayant une activité de facteur de stimulation des colonies de granulocytes humain est représenté par tout ou partie de la séquence d'acides aminés indiquée ci-dessous :

$(Met)_n$Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro
Gln	Ser	Phe	Leu	Leu	Lys	Cys	Leu	Glu	Gln	Val
Arg	Lys	Ile	Gln	Gly	Asp	Gly	Ala	Ala	Leu	Gln
Glu	Lys	Leu	(Val	Ser	Glu)$_m$	Cys	Ala	Thr	Tyr	Lys
Leu	Cys	His	Pro	Glu	Glu	Leu	Val	Leu	Leu	Gly
His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	Leu	Ser
Ser	Cys	Pro	Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly
Cys	Leu	Ser	Gln	Leu	His	Ser	Gly	Leu	Phe	Leu
Tyr	Gln	Gly	Leu	Leu	Gln	Ala	Leu	Glu	Gly	Ile
Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu	Asp	Thr	Leu
Gln	Leu	Asp	Val	Ala	Asp	Phe	Ala	Thr	Thr	Ile
Trp	Gln	Gln	Met	Glu	Glu	Leu	Gly	Met	Ala	Pro
Ala	Leu	Gln	Pro	Thr	Gln	Gly	Ala	Met	Pro	Ala
Phe	Ala	Ser	Ala	Phe	Gln	Arg	Arg	Ala	Gly	Gly
Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser	Phe	Leu

Glu	Val	Ser	Tyr	Arg	Val	Leu	Arg	His	Leu	Ala
Gln	Pro								

(avec la condition que m est 0 ou 1, et n est 0 ou 1).

8. Utilisation suivant l'une quelconque des revendications 1 à 5, dans laquelle la protéine ayant une activité de facteur de stimulation des colonies de granulocytes humain est une glycoprotéine contenant un polypeptide représenté par tout ou partie de la séquence des acides aminés indiquée ci-après, et une partie chaîne de sucre :

$(Met)_n$Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro
Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val
Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
Glu Lys Leu (Val Ser Glu)$_m$Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala
Gln Pro

(avec la condition que m est 0 ou 1, et n est 0 ou 1).