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(54) **USE OF G-CSF FOR THE EXTENSION OF THE THERAPEUTIC TIME-WINDOW OF THROMBOLYTIC STROKE THERAPY**

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(57) **ABSTRACT**

The present invention relates to the use of G-CSF and derivatives thereof for extending the therapeutic window of subsequent thrombolytic treatment of acute stroke, and thereby, allowing the diagnostic examinations which are necessary prior to the thrombolytic treatment in order to avoid hemorrhagic and other severe adverse side effects of the thrombolysis.

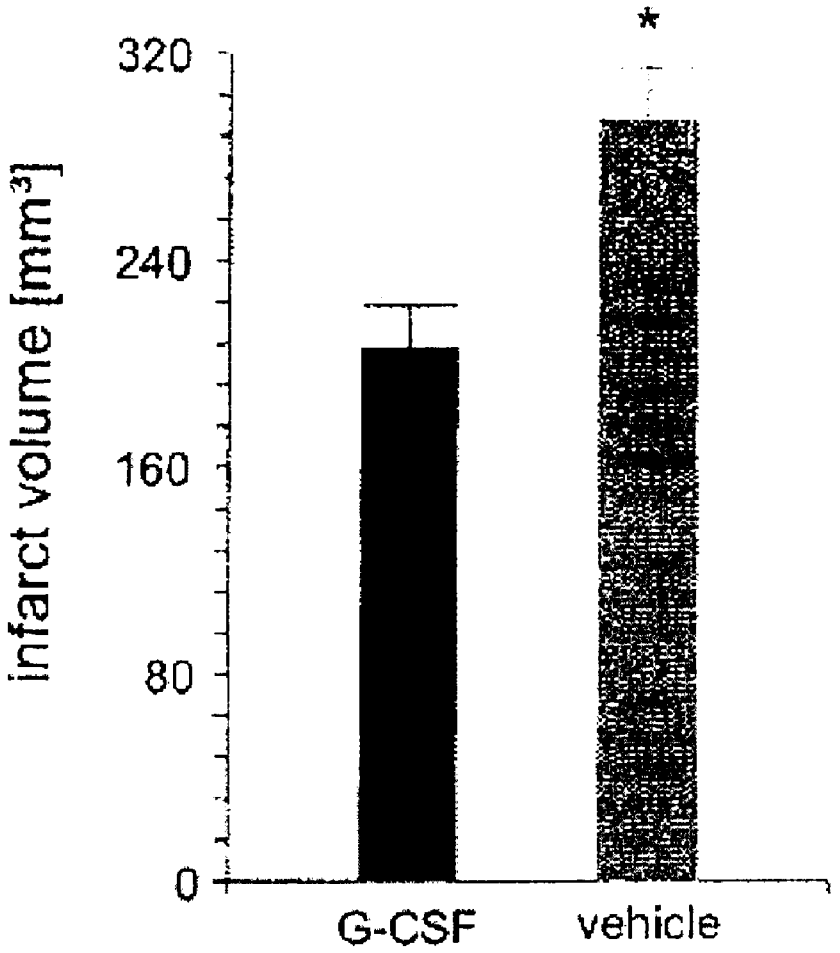

Fig. 1

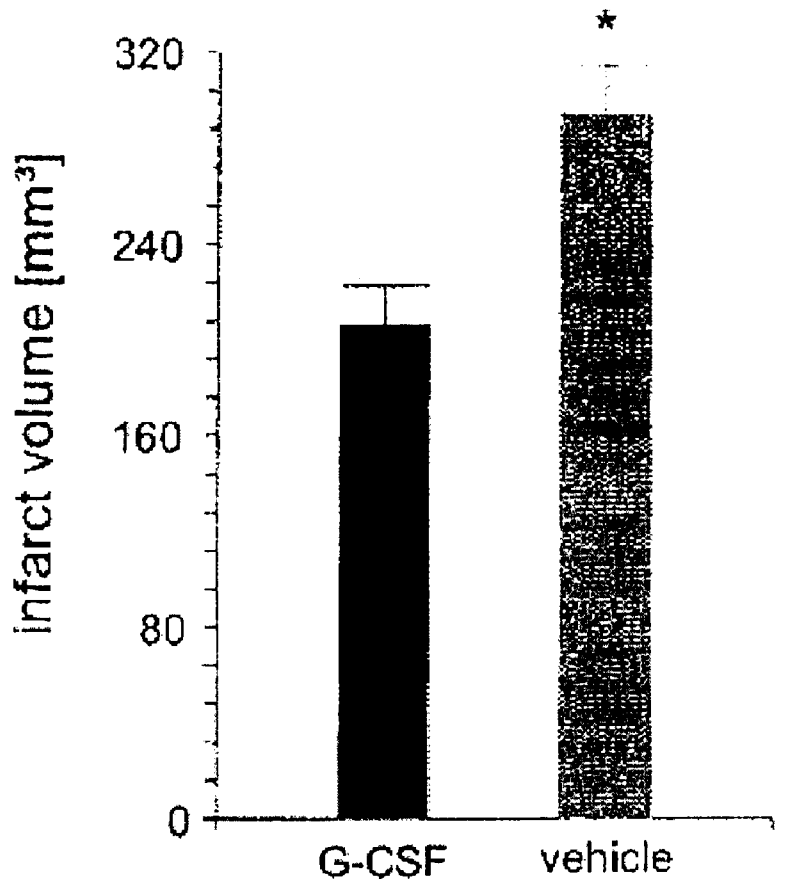

Fig. 2A

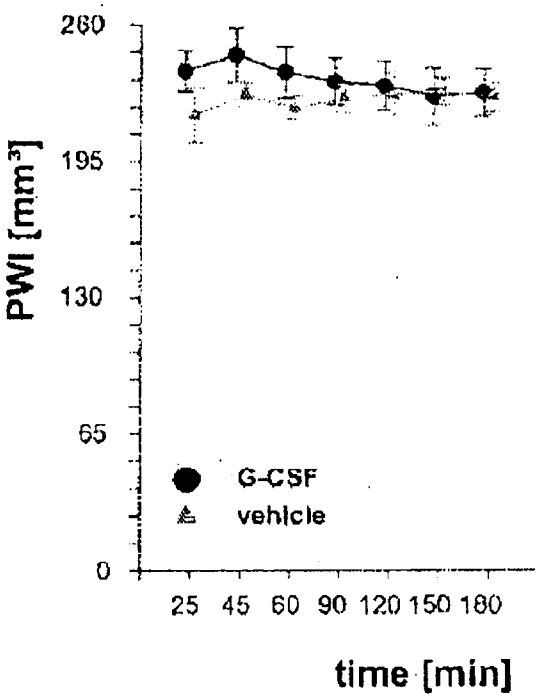
260
195
130
65
0
PWI [mm³]
G-CSF
vehicle
25 45 60 90 120 150 180
time [min]

Fig. 2B

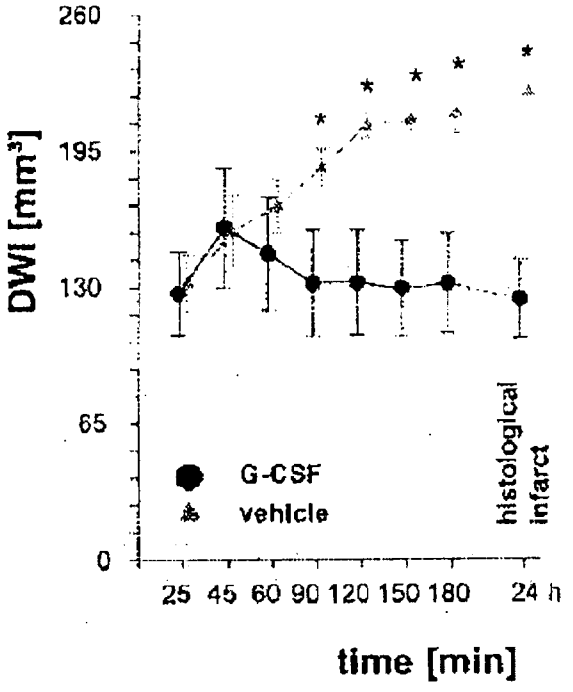
260
195
130
65
0
DWI [mm³]
G-CSF
vehicle
histological infarct
25 45 60 90 120 150 180 24 h
time [min]

Fig. 2C

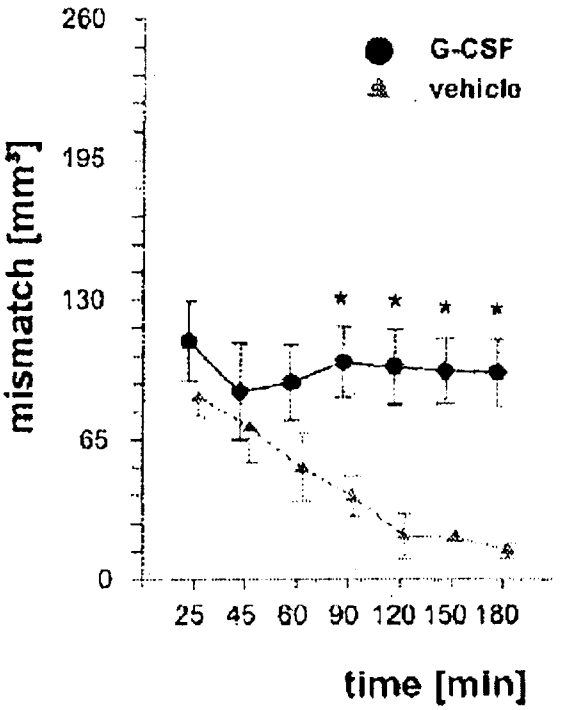
260
195
130
65
0
mismatch [mm³]
G-CSF
vehicle
*
*
*
*
25 45 60 90 120 150 180
time [min]

Fig. 2D

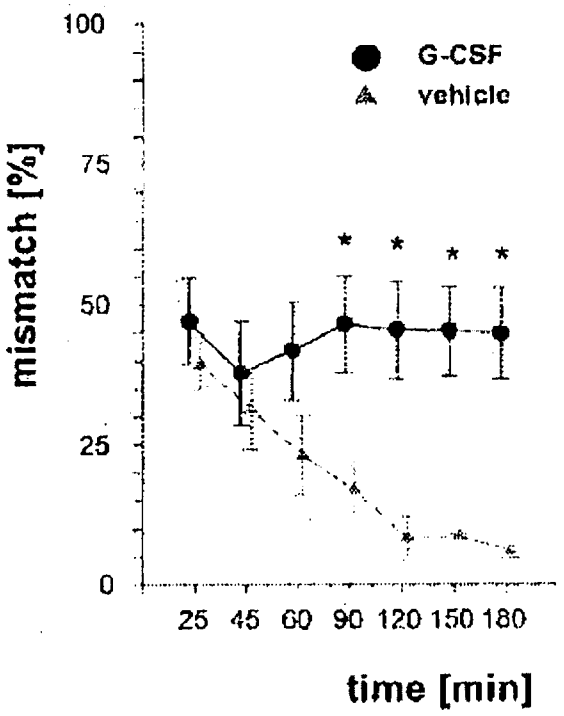
100
75
50
25
0
mismatch [%]
G-CSF
vehicle
*
*
*
*
25 45 60 90 120 150 180
time [min]

Fig. 3

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  1  M A G P A T Q S P M K L M A L Q L L L W H S A L W T V Q E A  hum G-CSF
  1  M A Q L S A Q R R M K L M A L Q L L L W Q S A L W S G R E A  mouse G-C
  1  - - - - - - - - - - M K L M A L Q L L L W H S A L W S G Q E A  rat G-CSF
  1  - - - - - - - - - - - K L M A L Q L L L W H S A L W M V Q E A  feline G-C
  1  - - - - - - - - - - M K L M V L Q L L L W H S A L W T V H E A  bovine G-C
  1  - - - - - - - - - - M K L M A L Q L L L W H I A L W M V P E A  pig G-CSF

 31  T P L G P A S S L P - - - - - - Q S F L L K C L E Q V R K I  hum G-CSF
 31  V P L V T V S A L P P S L P L P R S F L L K S L E Q V R K I  mouse G-C
 22  I P L L T V S S L P P S L P L P R S F L L K S L E Q V R K I  rat G-CSF
 21  T P L G P T S S L P - - - - - - Q S F L L K C L E Q V R K V  feline G-C
 22  T P L G P A R S L P - - - - - - Q S F L L K C L E Q V R K I  bovine G-C
 22  A P L S P A S S L P - - - - - - Q S F L L K C L E Q V R K I  pig G-CSF

 55  Q G D G A A L Q E K L V S E C A T Y K L C H P E E L V L L G  hum G-CSF
 61  Q A S G S V L L E Q L - - - C A T Y K L C H P E E L V L L G  mouse G-C
 52  Q A R N T E L L E Q L - - - C A T Y K L C H P E E L V L F G  rat G-CSF
 45  Q A D G T A L Q E R L - - - C A A H K L C H P E E L V L L G  feline G-C
 46  Q A D G A E L Q E R L - - - C A A H K L C H P E E L M L L R  bovine G-C
 46  Q A D G A E L Q E R L - - - C A T H K L C H P Q E L V L L G  pig G-CSF

 85  H S L G I P W A P L S S C P S Q A L Q L A G C L S Q L H S G  hum G-CSF
 88  H S L G I P K A S L S G C S S Q A L Q Q T Q C L S Q L H S G  mouse G-C
 79  H S L G I P K A S L S S C S S Q A L Q Q T K C L S Q L H S G  rat G-CSF
 72  H A L G I P Q A P L S S C S S Q A L Q L T G C L R Q L H S G  feline G-C
 73  H S L G I P Q A P L S S C S S Q S L Q L T S C L N Q L H G G  bovine G-C
 73  H S L G L P Q A S L S S C S S Q A L Q L T G C L N Q L H G G  pig G-CSF

115  L F L Y Q G L L Q A L E G I S P E L G P T L D T L Q L D V A  hum G-CSF
118  L C L Y Q G L L Q A L S G I S P A L A P T L D L L Q L D V A  mouse G-C
109  L F L Y Q G L L Q A L A G I S S E L A P T L D M L H L D V D  rat G-CSF
102  L F L Y Q G L L Q A L A G I S P E L A P T L D M L Q L D I T  feline G-C
103  L F L Y Q G L L Q A L A G I S P E L A P T L D T L Q L D V T  bovine G-C
103  L V L Y Q G L L Q A L A G I S P E L A P A L D I L Q L D V T  pig G-CSF

145  D F A T T I W Q Q M E E L G M A P A L Q P T Q G A M P A F A  hum G-CSF
148  N F A T T I W Q Q M E N L G V A P T V Q P T Q S A M P A F T  mouse G-C
139  N F A T T I W Q Q M E S L G V A P T V Q P T Q S T M P I F T  rat G-CSF
132  D F A I N I W Q Q M E D V G M A P A V P P T Q G T M P T F T  feline G-C
133  D F A T N I W L Q M E D L G A A P A V Q P T Q G A M P T F T  bovine G-C
133  D L A T N I W L Q M E D L R M A P A S L P T Q G T V P T F T  pig G-CSF

175  S A F Q R R A G G V L V A S H L Q S F L E V S Y R V L R H L  hum G-CSF
178  S A F Q R R A G G V L A I S Y L Q G F L E T A R L A L H H L  mouse G-C
169  S A F Q R R A G G V L V T S Y L Q S F L E T A H H A L H H L  rat G-CSF
162  S A F Q R R A G G T L V A S N L Q S F L E V A Y R A L R H F  feline G-C
163  S A F Q R R A G G V L V A S Q L H R F L E L A Y R G L R Y L  bovine G-C
163  S A F Q R R A G G V L V V S Q L Q S F L E L A Y R V L R Y L  pig G-CSF

205  A Q P                                                    hum G-CSF
208  A                                                        mouse G-C
199  P R P A Q K H F P E S L F I S I                           rat G-CSF
192  T K P                                                    feline G-C
193  A E P                                                    bovine G-C
193  A E P                                                    pig G-CSF
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USE OF G-CSF FOR THE EXTENSION OF THE THERAPEUTIC TIME-WINDOW OF THROMBOLYTIC STROKE THERAPY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Ser. No. 61/153,079, filed Feb. 17, 2006, herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the use of Granulocyte Colony Stimulating Factor (G-CSF) polypeptide in the prevention of neuronal cell death in the infarct penumbra after acute stroke. More particularly, the invention provides methods of enhancing the therapeutic window for thrombolytic treatment after acute stroke by the preceding administration of G-CSF polypeptide in conjunction with a subsequent thrombolytic therapy.

BACKGROUND OF THE INVENTION

[0003] Granulocyte colony stimulating factor (G-CSF) was originally identified as a hematopoietic factor in the myeloid lineage responsible for the generation of neutrophilic granulocytes. Recently, the presence and activity of this factor in the central nervous system was identified G-CSF and its receptor are up-regulated after cerebral ischemia, G-CSF acts anti-apoptotically on neurons, passes the intact blood-brain barrier, and reduces infarct size in experimental stroke models (Schneider et al., J Clin Invest. 2005, 115:2083; Zhao et al., Exp Neurol. 2007, 204:569; Schabitz et al., Stroke 2003, 34:745; Six et al., Eur J Pharmacol. 2003, 458:327; Shyu et al., Circulation 2004, 110:1847; Gibson et al., 2005, 25:431; Komine-Kobayashi et al., J Cereb Blood Flow Metab. 2006, 26:402; Schneider et al., BMC Biol. 2006, 4:36). This has led to a number of smaller clinical trials in acute ischemic stroke patients (reviewed in Schabitz et al., Stroke 2006, 37:1654; Schabitz et al., Trends Pharmacol Sci. 2007, 28:157). However, although meta-analysis of published data supports the broad basis for efficacy of this factor in experimental stroke models (Minnerup et al., Stroke 2008, 39:1856), the majority of experiments were done using transient ischemic models. In particular, no published data exist on embolic models.

[0004] Thrombolysis with recombinant tissue plasminogen activator (rt-PA) remains the only approved acute stroke therapy until now. Unfortunately, the use of rt-PA is limited by a relatively narrow time window. Efficacy was recently demonstrated up to 4.5 h following onset of stroke symptoms, but efficacy decline rapidly over time (Hacke et al., Lancet 2004, 363:768; Hacke et al., N Engl J Med. 2008, 359:1317). The biological reason for the reduced therapeutic efficiency over time likely lies in the progressing deterioration of cell viability with ongoing ischemia/hypoxia in hypoperfused brain areas. This may be paired with generation of free radicals during reperfusion (i.e., reperfusion injury). Clinically, this concept is supported by data that suggest that the presence of a perfusion/diffusion (PWI/DWI) mismatch on MRI identifies patients where thrombolysis may be efficacious later in the therapeutic time window (Fisher et al. Cerebrovasc Dis. 2006, 21 Suppl 2:64).

[0005] A strategy to extend the time window for thrombolysis may be to protect tissue at risk identified as the PWI/DWI mismatch region. Proof-of-concept for this hypothesis has been demonstrated with normobaric hyperoxia treatment (Henninger et al. J Cerb Blood Flow Metab. 2007, 27:1632) and stimulation of the sphenopalatine ganglion (Henninger and Fisher Stroke 2007, 38:2779).

BRIEF SUMMARY OF THE INVENTION

[0006] Cerebral infarcts caused by stroke comprise the infarct core (already irreversibly injured tissue) and the penumbra (tissue at risk but still salvageable). Thrombolysis, particularly with tissue plasminogen activator (t-PA), is known as an effective treatment of acute ischemic stroke but only if therapy is initiated within a short time period (therapeutic window) after the onset of stroke. The volume of salvageable penumbra tissues decreases strongly continuously over time-within the first hours of cerebral ischemia. Thereafter, the thrombolytic establishment of reperfusion is ineffective in preventing further neuronal cell death and ameliorating the clinical outcome or is even harmful. t-PA has to be administered within the first 4.5 h preferably 3 h, after stroke onset, whereas this time period is sometimes extended up to a total of 6 h by the physicians.

[0007] For this reason, early thrombolytic intervention is usually desired. On the other hand however, thrombolytic intervention may have severe hemorrhagic adverse side effects which worsen the clinical outcome of the stroke patient. Therefore, thrombolytic treatment requires neuroimaging to exclude a hemorrhage and assessment of basic coagulation parameters prior to administration of the thrombolytic agent. During that time however, neuronal cell death in the infarct penumbra continues and the therapeutic window for thrombolysis might close.

[0008] There is a need for a method or an agent capable to halt the neuronal cell death in the penumbra ("penumbra freezing") soon after the onset of the stroke and, thereby, extending the therapeutic window for later thrombolytic treatment which allows for the necessary careful diagnostic examinations and treatment decisions.

[0009] The inventors found that G-CSF when administered in a stroke model is capable to preserve the penumbra region and, thereby, prevent further extension of the infarct size. It is well accepted in the art that the extent of preserved penumbra tissue is crucial for the beneficial effect of a thrombolytic reperfusion. Since G-CSF is safe in acute ischemic stroke patients, and at least in animal models there is no indication that it might cause intracerebral hemorrhage, or increase the risk of systemic bleeding, it can be administrated to the stroke patient immediately with the begin of the intensive care and without extensive prior diagnostic examinations and even before admission in or transport to the hospital given by paramedicals or other qualified health professionals. G-CSF can be considered as an emergency drug that could be given in the ambulance to prolong the time-window for, and possibly improve outcome after thrombolysis, e.g by t-PA.

[0010] The present invention relates to the use of G-CSF for extending the therapeutic window of subsequent thrombolytic treatment of acute stroke, allowing the necessary pre-thrombolysis diagnostic examinations.

[0011] One aspect are methods of treating a patient suffering from acute stroke, comprising initial G-CSF administration, followed by diagnostic examinations, whereas said examinations allows the decision if a thrombolytic therapy is suitable to the patient, and, optionally, based on the results of the diagnostic examination, followed by a thrombolytic treat-

ment. Such diagnostic examinations can be e.g. the exclusion of a hemorrhagic stroke, which is a contra-indication for a thrombolytic therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. **1**: Infarct volume at 24 h after induction of an embolic ischemia by single clot injection. Shown are edema corrected volumes obtained from TTC-stained sections. Rats were treated with G-CSF at 1 h post clot injection (intravenously) and 4 h post clot injection (intraperitoneal), 120 μg/kg body weight each. G-CSF treatment resulted in significantly smaller infarcts compared to the vehicle group ($p<0.05$).

[0013] FIG. **2**: Spatiotemporal evolution of diffusion-weighted lesion within sMCAO model. Rats were subjected to permanent filament occlusion of the MCA, and monitored for 3 h after occlusion for the evolution of the diffusion-weighted lesion. G-CSF orvehicle solution were given at 60 min and at 4 h after occlusion onset. The 60 min dose was started before image acquisition at the 60 min time point. There were no statistical between- or within-group differences in CBF deficit. CBF was significantly larger than ADC at all time points except for 120 and 180 min in the vehicle group. The G-CSF group showed significantly smaller ADC volumes than the vehicle group starting at 90 min. Final infarct volume was also significantly smaller in the G-CSF group compared to the vehicle group ($p=0.007$). Shown are means+/−SEM; *: $p<0.05$; PWI volume (A), DWI and final infarct (B), absolute (C) and relative mismatch (D).

[0014] FIG. **3**: Alignment of G-CSF peptide sequences of various species (human (SEQ ID NO: 6), mouse (SEQ ID NO: 11), rat (SEQ ID NO: 12), feline (SEQ ID NO: 13), bovine (SEQ ID NO: 14), and pig (SEQ ID NO: 15)) shows the position of strongly and less conserved amino acids. Evolutionary strongly conserved amino acids are generally thought to be of major importance for the structure and function of the protein.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The inventors describe a finding that makes G-CSF ideally suited as a time-window extender in stroke treatment for any further therapy, preferably, thrombolytic stroke therapy (e.g. with rt-PA).

[0016] This is a very useful application, as a major issue that limits the usefulness of thrombolytic stroke therapy, e.g. rt-PA therapy is the limited time window due to loss of efficacy with time. rt-PA has to be administered usually during the initial 3 to 4.5 h after onset of the stroke based on clinical studies. Occasionally, it might be given by some physicians within up to 6 h. Since the possibility of hemorrhagic side effects of rt-PA has to be excluded for the individual patient to avoid worsening of the situation, it is often difficult to enable a safe thrombolytic therapy during this time-window. G-CSF could be given very soon after the suspicion of a cerebral insult has occurred, as it does not complicate a possible hemorrhagic stroke and as it is well-tolerated even in high doses.

[0017] The finding relates to the fact that in an animal model of stroke, permanent filament occlusion, G-CSF keeps the diffusion-weighted deficit stable in the presence of an ongoing ischemia. Such an effect is also known as "penumbra freezing". This means that damage to brain tissue can be delayed until a thrombolytic therapy can be applied to reopen the occluded vessels. In the cases where G-CSF alone might be not sufficient to enable complete recovery from the stroke, the unexpected finding according to the invention enables a combinational or consecutive therapy comprising an initial step of G-CSF administration to the subject and a later step of administration of an thrombolytic agent, e.g. rt-PA. The earlier G-CSF administration allows for a postponed onset of thrombolytic therapy within the first several days, preferably within the first 24 h, more preferably within the first 12 h after onset of the stroke. This allows for a closer diagnostic examination of the patient after stroke or after suspicion of stroke to ensure a safe and effective additional thrombolytic therapy. The advantage of such a combination of an early G-CSF administration and a postponed thrombolytic therapy (e.g. rt-PA administration) is to the inventors knowledge not disclosed previously.

[0018] Unexpectedly, G-CSF was effective in preserving the penumbra tissue even during the time the vessel was occluded.

[0019] According to the invention, G-CSF administration is started during the first 12 h, preferably during the first 6 h, and more preferably during the first 3 h after onset of the stroke. Preferred uses of G-CSF could be up to a time window of 24 h in doses of at least 10 μg/kg body weight, at least 90 μg/kg body weight, or at least 130 μg/kg body weight given intravenously (i.v.) or subcutaneously (s.c.) over 1-24 h.

[0020] According to the invention, it is included that the administration of G-CSF may either be completed before the administration of the thrombolytic agent or may be continued after the administration of the thrombolytic agent. Furthermore, it is also included within the present invention that G-CSF may be administered only once. Alternatively, G-CSF may also be administered in at least two separate steps.

[0021] Preferably human recombinant G-CSF, such as Filgrastim, is used according to the invention. Also functional G-CSF derivatives which are know to the person skilled in the art can be used according to the invention.

[0022] The method according to the invention is suitable for the therapy of mammals, preferably of humans suffering from stroke or give reason to suspect a stroke.

[0023] As one aspect of the invention, a method is provided for treating stroke of a mammalian subject, comprising the steps (a) starting the administration of G-CSF or a functionally active G-CSF derivative in a therapeutically active amount to the subject, and subsequently (b) administering to the subject a thrombolytic agent in a therapeutically active amount.

[0024] As another aspect of the invention, a method is provided for treating stroke of a mammalian subject, comprising the steps (a) administering to a subject G-CSF or a functionally active G-CSF derivative in a therapeutically active amount, and subsequently (b) administering to the subject a thrombolytic agent in a therapeutically active amount.

[0025] The mammalian subject can be a human being.

[0026] As one embodiment of the invention, a method as mentioned above is provided, wherein the subject undergoes after step (a) and before step (b) a diagnostic examination to exclude the risk of hemorrhagic or other adverse side effects during step (b).

[0027] The "thrombolytic agent" of above mentioned step (b) is meant to refer to any agent capable of dissolving at least partially a fibrin-platelet clot. Examples of thrombolytic agents include streptokinase, prourokinase, urokinase, desmoteplase and tissue-type plasminogen activator (t-PA). Although natural t-PA may be employed, it is preferable to employ recombinant t-PA (rt-PA, e.g. Alteplase). The invention may additionally employ hybrids, physiologically active

fragments or mutant forms of the above thrombolytic agents. The term "tissue-type plasminogen activator" as used herein is intended to include such hybrids, fragments and mutants, as well as both naturally derived and recombinantly derived tissue-type plasminogen activator.

[0028] As a further embodiment of the invention, a method as mentioned above is provided, wherein administration of said G-CSF or functionally active G-CSF derivative of step (a) starts within the first 6 h after onset of the stroke and/or administration of said thrombolytic agent of step (b) starts within the first 24 h after onset of the stroke or in the time period between 4.5 h and 24 h after onset of the stroke or between 6 h and 24 h after stroke.

[0029] As a still further embodiment of the invention, a method as mentioned above is provided, wherein G-CSF or functionally active G-CSF derivative of step (a) is administered to the subject within the first 6 h, the first 4.5 h, or the first 3 h after onset of the stroke and/or administration of said thrombolytic agent of step (b) starts within the first 24 h after onset of the stroke or in the time period between 4.5 h and 24 h after onset of the stroke or between 6 h and 24 h after stroke.

[0030] As one embodiment of the invention, a method as mentioned above is provided, wherein there is a time period of at least 0.5 h, at least 1.5 h, or at least 3 h between the administration, the start of the administration, or the end of the administration of G-CSF or functionally active G-CSF derivative of step (a) and the start of the administration of said thrombolytic agent of step (b). Preferably, this time period is used for diagnostic examination of the subject, assessing the risk of hemorrhagic or other adverse side effects of the thrombolytic therapy.

[0031] As a further aspect of the invention, a method is provided of treating a mammalian subject suffering from acute stroke, comprising an initial G-CSF administration or a start of a initial G-CSF administration, followed by diagnostic examinations, whereas said examinations assess the risk of a thrombolytic therapy for the subject, and, optionally, based on the results of the diagnostic examination, followed by a thrombolytic treatment. Such diagnostic examinations can be e.g. the exclusion of a hemorrhagic stroke, which is a counter-indication for a thrombolytic therapy.

[0032] As another embodiment of the invention, a method as mentioned above is provided, wherein said G-CSF of step (a) is given intravenously or subcutaneously in doses of at least 10 μg/kg body weight, at least 90 μg/kg body weight, or at least 130 μg/kg body weight.

[0033] As a further aspect of the invention, G-CSF or functionally active derivative thereof is provided for the preparation of a pharmaceutical composition for treating a mammalian subject suffering from acute stroke, wherein the subject is admitted to a stroke unit or a clinic within the initial 6 h after stroke onset or within the time period of 3 to 6 h after stroke onset or within the time period of 4.5 to 6 h after stroke onset, and wherein the expenditure of time for the diagnostic examination necessary to assess the subject's risk of hemorrhagic or other sever adverse side effects of a thrombolytic treatment would otherwise cause the expiration of the therapeutic window for thrombolytic treatment. The thrombolytic treatment in this context can be e.g. the administration of t-PA, such as rt-PA. The therapeutic window for thrombolytic treatment in this context can be within 3 h, within 4.5 h, or within 6 h after stroke onset. The diagnostic examination in this context can last at least 0.5 h, at least 1.5 h, or at least 3 h. The mammalian subject in this context can receive the G-CSF or functionally active derivative thereof immediately after admittance to the stroke unit or clinic, or within the first 6 h, within the first 4.5, or within the first 3 h after stroke onset. Further, the mammalian subject in this context can receive subsequently the thrombolytic treatment if the diagnostic examination permits such a treatment. The mammalian subject in this context can be a human being. The G-CSF in this context can be human G-CSF, preferably, Filgrastim.

[0034] The diagnostic examination of above described embodiments is meant to refer to any examination of the mammalian patient suffering from acute stroke which allows, improves, or supports the decision, whether a thrombolytic treatment, particularly thrombolytic treatment with t-PA, of the patient is indicated or contra-indicated. Such diagnostic examinations can be e.g., but without any claim of completeness: Medical imaging such as magnetic resonance imaging (MRI), analysis of blood parameters such as coagulation factors, or also survey of the patients anamnesis. Since patients suffering from acute stroke are frequently unconscious or confused, a survey of the patients anamnesis can be time consuming. Contraindications for thrombolytic treatment of acute stroke, particularly for t-PA treatment, which should be excluded by diagnostic examinations prior starting the treatment are e.g., but without any claim of completeness: Active internal bleeding, history of cerebrovascular accident, recent intracranial or intraspinal surgery or trauma, Intracranial neoplasm, arteriovenous malformation, or aneurysm, bleeding diathesis (including but not limited to current use of oral anticoagulants (e.g., warfarin sodium), an International Normalized Ratio (INR) >1.7, a prothrombin time (PT) >15 seconds, administration of heparin within 48 hours preceding the onset of stroke and elevated activated partial thromboplastin time (aPTT) at presentation, or platelet count $<100{,}000/mm^3$), uncontrolled hypertension at time of treatment (e.g., >185 mm Hg systolic or >110 mm Hg diastolic), intracranial hemorrhage, subarachnoid hemorrhage, recent (within 3 months) intracranial or intraspinal surgery, serious head trauma, previous stroke, history of intracranial hemorrhage, seizure at the onset of stroke

[0035] Granulocyte-colony stimulating factor (G-CSF) is a well known growth factor. The G-CSF that can be employed in the inventive methods described herein are human G-CSF (pro-form, short splice variant (SEQ ID NO: 2), mature form, short splice variant (SEQ ID NO: 4), pro-form, long splice variant (SEQ ID NO: 6), mature form, long splice variant (SEQ ID NO: 8), Filgrastim (SEQ ID NO: 10)) or various functional variants, muteins, and mimetics that are known and available. In the discussion that follows these are referred to as G-CSF derivatives.

[0036] Said G-CSF derivatives which can be employed in the present invention are proteins that are at least 70%, preferably at least 80%, more preferably at least 90% identical to human G-CSF amino acid sequences described herein. In another embodiment, the G-CSF that can be used are those that are encoded by polynucleotide sequence with at least 70%, preferably 80%, more preferably at least 90%, 95%, and 97% identity to the human G-CSF coding sequence (pro-form, short splice variant (SEQ ID NO: 1), mature form, short splice variant (SEQ ID NO: 3), pro-form, long splice variant (SEQ ID NO: 5), mature form, long splice variant (SEQ ID NO: 7), Filgrastim (SEQ ID NO: 9)), these polynucleotides will hybridize under stringent conditions to the coding polynucleotide sequence of the human G-CSF coding sequence. The terms "stringent conditions" or "stringent hybridization

conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides), for example, high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1 SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C. (see Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995)). Amino acid and polynucleotide identity, homology and/or similarity can be determined using the ClustalW algorithm, MEGALIGN™, Lasergene, Wis.)

[0037] Examples of the various G-CSF functional variants, muteins, and mimetics include functional fragments and variants (e.g., structurally and biologically similar to the wild-type protein and having at least one biologically equivalent domain), chemical derivatives of G-CSF (e.g., containing additional chemical moieties, such as polyethyleneglycol and polyethyleneglycol derivatives thereof, and/or glycosylated forms such as Lenogastrim™), and peptidomimetics of G-CSF (e.g., a low molecular weight compound that mimics a peptide in structure and/or function (see, e.g., Abell, Advances in Amino Acid Mimetics and Peptidomimetics, London: JAI Press (1997); Gante, Angew Chem. 1994, 106: 1780; Olson et al., J Med Chem. 1993, 36:3039).

[0038] Additional examples of G-CSF derivatives include a fusion protein of albumin and G-CSF (Albugranin™), or other fusion modifications such as those disclosed in U.S. Pat No. 6,261,250; PEG-G-CSF conjugates and other PEGylated forms; those described in WO 00/44785 and Viens et al., J Clin Oncology 2002, 6:24; norleucine analogues of G-CSF, those described in U.S. Pat. No. 5,599,690; G-CSF mimetics, such as those described in WO 99/61445, WO 99/61446, and Tian et al., Science 1998, 281:257; G-CSF muteins, where single or multiple amino acids have been modified, deleted or inserted, as described in U.S. Pat. Nos. 5,214,132 and 5,218, 092; those G-CSF derivatives described in U.S. Pat. No. 6,261,550 and U.S. Pat. No. 4,810,643; and chimeric molecules, which contain the full sequence or a portion of G-CSF in combination with other sequence fragments, e.g. Leridistim—see, for example, Streeter et al., Exp Hematol. 2001, 29:41, Monahan et al., Exp Hematol. 2001, 29:416, Hood et al., Biochemistry 2001, 40:13598, Farese et al., Stem Cells 2001, 19:514, Farese et al., Stem Cells 2001, 19:522, MacVittie et al., Blood 2000, 95:837. Additionally, the G-CSF derivatives include those with the cysteines at positions 17, 36, 42, 64, and 74 of SEQ ID NO: 4 or analogously of SEQ ID NO: 10, substituted with another amino acid, (such as serine) as described in U.S. Pat. No. 6,004,548, G-CSF with an alanine in the first (N-terminal) position; the modification of at least one amino group in a polypeptide having G-CSF activity as described in EP 0 335 423; G-CSF derivatives having an amino acid substituted or deleted in the N-terminal region of the protein as described in EP 0 272 703; derivatives of naturally occurring G-CSF having at least one of the biological properties of naturally occurring G-CSF and a solution stability of at least 35% at 5 mg/ml in which the derivative has at least Cys^{17} of the native sequence replaced by a Ser^{17} residue and Asp^{27} of the native sequence replaced by a Ser^{27} residue as described in EP 0 459 630; a modified DNA sequence encoding G-CSF where the N-terminus is modified for enhanced expression of protein in recombinant host cells, without changing the amino acid sequence of the protein as described in EP 0 459 630; a G-CSF which is modified by inactivating at least one yeast KEX2 protease processing site for increased yield in recombinant production using yeast as described in EP 0 243 153; lysine altered proteins as described in U.S. Pat. No. 4,904,584; cysteine altered variants of proteins as described in WO 90/12874 (U.S. Pat. No. 5,166,322); the addition of amino acids to either terminus of a G-CSF molecule for the purpose of aiding in the folding of the molecule after prokaryotic expression as described in AU-A-10948/92; substituting the sequence Leu-Gly-His-Ser-Leu-Gly-Ile (SEQ ID NO: 16) at position 50-56 of G-CSF of SEQ ID NO: 4 and position 53 to 59 of the G-CSF of SEQ ID No: 8 or/and at least one of the four histedine residues at positions 43, 79,156 and 170 of the mature G-CSF of SEQ ID NO: xx (174 form) or at positions 46, 82, 159, or 173 of the mature G-CSF of SEQ ID NO: 8 as described in AU-A-763 80/91; and a synthetic G-CSF-encoding nucleic acid sequence incorporating restriction sites to facilitate the cassette mutagenesis of selected regions and flanking restriction sites to facilitate the incorporation of the gene into a desired expression system as described in GB 2 213 821. Further examples of G-CSF analogs include SEQ ID NO: 17) and others described in U.S. Pat. No. 6,632,426. The contents of the above are incorporated herein by reference.

[0039] The various functional derivatives, variants, muteins and/or mimetics of G-CSF preferably retain at least 20%, preferably 50%, more preferably at least 75% and/or most preferably at least 90% of the biological activity of wild-type mammalian G-CSF activity—the amount of biological activity include 25%, 30%, 35%, 40%, 45%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 95%; and all values and subranges there between. Furthermore, the functional derivatives, variants, muteins and/or mimetics of G-CSF can also have 100% or more of the biological activity relative to wild-type mammalian G-CSF activity—the amount of biological activity including at least 105%, at least 110%), at least 125%, at least 150%, and at least 200%.

[0040] To measure the biological activity of G-CSF, several known assays can be employed singularly or in combination. One example of determining G-CSF function is illustrated in Example 1. Other methods for determining G-CSF function are known and include a colony formation assay employing murine bone marrow cells; stimulation of proliferation of bone marrow cells induced by G-CSF; specific bioassays with cells lines that depend on G-CSF for growth or that respond to G-CSF (e.g., AML-193; 32D; BaF3; GNFS-60; HL-60; Ml; NFS-60; OCl/AMLIa; and WEHI-3B). These and other assays are described in Braman et al., Am J Hematology 1992, 39:194; Clogston et al., Anal Biochem. 1992, 202:375; Hattori et al., Blood 1990, 75:1228; Kuwabara et al., J Pharmacobiodyn. 1992, 15:121; Motojima et al., J Immunological Methods 1989, 118:187; Sallerfors and Olofsson, Eur J Haematology 1992, 49:199; Shorter et al., Immunology 1992, 75:468; Tanaka and Kaneko, J Pharmacobiodyn. 1992, 15:359; Tie et al., J Immunological Methods 1992, 149:115; Watanabe et al., Anal Biochem. 1991, 195:38.

[0041] In one embodiment, the G-CSF is modified or formulated, or is present as a G-CSF mimetic that increases its ability to cross the blood-brain barrier, or shift its distribution coefficient towards brain tissue. An example of such a modification is the addition of PTD or TAT sequences (Cao et al., J Neurosci. 2002, 22:5423; Mi et al., Mol Ther. 2000, 2:339; Morris et al., Nat Biotechnol. 2001, 19:1173; Park et al., J Gen Virol. 2002, 83:1173). These sequences can also be used in mutated forms, and added with additional amino acids at the amino- or carboxy-terminus of proteins. Also, adding bradykinin, or analogous substances to an intravenous application of any G-CSF preparation will support its delivery to the brain, or spinal cord (Emerich et al., Clin Pharmacokinet. 2001, 40:105; Siegal et al., Clin Pharmacokinet. 2002, 41:171).

[0042] In one embodiment the biological activity of G-CSF is enhanced by fusion to another hematopoietic factor. The enhanced activity can be measured in a biological activity assay as described above. Such a preferred modification or formulation of G-CSF leads to an increased antiapoptotic effect and/or an increase in neurogenesis. An example for such a modification is Myelopoietin-1, a G-CSF/IL-3 fusion protein (McCubrey et al., Leukemia 2001, 15:1203) or Progenipoietin-1 (ProGP-1) is a fusion protein that binds to the human fetal liver tyrosine kinase flt-3 and the G-CSF receptor.

EXAMPLES

Example 1

[0043] G-CSF Decreases Infarct Size within Embolic Model

[0044] Embolic models of cerebral ischemia possibly present a stroke model that is closer to the human situation compared to the filament model. So far, efficacy of G-CSF has not been shown in embolic models. Here, embolic stroke was modeled by injection of a preformed blood clot into the internal carotid artery of rats.

[0045] Male Wistar rats (n=20) weighing approximately 320 g were anesthetized with isoflurane (5% for induction, 2% for surgery, 1.2% for maintenance). PE-50 polyethylene tubing was inserted into the femoral artery for monitoring of mean arterial blood pressure (MABP) and for obtaining blood samples to measure blood gases (pH, PaO_2, $PaCO_2$), electrolytes (Na^+, K^+, Ca^{2+}), and plasma glucose. Body temperature was monitored continuously with a rectal probe and maintained at 37.0+/−0.3° C. with a thermostatically controlled heating lamp. For embolic stroke (ES) one red blood clot (diameter=0.35 mm, length=18 mm) was injected into the internal caroted artery (ICA) of 20 animals over approx. 1 s at the bifurcation of the pterygopalatanine artery (PPA) and ICA. Laser Doppler Flowmetry was used to monitor occlusion success.

[0046] Verum (G-CSF, Filgrastim (SEQ ID NO: 10)) and vehicle (buffer solution (250 mM Sorbitol, 0.004% Tween-80, and 10 mM sodium-acetate buffer (pH 4)) groups received two injections: an intravenous infusion (120 μg/kg body weight over 30 min) at 1 h after clot injection, and an intraperitoneal bolus (120 μg/kg body weight) at 4h after clot injection. At 24 h animals were neurologically scored as previously described (rating scale: 0: no deficit, 1: failure to extend the left forepaw, 2: decreased grip strength of left forepaw, 3: circling to paretic side by pulling the tail, 4: spontaneous contralateral circling, and 5:death; Menzies et al., Neurosurgery 1992, 31:100) and sacrificed to determine infarct volumes by 2,3,5-triphenyltetrazolium chloride (TTC) staining with edema correction (Meng et al., Ann Neurol. 2004, 55:207).

[0047] Physiological parameters (blood pH, partial pressure of blood gases ($PaCO_2$, PaO_2), plasma concentrations of electrolytes (Na^+, K^+, CA^{2+}) and of glucose) were not significantly changed by treatment. Also, MABP was not influenced by treatment ($p>0.05$ by repeated measures ANOVA), however there was a significant group-independent drop in MABP at 30 min, after which the blood pressure rose again.

[0048] 12 of 20 animals died prematurely between 16 and 24 h post ES and were therefore included in the TTC analyses. Infarct volumes determined by postmortem TTC staining were 295+/−20 mm^3 (vehicle) vs. 206+/−16 mm^3 (G-CSF, means+/−SEM; P=0.003) (FIG. **1**). This considerable decrease in infarct size was however not reflected in the neuroscore at 24 h, which did not show any difference between treatments (vehicle: 4.0+/−1.33; G-CSF: 4.2+/−1.32), likely reflecting the insensitivity of that scale for larger infarcts.

Example 2

[0049] G-CSF Halts the Evolution of a DWI Lesion in the Presence of a Permanent Perfusion Deficit

[0050] Permanent filament occlusion of the MCA was performed as previously described using 4-0 silicone-coated nylon filament sutures (suture occlusion of the right middle cerebral (sMCAO; Bouley et al., Neurosci Lett. 2007, 412:185). Wistar rats (n=15) weighing 320+/−19 g were anesthetized with isoflurane (5% for induction, 2% for surgery, 1.2% for maintenance) in room air. PE-50 polyethylene tubing was inserted into the femoral artery for monitoring of mean arterial blood pressure (MABP) and for obtaining blood samples to measure blood gases (pH, PaO_2, $PaCO_2$), electrolytes (Na^+, K^+, Ca^{2+}), and plasma glucose at prior to as well as 30, 60, 90, 120, 180 min after middle cerebral artery occlusion (MCAO). Body temperature was monitored continuously with a rectal probe and maintained at 37.0+/−0.3° C. with a thermostatically controlled heating lamp.

[0051] The perfusion deficit and DWI lesion was monitored over a time period of 180 min by MRI measurements. These MRI experiments were performed on a 4.7 T/40 cm horizontal magnet equipped with a Biospec Bruker console (Billerica, Mass., USA), and a 20 G/cm gradient insert (ID=12 cm, 120 ps rise time). A surface coil (ID=2.3 cm) was used for brain imaging and an actively decoupled neck coil for perfusion labelling (Meng et al., Ann Neurol. 2004, 55:207). Animals were imaged at 25, 45, 60, 90, 120, 150 and 180 min post-sMCAO. Three ADC maps were separately acquired with diffusion-sensitive gradients applied along the x, y, or z direction. Single shot, echo-planar images (EPI) were acquired over 3 min with matrix=64×64, spectral width=200 kHz, TR=2 s (90° flipangle), TE=37.5 ms, b=8 and 1,300 s/mm^2, Δ=24 ms, δ=4.75 ms, field of view (FOV)=2.56×2.56 cm, seven 1.5 mm slices, and 16 averages. Quantitative CBF measurements were made using the continuous arterial spin-labeling technique with single-shot, gradient-echo, EPI acquisition. Sixty paired images (for signal averaging) were acquired over 4 min, alternately, one with arterial spin labeling and the other (control) without spin-labeling preparation. The MRI parameters were similar to ADC measurements except TE=13.5 milliseconds. Arterial spin labeling utilized a 1.78-second, square radiofrequency pulse in the presence of 1.0 Gauss/cm gradient along the flow direction. The sign of the frequency offset was switched for nonlabeled images.

[0052] Final infarct volumes were determined at 24 h after onset of occlusion, whereas brains were removed and sectioned coronally into seven 1.5 mm-thick slices corresponding to the MR slices and stained with TTC.

[0053] Rats were treated with vehicle (buffer solution (250 mM Sorbitol, 0.004% Tween-80, and 10 mM sodium-acetate buffer (pH 4)), n=5) or G-CSF (Filgrastim, SEQ ID NO: 10; n=10) at 1 h after occlusion (intravenously; 120 μg/kg body weight over 30 min) and 4 h after occlusion (intraperitoneally; 120 μg/kg body weight as bolus).

[0054] Animals surviving for more than 16 hours were prespecified to be included in the study while those dying before 16 hours were excluded. Effects of G-CSF on apparent diffusion coefficient (ADC) and cerebral blood flow (CBF) characteristics as well as the spatiotemporal evolution of the ischemic lesion were evaluated.

[0055] Blood gases, electrolytes, pH, and blood glucose levels did not differ between the two groups. MABP was also not significantly different between treatment groups in both experiments ($p>>0.05$ by repeated measures ANOVA), however there was a group-independent significant rise over the course of the experiment ($p<0.05$ for factor time by repeated measures ANOVA). 2 of 15 animals died between 16 to 24 h.

[0056] Images were analyzed using Quickvol II (Schmidt et al., J Neurooncol. 2004, 68:207). Quantitative CBF and ADC maps and their corresponding threshold-derived lesion volumes were calculated as described previously (Meng et al., Ann Neurol. 2004, 55:207). The thresholds used to define abnormal DWI and PWI regions were a reduction to 0.53×10^{-3} mm^2/s for ADC and 0.3 mL/g/min for CBF as previously validated (Meng et al., Ann Neurol. 2004, 55:207). FIG. **2** summarizes the spatiotemporal evolution of threshold-derived ADC and CBF lesion volumes. The CBF lesion volume did not differ between groups (vehicle and G-CSF) and remained relatively constant over time at about 230 mm^3 (FIG. **2**A).

[0057] The ADC-derived lesion in the vehicle-treated animals increased with time in a linear fashion until 120 min, when the curve flattened. The final infarct volume determined at 24 h by the TTC method lay slightly above the last DWI volume measured at 180 min post occlusion. In G-CSF-treated animals, the DWI lesion grew from 25 min to 45 min post occlusion identical to the vehicle situation. However, when the MRI data were obtained at the 60 min time point after application of G-CSF, the increase seemed to begin to reverse. At 90 min, the DWI lesion in the G-CSF-treated animals became significantly smaller compared to the vehicle-treated rats (repeated measures ANOVA: $p<0.0001$ for the interaction treatment-time followed by Tukey-Kramer post-hoc test). For the following time points measured, the lesion remained stable until the end of the MRI data acquisition at 180 min, and resulted in a final infarct at 24 h of approximately the same size (FIG. **2**B).

[0058] The TTC-defined infarct volumes were significantly different between the treatment groups ($223+/-7$ mm^3 (vehicle) vs. $124+/-19$ mm^3 (G-CSF; $p=0.007$), and correspond well to the 3 h ADC lesion volumes in both groups and to the 3 h CBF in the vehicle group (FIGS. **2**B and **2**A).

[0059] FIGS. **2**C and **2**D show the absolute and relative mismatch between CBF and ADC derived volumes. All two measures also became significantly different at 90 min following occlusion ($p<0.05$; repeated measures ANOVA followed by Tukey Kramer post hoc test). Employing an alternative statistical approach and comparing DWI volume behaviour over time relative to PWI volume and treatment by a multiple linear regression model (factors: PWI, ANIMAL (random factor), TREATMENT, TIME, TIME×TREATMENT interaction) showed the treatment effect to become significant at 84 min post sMCAO. The present experiment shows that the action of G-CSF must be immediate to allow for a significant effect on the DWI deficit volume at least at 90 min post onset of occlusion. Induction of anti-apoptotic cascades in vitro is immediate, with phosphorylation and activation of Akt within 5 min after addition of G-CSF to the neurons 9. In contrast, an indirect effect mediated by bone-marrow derived cells would require release of those cells from the bone marrow into the bloodstream, passage of the blood-brain barrier, and tissue invasion, possibly followed by release of protective factors. This is unlikely to be rapid enough for the effect observed in the current experiment.

[0060] Significant between-group differences were not detected in Menzies neurological scores at 4 and 24 h, respectively, likely reflecting the insensitivity of that scale for larger infarcts.

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Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu
        35                  40                  45

gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag gag aag      192
Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys
    50                  55                  60

ctg gtg agt gag tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg      240
Leu Val Ser Glu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
65                  70                  75                  80

gtg ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg agc agc      288
Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
                85                  90                  95

tgc ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat      336
Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
            100                 105                 110

agc ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg atc      384
Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
        115                 120                 125
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```
tcc ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc      432
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
    130                 135                 140

gac ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc      480
Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
145                 150                 155                 160

cct gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct      528
Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
                165                 170                 175

ttc cag cgc cgg gca gga ggg gtc ctg gtt gcc tcc cat ctg cag agc      576
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser
            180                 185                 190

ttc ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcc cag ccc          621
Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
        195                 200                 205


<210> SEQ ID NO 6
<211> LENGTH: 207
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys Leu Met Ala Leu Gln
1               5                   10                  15

Leu Leu Leu Trp His Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro
            20                  25                  30

Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu
        35                  40                  45

Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys
    50                  55                  60

Leu Val Ser Glu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
65                  70                  75                  80

Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
                85                  90                  95

Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
            100                 105                 110

Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
        115                 120                 125

Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
    130                 135                 140

Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
145                 150                 155                 160

Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
                165                 170                 175

Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser
            180                 185                 190

Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
        195                 200                 205


<210> SEQ ID NO 7
<211> LENGTH: 531
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(531)
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<400> SEQUENCE: 7

```
acc ccc ctg ggc cct gcc agc tcc ctg ccc cag agc ttc ctg ctc aag       48
Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
1               5                   10                  15

tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag       96
Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
            20                  25                  30

gag aag ctg gtg agt gag tgt gcc acc tac aag ctg tgc cac ccc gag      144
Glu Lys Leu Val Ser Glu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu
        35                  40                  45

gag ctg gtg ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg      192
Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu
    50                  55                  60

agc agc tgc ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa      240
Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
65                  70                  75                  80

ctc cat agc ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa      288
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu
                85                  90                  95

ggg atc tcc ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac      336
Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp
            100                 105                 110

gtc gcc gac ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga      384
Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly
        115                 120                 125

atg gcc cct gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc      432
Met Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala
    130                 135                 140

tct gct ttc cag cgc cgg gca gga ggg gtc ctg gtt gcc tcc cat ctg      480
Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu
145                 150                 155                 160

cag agc ttc ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcc cag      528
Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln
                165                 170                 175

ccc                                                                  531
Pro
```

<210> SEQ ID NO 8
<211> LENGTH: 177
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

```
Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
1               5                   10                  15

Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
            20                  25                  30

Glu Lys Leu Val Ser Glu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu
        35                  40                  45

Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu
    50                  55                  60

Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
65                  70                  75                  80

Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu
                85                  90                  95

Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp
```

-continued

```
            100                 105                 110

Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly
        115                 120                 125

Met Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala
    130                 135                 140

Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu
145                 150                 155                 160

Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln
                165                 170                 175

Pro
```

<210> SEQ ID NO 9
<211> LENGTH: 525
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(525)

<400> SEQUENCE: 9

```
atg acc ccc ctg ggc cct gcc agc tcc ctg ccc cag agc ttc ctg ctc       48
Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu
1               5                   10                  15

aag tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc       96
Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu
            20                  25                  30

cag gag aag ctg tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg      144
Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
        35                  40                  45

gtg ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg agc agc      192
Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
    50                  55                  60

tgc ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat      240
Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
65                  70                  75                  80

agc ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg atc      288
Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
                85                  90                  95

tcc ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc      336
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
            100                 105                 110

gac ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc      384
Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
        115                 120                 125

cct gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct      432
Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
    130                 135                 140

ttc cag cgc cgg gca gga ggg gtc ctg gtt gcc tcc cat ctg cag agc      480
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser
145                 150                 155                 160

ttc ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcc cag ccc          525
Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
                165                 170                 175
```

<210> SEQ ID NO 10
<211> LENGTH: 175
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 10

Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu
1 5 10 15

Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu
20 25 30

Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
35 40 45

Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
50 55 60

Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
65 70 75 80

Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
85 90 95

Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
100 105 110

Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
115 120 125

Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
130 135 140

Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser
145 150 155 160

Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
165 170 175

<210> SEQ ID NO 11
<211> LENGTH: 208
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 11

Met Ala Gln Leu Ser Ala Gln Arg Arg Met Lys Leu Met Ala Leu Gln
1 5 10 15

Leu Leu Leu Trp Gln Ser Ala Leu Trp Ser Gly Arg Glu Ala Val Pro
20 25 30

Leu Val Thr Val Ser Ala Leu Pro Pro Ser Leu Pro Leu Pro Arg Ser
35 40 45

Phe Leu Leu Lys Ser Leu Glu Gln Val Arg Lys Ile Gln Ala Ser Gly
50 55 60

Ser Val Leu Leu Glu Gln Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
65 70 75 80

Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Lys Ala Ser
85 90 95

Leu Ser Gly Cys Ser Ser Gln Ala Leu Gln Gln Thr Gln Cys Leu Ser
100 105 110

Gln Leu His Ser Gly Leu Cys Leu Tyr Gln Gly Leu Leu Gln Ala Leu
115 120 125

Ser Gly Ile Ser Pro Ala Leu Ala Pro Thr Leu Asp Leu Leu Gln Leu
130 135 140

Asp Val Ala Asn Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Asn Leu
145 150 155 160

Gly Val Ala Pro Thr Val Gln Pro Thr Gln Ser Ala Met Pro Ala Phe
165 170 175

-continued

```
Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Ala Ile Ser Tyr
            180                 185                 190

Leu Gln Gly Phe Leu Glu Thr Ala Arg Leu Ala Leu His His Leu Ala
        195                 200                 205


<210> SEQ ID NO 12
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 12

Met Lys Leu Met Ala Leu Gln Leu Leu Leu Trp His Ser Ala Leu Trp
1               5                   10                  15

Ser Gly Gln Glu Ala Ile Pro Leu Leu Thr Val Ser Ser Leu Pro Pro
            20                  25                  30

Ser Leu Pro Leu Pro Arg Ser Phe Leu Leu Lys Ser Leu Glu Gln Val
        35                  40                  45

Arg Lys Ile Gln Ala Arg Asn Thr Glu Leu Leu Glu Gln Leu Cys Ala
    50                  55                  60

Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Phe Gly His Ser
65                  70                  75                  80

Leu Gly Ile Pro Lys Ala Ser Leu Ser Ser Cys Ser Ser Gln Ala Leu
                85                  90                  95

Gln Gln Thr Lys Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr
            100                 105                 110

Gln Gly Leu Leu Gln Ala Leu Ala Gly Ile Ser Ser Glu Leu Ala Pro
        115                 120                 125

Thr Leu Asp Met Leu His Leu Asp Val Asp Asn Phe Ala Thr Thr Ile
    130                 135                 140

Trp Gln Gln Met Glu Ser Leu Gly Val Ala Pro Thr Val Gln Pro Thr
145                 150                 155                 160

Gln Ser Thr Met Pro Ile Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly
                165                 170                 175

Gly Val Leu Val Thr Ser Tyr Leu Gln Ser Phe Leu Glu Thr Ala His
            180                 185                 190

His Ala Leu His His Leu Pro Arg Pro Ala Gln Lys His Phe Pro Glu
        195                 200                 205

Ser Leu Phe Ile Ser Ile
    210


<210> SEQ ID NO 13
<211> LENGTH: 194
<212> TYPE: PRT
<213> ORGANISM: Felis catus

<400> SEQUENCE: 13

Lys Leu Met Ala Leu Gln Leu Leu Leu Trp His Ser Ala Leu Trp Met
1               5                   10                  15

Val Gln Glu Ala Thr Pro Leu Gly Pro Thr Ser Ser Leu Pro Gln Ser
            20                  25                  30

Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Val Gln Ala Asp Gly
        35                  40                  45

Thr Ala Leu Gln Glu Arg Leu Cys Ala Ala His Lys Leu Cys His Pro
    50                  55                  60
```

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```
Glu Glu Leu Val Leu Leu Gly His Ala Leu Gly Ile Pro Gln Ala Pro
65                  70                  75                  80

Leu Ser Ser Cys Ser Ser Gln Ala Leu Gln Leu Thr Gly Cys Leu Arg
                85                  90                  95

Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu
            100                 105                 110

Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Met Leu Gln Leu
        115                 120                 125

Asp Ile Thr Asp Phe Ala Ile Asn Ile Trp Gln Gln Met Glu Asp Val
    130                 135                 140

Gly Met Ala Pro Ala Val Pro Pro Thr Gln Gly Thr Met Pro Thr Phe
145                 150                 155                 160

Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly Thr Leu Val Ala Ser Asn
                165                 170                 175

Leu Gln Ser Phe Leu Glu Val Ala Tyr Arg Ala Leu Arg His Phe Thr
            180                 185                 190

Lys Pro


<210> SEQ ID NO 14
<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Bos taurus

<400> SEQUENCE: 14

Met Lys Leu Met Val Leu Gln Leu Leu Leu Trp His Ser Ala Leu Trp
1               5                   10                  15

Thr Val His Glu Ala Thr Pro Leu Gly Pro Ala Arg Ser Leu Pro Gln
            20                  25                  30

Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Ala Asp
        35                  40                  45

Gly Ala Glu Leu Gln Glu Arg Leu Cys Ala Ala His Lys Leu Cys His
    50                  55                  60

Pro Glu Glu Leu Met Leu Leu Arg His Ser Leu Gly Ile Pro Gln Ala
65                  70                  75                  80

Pro Leu Ser Ser Cys Ser Ser Gln Ser Leu Gln Leu Thr Ser Cys Leu
                85                  90                  95

Asn Gln Leu His Gly Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala
            100                 105                 110

Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Thr Leu Asp Thr Leu Gln
        115                 120                 125

Leu Asp Val Thr Asp Phe Ala Thr Asn Ile Trp Leu Gln Met Glu Asp
    130                 135                 140

Leu Gly Ala Ala Pro Ala Val Gln Pro Thr Gln Gly Ala Met Pro Thr
145                 150                 155                 160

Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser
                165                 170                 175

Gln Leu His Arg Phe Leu Glu Leu Ala Tyr Arg Gly Leu Arg Tyr Leu
            180                 185                 190

Ala Glu Pro
        195


<210> SEQ ID NO 15
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-continued

<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Sus scrofa

<400> SEQUENCE: 15

Met Lys Leu Met Ala Leu Gln Leu Leu Leu Trp His Ile Ala Leu Trp
1 5 10 15

Met Val Pro Glu Ala Ala Pro Leu Ser Pro Ala Ser Ser Leu Pro Gln
20 25 30

Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Ala Asp
35 40 45

Gly Ala Glu Leu Gln Glu Arg Leu Cys Ala Thr His Lys Leu Cys His
50 55 60

Pro Gln Glu Leu Val Leu Leu Gly His Ser Leu Gly Leu Pro Gln Ala
65 70 75 80

Ser Leu Ser Ser Cys Ser Ser Gln Ala Leu Gln Leu Thr Gly Cys Leu
85 90 95

Asn Gln Leu His Gly Gly Leu Val Leu Tyr Gln Gly Leu Leu Gln Ala
100 105 110

Leu Ala Gly Ile Ser Pro Glu Leu Ala Pro Ala Leu Asp Ile Leu Gln
115 120 125

Leu Asp Val Thr Asp Leu Ala Thr Asn Ile Trp Leu Gln Met Glu Asp
130 135 140

Leu Arg Met Ala Pro Ala Ser Leu Pro Thr Gln Gly Thr Val Pro Thr
145 150 155 160

Phe Thr Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Val Ser
165 170 175

Gln Leu Gln Ser Phe Leu Glu Leu Ala Tyr Arg Val Leu Arg Tyr Leu
180 185 190

Ala Glu Pro
195

<210> SEQ ID NO 16
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide for GCSF

<400> SEQUENCE: 16

Leu Gly His Ser Leu Gly Ile
1 5

<210> SEQ ID NO 17
<211> LENGTH: 174
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polypeptide variant of human G-CSF

<400> SEQUENCE: 17

Ala Pro Thr Thr Arg Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
1 5 10 15

Ser Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
20 25 30

-continued

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Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
        35                  40                  45

Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
    50                  55                  60

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
65                  70                  75                  80

Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
                85                  90                  95

Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
            100                 105                 110

Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
        115                 120                 125

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
    130                 135                 140

Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
145                 150                 155                 160

Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
                165                 170
```

1. A method for treating stroke of a mammalian subject, comprising:

(a) starting the administration of G-CSF or a functionally active G-CSF derivative in a therapeutically active amount to the subject; and subsequently

(b) administering to the subject a thrombolytic agent in a therapeutically active amount.

2. The method of claim **1**, wherein the subject undergoes after the starting (a) and before the administering (b) a diagnostic examination to exclude the risk of hemorrhagic or other adverse side effects during the administering (b).

3. The method of claim **1**, wherein human G-CSF is used administered in (a).

4. The method of claim **1**, wherein the thrombolytic agent in (b) comprises rt-PA.

5. The method of claim **1**, wherein the administration of G-CSF or a functionally active G-CSF derivative starts within the first 6 h after onset of the stroke.

6. The method of claim **1**, wherein the thrombolytic agent is administered later than 6 h after onset of the stroke.

7. The method of claim **1**, wherein the administration of G-CSF or a functionally active G-CSF derivative is completed within the first 6 h after onset of the stroke.

8. The method of claim **1**, wherein the thrombolytic agent is administered at least 0.5 h after the starting of the administration of G-CSF or a functionally active G-CSF derivative.

9. The method of claim **2**, wherein the diagnostic examination lasts at lest least 0.5 h.

10. The method of claim **1**, wherein G-CSF is given intravenously or subcutaneously in a dose of at least 90 μg/kg body weight.

11. The method of claim **1**, wherein the stroke is an acute stroke,

wherein the subject is admitted to a stroke unit or a clinic within the initial 6 h after stroke onset, and

wherein an expenditure of time for a diagnostic examination necessary to assess the subject's risk of hemorrhagic or at least one other severe adverse side effect of a thrombolytic treatment would otherwise cause an expiration of a therapeutic window for thrombolytic treatment.

12. The method of claim **11**, wherein the therapeutic window for thrombolytic treatment is 3 h after stroke onset.

13. The method of claim **11**, wherein the diagnostic examination lasts at least 0.5 h

14. The method of claim **11**, wherein the mammalian subject receives the G-CSF or functionally active G-CSF derivative immediately after admittance to the stroke unit or clinic.

15. The method of claim **11**, wherein the G-CSF is human G-CSF.

16. The method of claim **1**, wherein a stroke onset has been observed in the subject within six hours before the administration of G-CSF and wherein apart from observing the stroke onset$_s$ no further stroke diagnosis has been performed before the administration of G-CSF.

17. The method of claim **1**, wherein a stroke onset has been observed in the subject within six hours before the administration of G-CSF and wherein after the administration of G-CSF a diagnostic examination of the subject to exclude the risk of hemorrhagic or other adverse side effects due to the administration of the thrombolytic agent is performed,

18. A thrombolytic agent, suitable for treating stroke in a subject, wherein the subject has been administered with G-CSF within 6 hours after the onset of stroke in the subject and wherein after administration of the G-CSF a diagnostic examination of the subject to exclude a risk of hemorrhagic or at least one other adverse side effect due to the administration of the thrombolytic agent has been performed.

19. The thrombolytic agent of claim **18**, suitable for administration later than 6 h after onset of the stroke.

20. A composition, comprising:

a G-CSF or a functionally active G-CSF derivative; and

a pharmaceutically acceptable carrier.

* * * * *