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(57) Abstract

The present invention relates to a (pharmaceutical) composition comprising a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule and, optionally, a pharmaceutically acceptable carrier and/or diluent. Furthermore, the present invention relates to the use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the preparation of a (pharmaceutical) composition for enhancing collateral growth of collateral arteries and/or arteries from pre-existing arteriolar connections and/or preventing and/or treating an occlusive disease. The present invention also relates to a method for enhancing collateral growth of collateral arteries and/or arteries from pre-existing arteriolar connections, and/or preventing and/or treating an occlusive disease, said method comprising administering to a subject in need thereof an effective amount of circulating blood cells, preferably monocytes loaded with a therapeutically active molecule. Also described are (pharmaceutical) kits, diagnostic compositions, their preparation and use as well as diagnostic methods.

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Pharmaceutical compositions comprising circulating blood cells, preferably monocytes and uses thereof

The present invention relates to a (pharmaceutical) composition comprising a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule and, optionally, a pharmaceutically acceptable carrier and/or diluent. Furthermore, the present invention relates to the use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the preparation of a (pharmaceutical) composition for enhancing collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections and/or preventing and/or treating an occlusive disease. The present invention also relates to a method for enhancing collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections, and/or preventing and/or treating an occlusive disease, said method comprising administering to a subject in need thereof an effective amount of circulating blood cells, preferably monocytes loaded with a therapeutically active molecule. Also described are (pharmaceutical) kits, diagnostic compositions, their preparation and use as well as diagnostic methods.

Preexisting collateral arteries are found in many regions of the body (hind limbs, heart, brain etc.). These vessels have the ability to markedly increase their lumen by growth so as to provide enhanced perfusion to the jeopardized ischemic regions following acute and chronic arterial occlusions. This process is called arteriogenesis and is not a process of passive dilatation but one of active proliferation and remodeling. Under normal flow conditions and depending on the pressure. gradient between the interconnecting arterial networks there is only minimal net forward flow, but small amounts of flow may oscillate within the network. In case of a sudden arterial occlusion or a slowly progressing stenosis, a steep pressure gradient along the shortest path within the interconnecting network develops which increases the

blood flow velocity and hence fluid shear stress in these vessels that now assume the new function as "collaterals". The effect of this sustained increase in shear is the upregulation of distinct processes in the collateral arteries: upregulation of adhesion molecules [e.g. VCAM, ICAM]; increased endothelial production of several cytokines [MCP-1, GM-CSF, TNF-α]; attraction of circulating blood cells, preferably monocytes along the chemotactic gradient to the activated endothelium; adhesion and invasion of circulating blood cells, preferably monocytes and maturation to macrophages. These in turn create an inflammatory environment and produce fairly large amounts of growth factors, in particular fibroblast growth factor-2 (FGF-2). The invasion of circulating blood cells, preferably monocytes is soon followed by the first wave of mitosis of the endothelial- and smooth muscle cells (proliferating phase). Besides mitosis the perivascular inflammation creates the space for the greatly expanding collateral vessel that can increase its diameter for up to 20 times. The old structure is for a large part dismantled and replaced largely by new intimal and medial smooth muscle cells (remodelling phase). Finally arteriogenesis results in a functional artery originating from a preexisting arteriole. In previous studies it could be shown that chronic intraarterial infusion of the monocyte chemoattractant protein-1 [MCP-1] greatly increased the development of arterial collateral blood vessels following femoral artery occlusion. These collaterals were more numerous on angiograms and their ability to conduct blood had increased by a factor of six-fold. The histological appearance of these typical corkscrew vessels was that of muscular arteries. In another study a single dose of lipopolysaccharide [LPS] was injected intravenously into New Zealand White Rabbits 3 days after ligation of the femoral artery. This potent stimulator of Tumor-Necrosis-Factor-alpha [TNF-α] also markedly enhanced the number of monocyte-derived macrophages that accumulated around growing collateral arteries. Peripheral and collateral conductances were markedly increased. Histological and mRNA studies showed an upregulation of ICAM-1 on the endothelium of the newly recruited collateral arteries as compared to normal arteries. In another study it could be shown that the process of rapidly proliferating collateral arteries (arteriogenesis) can be inhibited with monoclonal antibodies against ICAM-1. This indicates that monocyte invasion, the sequel of ICAM-1 upregulation on the endothelial side and the first detectable morphological event in early collateral artery formation, is an obligatory step in arteriogenesis.

In the treatment of subjects with arterial occlusive diseases most of the current treatment strategies aim at ameliorating their effects. The only curative approaches

involve angioplasty (balloon dilatation) or bypassing surgery. The former carries a high risk of restenosis and can only be performed in certain arterial occlusive diseases, like ischemic heart disease. The latter is invasive and also restricted to certain kinds of arterial occlusive diseases. There is no established treatment for the enhancement of collateral growth.

Thus, the technical problem underlying the present invention is to provide (pharmaceutical) compositions and methods for the enhancement of the growth of collateral arteries and/or other arteries from preexisting arteriolar connections.
The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a composition, preferably a pharmaceutical composition comprising a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule and, optionally, a pharmaceutically acceptable carrier and/or diluent.
As is well known in the art, monocytes descend from bone marrow derived myeloid progenitors and belong to the mononuclear phagocyte system. They circulate in the blood and have the ability to adhere to the endothelium. Surviving apoptosis they ultimately transmigrate through the endothelial layer and differentiate into macrophages (for details see, e.g., Roitt, I. et al., Immunology, Gower Medical Publishing Ltd., London, 1985). As used in accordance with the present invention, the term "monocyte" comprises monocytes as well as cells that display essentially the same biological properties/activities as monocytes. Such properties are invasion into proliferating collateral arteries and production of a variety of growth factors as indicated in the pertinent literature. Also, for putting the present invention into action other circulating blood cells that are also involved in arteriogenesis such as mast cells, lymphocytes, or granulocytes as well as cells that display essentially the same biological properties/activities as mast cells, lymphocytes, or granulocytes may be used and, thus, are encompassed by the present invention. In accordance with the present invention, it has surprisingly been found that circulating blood cells, preferably monocytes when isolated from the blood of a subject and treated in vitro so as to take up a desired molecule retain their biological properties/activities when returned to said subject. In particular, it could be demonstrated that such circulating blood cells, preferably monocytes keep the ability to be attracted by chemotactic

molecules and, more importantly, that these loaded cells are able to transmigrate through the endothelium of proliferating collateral arteries. Rather, the person skilled in the art would have expected that the such loaded circulating blood cells would have been selected against in the spleen and then been discarded. Further, in vitro manipulation of cells often leads to adverse affection of membranes that often renders them unsuitable for therapeutic in vivo applications.

In accordance with the invention, it was found that after ligation of the femoral artery accumulation of small fluorescent microspheres in the hindlimb muscle tissue only occurred when they were loaded into monocytes. Furthermore, in these experiments the quantified number of monocytes was significantly higher in the ligated hindlimb as compared to the unligated hindlimb (Figure 1). This indicates that monocyte invasion is an obligatory and highly selective step in collateral artery formation and that circulating monocytes play a crucial role in early arteriogenesis. Furthermore, these data demonstrate that microspheres can be selectively transported on a biological route to the place of action, namely proliferating collateral arteries.

Therefore, it is envisaged in accordance with the present invention that circulating blood cells, preferably monocytes are used as vehicles for therapeutically active molecules, wherein said circulating blood cells, preferably monocytes can deliver said therapeutically active molecules selectively to a desired site. Since said therapeutically active molecules are carried within the circulating blood cells, preferably monocytes and are only released therefrom at the desired site (due to the fact that circulating blood cells, preferably monocytes attach to and penetrate shear stress activated endothelium, for example, due to the upregulation of ICAM-1), the application of the (pharmaceutical) compositions of the present invention advantageously results in low systemic concentrations of said therapeutically active molecules but in very high local concentrations. Thereby it is possible to avoid systemic side effects of the applied substance in patients without decreasing efficacy of the applied substance at the place of interest.

The (pharmaceutical) composition of the present invention may further comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable (pharmaceutical) carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These (pharmaceutical) compositions can be administered to the subject at a suitable dose. Administration of

the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 μg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the (pharmaceutical) composition should be in the range of 1 μg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 μg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the (pharmaceutical) composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the (pharmaceutical) composition.

In another embodiment, the present invention relates to the use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the

preparation of a (pharmaceutical) composition for enhancing collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections.
For the purpose of the present invention the growth of arteries from preexisting arteriolar connections is also called "arteriogenesis". In particular, "arteriogenesis" is the in situ growth of arteries by proliferation of endothelial and smooth muscle cells from preexisting arteriolar connections supplying blood to ischemic tissue, tumor or sites of inflammation. These vessels largely grow outside the affected tissue but are much more important for the delivery of nutrients to the ischemic territory, the tumor or the site of inflammation than capillaries sprouting in the diseased tissue by angiogenic processes.

The present invention also relates to the use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the preparation of a (pharmaceutical) composition for preventing and/or treating an occlusive disease.

In another embodiment, the present invention relates to a method for enhancing collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections, and/or preventing and/or treating an occlusive disease, said method comprising administering to a subject in need thereof an effective amount of circulating blood cells, preferably monocytes loaded with a therapeutically active molecule.

In a preferred embodiment of the use or the method of the present invention, said occlusive disease is an arterial occlusive disease.

In a more preferred embodiment said arterial occlusive disease is a coronary artery disease, cerebral occlusive disease, peripheral occlusive disease, visceral occlusive disease, renal artery disease or mesenterial arterial insufficiency.

In another preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention said therapeutically active molecule is a nucleic acid molecule encoding an arteriogenic polypeptide or functionally equivalent fragment thereof, or is an arteriogenic polypeptide or functionally equivalent fragment thereof or a lipopolysaccharide-like molecule.

The term "lipopolysaccharide-like molecule" is intended to comprise molecules that display the antigenicity but not the toxicity of LPS. Also comprised in certain embodiments of the invention is the employment of substances that enhance adhesion of the monocyte and transmigration to the endothelium of proliferating collateral arteries like, e.g., cell adhesion molecules like selectins and/or integrins such as the Mac-1 Receptor, etc.

In a more preferred embodiment, said nucleic acid molecule is DNA.
However, other nucleic acid molecules like, e.g., RNA, peptide nucleic acid (PNA) or nucleic acid molecules comprising two or more of the above mentioned nucleic acid molecules may also be used in accordance with the present invention.

In an additional more preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention, said nucleic acid molecule is comprised in a vector.
Vectors that may be used in accordance with the present invention comprise, e.g., plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering. Preferably, said vector is an expression vector and/or a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the nucleic acid molecule of the invention into targeted cell populations. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel et al., Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the nucleic acid molecule of the invention can be reconstituted into liposomes for delivery to target cells. The vector comprising the nucleic acid molecule of the invention can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas, e.g., calcium phosphate, liposome or DEAE-Dextran mediated transfection or electroporation may be used for other cellular hosts; see Sambrook, supra.
Such vectors may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions.

In a further more preferred embodiment, said nucleic acid molecule is operatively linked to an expression control sequence.

Said expression control sequence allows expression in prokaryotic or preferably in eukaryotic cells. Expression of said nucleic acid molecule comprises transcription of the nucleic acid molecule into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and, optionally, a poly-A signal ensuring termination of transcription and stabilization of the transcript, and/or an intron further enhancing expression of said polynucleotide. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the nucleic acid molecule. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the nucleic acid molecule of the invention and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an C- or N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL)) or pCI (Promega).

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used.
As mentioned above, the vector of the present invention may also be a gene transfer or targeting vector. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The nucleic acid molecule and vector of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) into the cell.

In a most preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention, said expression control sequence is constitutively active or inducible.
Expression control sequences that are constitutively active and that may be used in accordance with the present invention have been discussed hereinabove. Such expression control sequence may be used if, e.g., expression of the arteriogenic polypeptide or functionally equivalent fragment thereof at sites other than the desired site is tolerable.
A "desired site" in accordance with the present invention may be, e.g., a site of vessel occlusion or, generally, a site where collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections shall be enhanced like, e.g., the collateral circulation of the brain or the arteries of the neck (stenosis of the carotid arteries).
However, if constitutive expression of the arteriogenic polypeptide or functionally equivalent fragment thereof is not desirable, inducible expression control sequences may advantageously be used that ensure expression only at the target site. Examples of such inducible expression control sequences include sequences that are sensitive to either a biological signal (such as increased levels of shear stress within collateral arteries or elevated concentrations of, e.g., a cytokine and/or

chemokine) and/or are sensitive to the signal of an externally located device producing a physical, chemical, biological and/or other signal (see below).

In another more preferred embodiment, said arteriogenic polypeptide attracts and/or activates circulating arteriogenic cells and/or influences the survival rate of circulating and resident arteriogenic cells or is VCAM, ICAM, FGF-2, J-309, or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 and/or Platelet-activating factor (PAF) or a functionally equivalent fragment thereof.

In a most preferred embodiment, said arteriogenic polypeptide attracting circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, or a functionally equivalent fragment thereof, said arteriogenic polypeptide activating circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, Tumor Necrosis Factor alpha (TNF-alpha), or a functionally equivalent fragment thereof, and/or said arteriogenic polypeptide influencing the survival rate of circulating and resident arteriogenic cells is selected from the group consisting of colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF or a functionally equivalent fragment thereof.

In a further preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention, said therapeutically active molecule is reversibly coupled to a microsphere.
Advantageously, coupling of the therapeutically active molecule to a microsphere allows the selective transport of these molecules. Moreover, these molecules stay inactive as long as they are coupled to the microspheres. An example of such a reversible coupling is a hydrophobic or van-der-Waals coupling reaction. Only after the release from said microsphere these molecules turn active. The person skilled in the art is well aware of corresponding coupling methods. The complex of therapeutically active molecules and microspheres is preferably taken up by, e.g., the monocytes by phagocytosis.

In a more preferred embodiment, said microsphere is a latex particle, lipid-comprising structure, sponge-comprising structure, microbubble, polystyrene microsphere, biotinylated polystyrene microsphere, protein or dextran conjugate with different sizes and surface properties or any other substance that can bind said therapeutically active molecule.

In an additional preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention, loading of the circulating blood cell, preferably a monocyte is effected by phagocytosis, diffusion, transfection/transformation, receptor mediated uptake of molecules, electroporation and/or other mechanical methods of uptake.

In a more preferred embodiment, prior to said loading the lysosomal activity of said circulating blood cell, preferably a monocyte is inhibited.
This inhibition of activity can be achieved by several (pharmaceutical) and/or other substances decreasing the lyosomal activity like, e.g., gangliosides, protease-inhibitors, etc., that are well known in the art. Inhibition of the lysosomal activity may be necessary in cases where said therapeutically active molecule is prone to rapid degradation.

In a preferred embodiment of the (pharmaceutical) composition, the use or the method of the present invention, said therapeutically active molecule is released from the circulating blood cell, preferably a monocyte at the desired site.

In a more preferred embodiment, prior to said release from said circulating blood cell, preferably a monocyte said therapeutically active molecule is uncoupled from said microsphere.
Advantageously, this uncoupling is effected by an external device, which is located close to or directly on the skin in regions of proliferating collateral arteries and which influences the binding of the molecule, DNA etc. to the microsphere. An externally controlled release further guarantees that only microsphere loaded circulating blood cells, preferably monocytes in the region of interest may release the molecule and/or DNA and/or other substance. Other circulating blood cells, preferably monocytes,

attaching, e.g., to atherosclerotic plaques etc. may invade these regions but cannot release the molecule.
Accordingly, it is, for example, envisaged in accordance with the present invention that the therapeutically active molecule after uncoupling is directly secreted from the circulating blood cell, preferably a monocyte and delivered to the desired target site. If, e.g., the therapeutically active molecule is a nucleic acid molecule encoding an arteriogenic polypeptide or functionally equivalent fragment thereof, said acid molecule may be delivered to the described target cells where said arteriogenic polypeptide or functionally equivalent fragment thereof is expressed. Alternatively or additionally, after uncoupling said nucleic acid molecule may be delivered to the nucleus of the circulating blood cell, preferably a monocyte and said arteriogenic polypeptide or functionally equivalent fragment thereof be expressed in the circulating blood cell, preferably a monocyte and secreted.

In a most preferred embodiment, uncoupling is effected by a pH-change, echocardiographic signal, magnetic resonance signal, ultrasound, and/or thermographic signal.

In another preferred embodiment of the use of the present invention, said (pharmaceutical) composition is administered after local administration of a circulating blood cell monocyte chemotactic protein, preferably a monocyte chemotactic protein.

In a further preferred embodiment of the method of the present invention, prior to administering said effective amount of circulating blood cells, preferably monocytes loaded with a therapeutically active molecule, an effective amount of a circulating blood cell monocyte chemotactic protein, preferably a monocyte chemotactic protein is administered.

The present invention further relates to a (pharmaceutical) kit comprising the (pharmaceutical) composition of the present invention and, in a different compartment, another (pharmaceutical) composition comprising a circulating blood cell, preferably a monocyte chemotactic protein.

In a more preferred embodiment of the use, the (pharmaceutical) kit or the method of the present invention, said circulating blood cell monocyte chemotactic protein, preferably a monocyte chemotactic protein attracts and/or activates circulating arteriogenic cells and/or influences the survival rate of circulating and resident arteriogluic cells or is a lipopolysaccharide-like molecule, J-309, or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 and/or Platelet-activating factor (PAF).

In a most preferred embodiment, said monocyte chemotactic protein attracting circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, or a functionally equivalent fragment thereof, said monocyte chemotactic protein activating circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, Tumor Necrosis Factor alpha (TNF-alpha), or a functionally equivalent fragment thereof, and/or said monocyte chemotactic protein influencing the survival rate of circulating and resident arteriogenic cells is selected from the group consisting of colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF or a functionally equivalent fragment thereof.

In another embodiment, the present invention relates to a diagnostic composition comprising a circulating blood cell, preferably a monocyte loaded with a detectable molecule.
The diagnostic composition optionally comprises suitable means for detection. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

The present invention also relates to the use of a circulating blood cell, preferably a monocyte loaded with a detectable molecule for the preparation of a diagnostic

composition for detecting collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections.

Furthermore, the present invention relates to the use of a circulating blood cell, preferably a monocyte loaded with a detectable molecule for the preparation of a diagnostic composition for detecting a vessel occlusion.

In a further embodiment, the present invention relates to a method for detecting collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections, and/or detecting and/or diagnosing a vessel occlusion, comprising the detection of microspheres originally contained in the circulating blood cells, preferably monocytes in said arteries or occlusions.
Additionally, occlusion can then be measured by clinical parameters such as Doppler echo or contrast media echo etc.

In a preferred embodiment of the diagnostic composition, the use or the method of the present invention said detectable molecule is a substance that attracts and/or activates circulating arteriogenic cells and/or influences the survival rate of circulating and resident arteriogluic cells or is a lipopolysaccharide-like molecule, J-309, or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 or Platelet-activating factor (PAF) or an expressible nucleic acid molecule encoding one or more of the above mentioned (poly)peptides.

In a more preferred embodiment, said substance attracting circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, or a functionally equivalent fragment thereof, said substance activating circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, Tumor Necrosis Factor alpha (TNF-alpha), or a functionally equivalent fragment thereof, and/or said substance influencing the survival rate of circulating and resident arteriogenic cells is selected from the group consisting of colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or

M-CSF or a functionally equivalent fragment thereof, or an expressible nucleic acid molecule encoding one or more of the above mentioned (poly)peptides.

In another preferred embodiment, said detectable molecule is coupled to a microsphere.

In a more preferred embodiment, said microsphere is a latex particle, lipid-comprising structure, sponge-comprising structure, microbubble, polystyrene microsphere, biotinylated polystyrene microsphere, protein or dextran conjugate with different sizes and surface properties or any other substance that can bind said detectable molecule.

In a further preferred embodiment of the diagnostic composition, the use or the method of the present invention, loading of the circulating blood cell, preferably a monocyte is effected by phagocytosis, diffusion or transfection.
If the detectable molecule is a nucleic acid molecule, what has been discussed hereinabove with respect to expression of nucleic acid molecules, i.e. endogenous production of therapeutic substances, methods of introducing nucleic acid molecules into cells (e.g. calcium phosphate, liposome or DEAE-Dextran mediated transfection or electroporation), etc., also applies here.

The components of the kit, the (pharmaceutical) and/or diagnostic composition of the present invention may be packaged in containers such as vials, optionally in buffers and/or solutions. If appropriate, one or more of said components may be packaged in one and the same container.

The references cited in the present specification are herewith incorporated by reference.

The figures show:

Figure 1:

Number of monocytes/gram muscle tissue: direct infusion of microspheres= 1000±725, unligated hindlimb =35000±7200; ligated hindlimb = 120000±10500. All differences were significant.

Figure 2:

Total number of monocytes/gram tissue: unligated; 35000 ± 7200, ligated; 120000 ± 10500, MCP-1 treatment: 320000 ± 28000, MCP-1 treatment and anti-ICAM antibodies;132000 ± 11000. The number of monocytes/gram tissue was significantly different for all groups except for the ligated group and the group treated with the combination of MCP-1 and anti-ICAM antibodies.

The examples illustrate the invention.

Example 1: Animal model

The present study was performed with the permission of the State of Hessen, Regierungspraesidium Darmstadt, according to section 8 of the *German Law for the Protection of Animals.* It conforms with the *Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health* (NIH Publication No. 85-23, revised 1985).

The surgical procedure was performed under sterile conditions. Femoral arteries were exposed and cannulated with a sterile polyethylene catheter (inner diameter: 1mm; outer diameter: 1.5mm) pointing upstream, with the tip of the catheter positioned distal to the branching of the arteria circumflexa femoris. The catheter itself occludding the artery was connected to an osmotic minipump (2ML-2, Alza Corporation, Palo Alto, CA), which was implanted under the skin of the lower right abdomen. After closure of the incision and subcutaneous application of antibiotics, the animals were outfitted with plastic collars that allowed them to move freely but prevented self-mutilation. The rabbits were housed individually with free access to water and chow to secure mobility. Hematocrit and serum values of total protein,

albumin, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase were not significantly changed by the treatment. For hemodynamic measurement the animals were again anesthetized with an intramuscular injection of ketamine hydrochloride and xylazine for tracheostomy and artificial ventilation. Anesthesia was deepened with pentobarbital (12 mg/kg body weight per hour). The carotid artery was cannulated for continuous pressure monitoring. The arteria saphena magna (anterior tibial artery in humans and main arterial supply to the lower limb and foot in the rabbit) was exposed just above the ankle and cannulated with sterile polyethylene heparinized tubing (inner diameter 0.58mm; outer diameter 0.96mm). These tubings were connected to a Statham P23DC pressure transducer (Statham, Spectramed) for measurement of peripheral pressures (PP). After heparinization with 5000 Units heparin, the left femoral artery was exposed and cannulated with sterile polyethylene catheter (inner diameter: 1mm; outer diameter: 1.5mm) for the microsphere reference sample. After cannulation of the abdominal aorta a pump-driven shunt was installed to ensure oxygenated blood flow from the carotid artery via the canula in the abdominal aorta into the right and left legs. A flow probe was installed to measure total flow to both hindlimbs. The absence of any residual volume in the minipumps (<3%) after the experiment verified delivery of the contents.

Example 2: In vivo Pressure-Flow Relations

Maximum vasodilation was achieved by injecting adenosine (1 mg/ml; Sigma Chemical Company, St. Louis, MO) to the shunt at a flow rate of 0.5 ml/min. After stabilization of peripheral and central pressures both legs were perfused at six different pressures.

The six perfusion pressures levels (40,50,60,70,80,90 mmHg) were generated in vivo with a roller pump (Stoeckert) installed in the above mentioned shunt between carotid artery and abdominal aorta. Peripheral pressures and collateral flows were measured under maximal vasodilation (adenosine). For each pressure level microspheres with a different fluorescent color (either scarlet, crimson, red, blue-green, yellow-green or orange; diameter: 15 μm; Molecular Probes, Eugene, Oregon, U.S.) were injected into the mixing chamber, which was installed in the carotid-abdominal aortic shunt. All recordings were stored on a computerized recordings system (MacLab, Macintosh) from which they were retrieved for further processing.

Example 3: Counting of Microspheres

The Quadriceps-, adductor longus-, adductor magnus-, gastrocnemius-, soleus, and peroneal muscles were dissected and each muscle was divided into 3 consecutive pieces from the proximal to the distal end. The whole muscle and afterwards each sample were weight and cut into small pieces (1-2g). The muscle samples were then packed loosely into 12mm x 75mm polystyrene tubes (Becton Dickinson & Co, Lincoln Park, NJ) containing 3 ml of SDS solution [SDS solution (Boehringer Mannheim Corp.): 1% SDS, 0.5% sodium azide (Sigma Chemical Company, St. Louis, MO), and 0.8% tween-80 (Fisher Scientific, Fairlawn, NJ) in 50 millimolar pH 8 tris buffer (Sigma Chemical Company, St. Louis, MO)], proteinase K solution (2 mg/ml; Boehringer Mannheim Corp.) and 1 ml of suspended blue microspheres as internal standard (diameter: 15µm, Molecular Probes, Eugene, Oregon, U.S.). Each tube was capped and secured in a shaking water bath for 24-48 hours. The samples were then centrifuged at 1000g for 45 minutes, the supernatant was pipetted off and the pellet was resuspended in 1 ml PBS (pH 7.4). The probes were rigorously shaken before FACScan analysis. The microspheres were counted using a flow cytometer (FACS-Calibur) equipped with a second laser and a detector for a fourth fluorescence. Flows for each sample were calculated from the number of microspheres in the sample (m^S), the respective microspheres count in the reference sample (m^{rS}), the internal standard in the sample (IS^S), internal standard in the reference sample (IS^{rS}), the weight of the reference sample (W) and the time during which the reference sample was withdrawn using following equation:

$$\text{flow [mg/ml]} = \frac{m^s \cdot IS^{rs}}{IS^s \cdot m^{rs}} \cdot \frac{w}{t}$$

m^s = sample microsphere
IS^{rs} = internal standard reference
IS^s = internal standard
m^{rs} = microsphere reference sample
w = weight
t = time

Example 4: Calculation of Conductances

We measured systemic pressure [SP] and peripheral pressure [PP]. Venous pressure was equal to atmospheric pressure [AP] (zero in our case). Since arterial resistances are much lower than collateral and peripheral resistances, they can be neglected. SP represent the pressure at the stem region of the collateral arteries. PP is the pressure at the reentry region and is identical to the pressure head of the circulation in the lower leg; AP is the pressure at the venous end of the peripheral circulation. Collateral flow is equal to the sum of flows to the tissue of the distal adductor plus the flow to the tissue of the lower leg. Collateral resistance was defined as pressure difference between SP and PP divided by the flow going to the distal adductor an the lower leg. The reciprocal values of these resistances represent collateral-, peripheral-, and bulk conductance. Because a positive pressure intercept is observed even at maximal vasodilation, all conductances were calculated from the slope of pressure-flow relations.

Example 5: Postmortem Angiography

After maximal vasodilatation (adenosine) legs were perfused with Krebs-Henseleit buffered saline in a warmed waterbath of 37°C for 1 minute at a pressure of 80 mmHg, followed by perfusion for 8-10 minutes at 80 mmHg with contrast medium based on bismuth and gelatin according to a formula developed by Fulton. Subsequently, the contrast medium was allowed to gel by placing the limbs on crushed ice for 45 minutes. Angiograms were taken at two different angles in a "soft X-ray" Balteau radiography apparatus (Machlett Laboratories) at low accelerating voltage (20kEV) using a single-enveloped Structurix D7DW film (AGFA). The resulting stereoscopic images allowed analysis of collateral growth in three dimensions.

Example 6: Quantification of collateral arteries

To differentiate between collateral vessels and muscle vessels for further quantification, we used Longland's definition of collateral arteries (Longland et. al. 1954). Stem, midzone and reentry were identified under stereoscopic viewing using a 3-fold magnification of our angiograms. Collateral arteries then were divided in two groups: group one consisted of vessels whose stem branched from the arteria circumflexa femoris lateralis. Group two of the arteries originated from the arteria profunda femoris. The length of the midzone in each group was almost the same, so their measurement did not give any further information. Re-entry of the collaterals from the first group usually descended into the arteria genus descendens, the second group into the arteria caudalis femoris. Only about 10% of the collateral arteries originate from other vessels, e.g., from the a. iliaca externa or from the a. iliaca interna. Collateral vessels were marked after counting to make sure, that no vessel was counted twice. We used a further 3-fold magnification to measure the diameter of the vessels with an accuracy of ±0.1 mm.

Example 7: Statistical Analysis

Data are described as mean ± SD. Differences were assessed using unpaired Student's t-test for intergroup comparisons and the Mann-Whithney rank-sum test for unequal variances. Values of $p \leq .05$ were accepted for statistical significance.

Example 8: Evaluation of the kinetics of monocytes in collateral arteries via monocyte loading with fluorescent microspheres

The right femoral artery was ligated in 12 rabbits. Isolated rabbit monocytes were loaded with fluorescent microspheres (∅ 2µm) and reinfused intravenously. 48 hours after reinfusion animals were killed and biopsies were taken of the adductor and quadriceps muscle in both hindlimbs whereby the biopsies of the left hindlimb served as control. In a second control group microspheres were directly infused. With FACS analysis the total number of monocytes per gram muscle tissue was quantified (Figure 1).

Example 9: Attraction of loaded monocytes via MCP-1

12 rabbits were treated with local infusion of either MCP-1 alone or MCP-1 plus monoclonal ICAM-antibodies via osmotic minipumps after ligation of the a. femoralis. Non-ligated animals and ligated animals infused with PBS served as control. Isolated rabbit monocytes were loaded with fluorescent microspheres (∅ 2μm) and reinfused intravenously. 48 hours after reinfusion animals were killed and biopsies were taken of the adductor and quadriceps muscle in both hindlimbs. With FACS analysis the total number of monocytes/gram tissue was quantified (Figure 2).
Like in example 8, all New Zealand White rabbits were clinically healthy and showed no obvious defects. None of the animals were lost during or after femoral artery ligation. We also did not observe any gangrene or gross impairment of hindlimb function after femoral artery occlusion.

Claims

1. A composition, preferably a pharmaceutical composition comprising a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule and, optionally, a pharmaceutically acceptable carrier and/or diluent.

2. Use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the preparation of a (pharmaceutical) composition for enhancing collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections.

3. Use of a circulating blood cell, preferably a monocyte loaded with a therapeutically active molecule for the preparation of a (pharmaceutical) composition for preventing and/or treating an occlusive disease.

4. The use of claim 3, wherein said occlusive disease is an arterial occlusive disease.

5. The use of claim 4, wherein said arterial occlusive disease is a coronary artery disease, cerebral occlusive disease, peripheral occlusive disease, visceral occlusive disease, renal artery disease or mesenterial arterial insufficiency.

6. The (pharmaceutical) composition of claim 1, or the use of any one of claims 2 to 5, wherein said therapeutically active molecule is a nucleic acid molecule, a nucleic acid molecule encoding an arteriogenic polypeptide or functionally equivalent fragment thereof, or is an arteriogenic polypeptide or functionally equivalent fragment thereof or a lipopolysaccharide-like molecule.

7. The (pharmaceutical) composition or the use of claim 6, wherein said nucleic acid molecule is DNA.

8. The (pharmaceutical) composition or the use of claim 6 or 7, wherein said nucleic acid molecule is comprised in a vector.

9. The (pharmaceutical) composition or the use of any one of claims 6 to 8, wherein said nucleic acid molecule is operatively linked to an expression control sequence.

10. The (pharmaceutical) composition or the use of claim 9, wherein said expression control sequence is constitutively active or inducible.

11. The (pharmaceutical) composition or the use of any one of claims 6 to 10, wherein said arteriogenic polypeptide attracts and/or activates circulating arteriogenic cells and/or influences the survival rate of circulating and resident arteriogenic cells or is VCAM, ICAM, FGF-2, J-309, and/or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 and/or Platelet-activating factor (PAF) or a functionally equivalent fragment thereof.

12. The (pharmaceutical) composition or the use of claim 11, wherein said arteriogenic polypeptide attracting circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, or a functionally equivalent fragment thereof, said arteriogenic polypeptide activating circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, Tumor Necrosis Factor alpha (TNF-alpha), or a functionally equivalent fragment thereof, and/or said arteriogenic polypeptide influencing the survival rate of circulating and resident arteriogenic cells is selected from the group consisting of colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF or a functionally equivalent fragment thereof.

13. The (pharmaceutical) composition of any one of claims 1, or 6 to 12, or the use of any one of claims 2 to 12, wherein said therapeutically active molecule is reversibly coupled to a microsphere.

14. The (pharmaceutical) composition or the use of claim 13, wherein said microsphere is a latex particle, lipid-comprising structure, sponge-comprising structure, microbubble, polystyrene microsphere, biotinylated polystyrene microsphere, protein or dextran conjugate with different sizes and surface properties or any other substance that can bind said therapeutically active molecule.

15. The (pharmaceutical) composition of any one of claims 1, or 6 to 14, or the use of any one of claims 2 to 14, wherein loading of the circulating blood cell, preferably a monocyte is effected by phagocytosis, diffusion, transfection/transformation, receptor mediated uptake of molecules, electroporation and/or other mechanical methods of uptake.

16. The (pharmaceutical) composition or the use of claim 15, wherein prior to said loading the lysosomal activity of said circulating blood cell, preferably said monocyte is inhibited.

17. The (pharmaceutical) composition of any one of claims 1, or 6 to 16, or the use of any one of claims 2 to 16, wherein at the desired site said therapeutically active molecule is released from the circulating blood cell, preferably the monocyte.

18. The (pharmaceutical) composition or the use of claim 17, wherein prior to said release from said circulating blood cell, preferably said monocyte said therapeutically active molecule is uncoupled from said microsphere.

19. The (pharmaceutical) composition or the use of claim 18, wherein uncoupling is effected by a pH-change, echocardiographic signal, magnetic resonance signal, ultrasound, and/or thermographic signal.

20. The use of any one of claims 2 to 19, wherein said (pharmaceutical) composition is administered after local administration of a circulating blood cell, preferably a monocyte chemotactic protein.

21. (Pharmaceutical) kit comprising the (pharmaceutical) composition of any one of claims 1, or 6 to 19 and, in a different compartment, another (pharmaceutical) composition comprising a circulating blood cell, preferably a monocyte chemotactic protein.

22. The use of claim 20 or the (pharmaceutical) kit of claim 21, wherein said monocyte chemotactic protein is MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, RANTES, J-309, and/or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 and/or Platelet-activating factor (PAF).

23. A diagnostic composition comprising a circulating blood cell, preferably a monocyte loaded with a detectable molecule.

24. Use of a circulating blood cell, preferably a monocyte loaded with a detectable molecule for the preparation of a diagnostic composition for detecting collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections.

25. Use of a circulating blood cell, preferably a monocyte loaded with a detectable molecule for the preparation of a diagnostic composition for detecting a vessel occlusion.

26. A method for detecting collateral growth of collateral arteries and/or arteries from preexisting arteriolar connections, and/or detecting and/or diagnosing a vessel occlusion, comprising the detection of microspheres originally contained in the circulating blood cells, preferably monocytes in said arteries or occlusions.

27. The diagnostic composition of claim 23 or the use of claim 24 or 25, wherein said detectable molecule is a substance that attracts and/or

activates circulating arteriogenic cells and/or influences the survival rate of circulating and resident arteriogluic cells or is a lipopolysaccharide-like molecule, J-309, or any other CC-chemokine or classical chemoattractant like N-farnesyl peptides, C5a, leukotrine B4 or Platelet-activating factor (PAF).

28. The diagnostic composition or the use of claim 27, wherein said substance attracting circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, or a functionally equivalent fragment thereof, said substance activating circulating arteriogenic cells is selected from the group consisting of chemokines, preferably MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Fraktalkines, MIP-1-alpha, and/or MIP-1 beta, interleukins, colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF, Tumor Necrosis Factor alpha (TNF-alpha), or a functionally equivalent fragment thereof, and/or said substance influencing the survival rate of circulating and resident arteriogenic cells is selected from the group consisting of colony stimulating factors, preferably GM-CSF, G-CSF, CSF-1, and/or M-CSF or a functionally equivalent fragment thereof.

29. The diagnostic composition of any one of claims 23, 27 or 28, or the use of any one of claims 24 to 27, wherein said detectable molecule is coupled to a microsphere.

30. The diagnostic composition or the use of claim 29, wherein said microsphere is a latex particle, lipid-comprising structure, sponge-comprising structure, microbubble, polystyrene microsphere, biotinylated polystyrene microsphere, protein or dextran conjugate with different sizes and surface properties or any other substance that can bind said detectable molecule.

31. The diagnostic composition of any one of claims 23, or 27 to 30, or the use of any one of claims 24 to 30, wherein loading of the circulating blood cell, preferably the monocyte is effected by phagocytosis, diffusion or transfection.

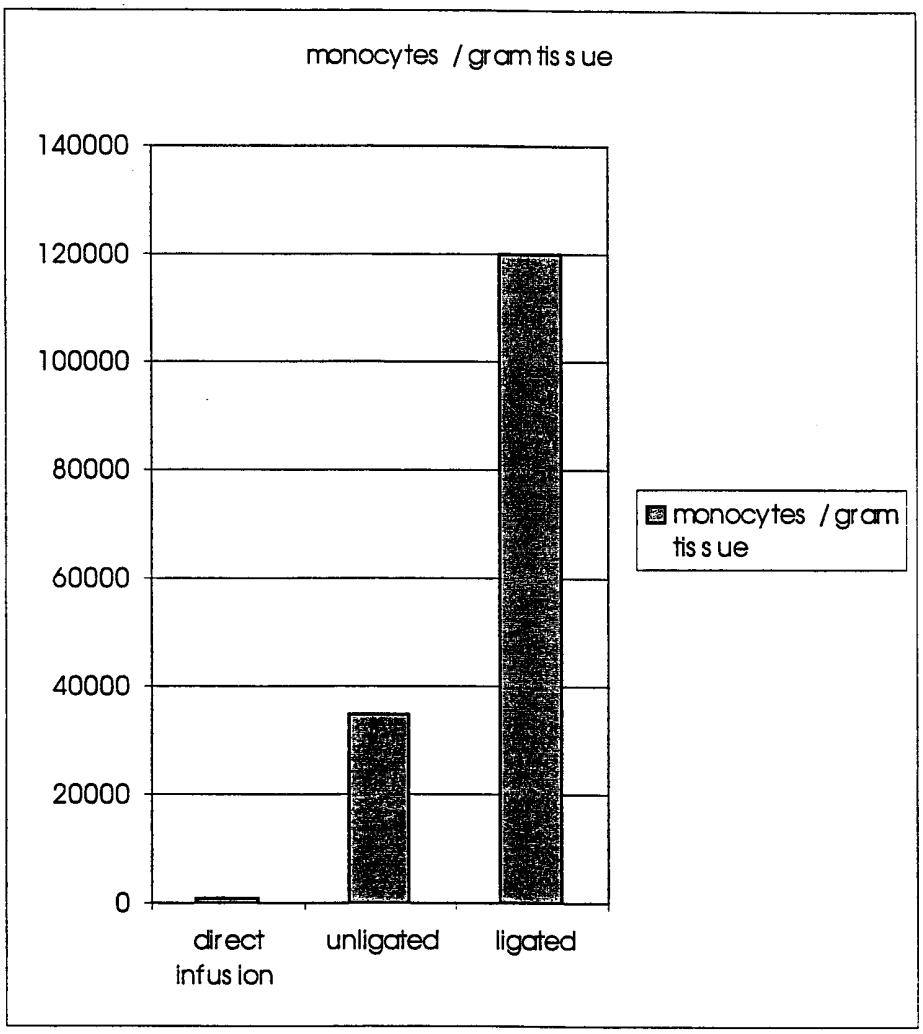
monocytes / gram tissue
140000
120000
100000
80000
60000
40000
20000
0
direct infusion
unligated
ligated
monocytes / gram tissue

Figure 1

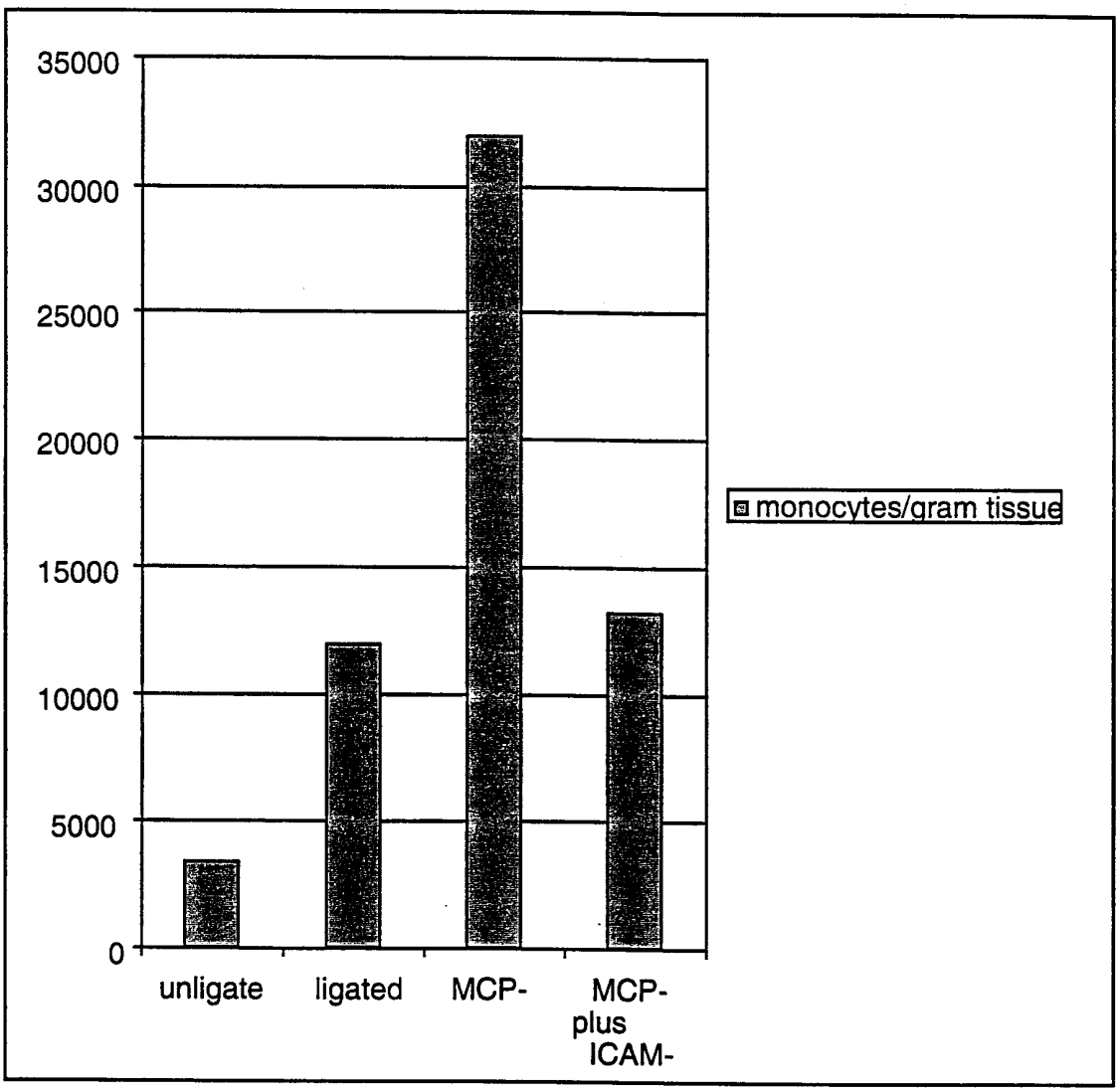
35000
30000
25000
20000
15000
10000
5000
0
unligate
ligated
MCP-
MCP-plus ICAM-
monocytes/gram tissue

Figure 2

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 00/03087

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10 C12N5/06 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 13054 A (BARTHOLEYNS JACQUES ;DREYFUS PATRICK A (FR); IDM IMMUNO DESIGNED M) 18 March 1999 (1999-03-18) page 2, line 1 - line 28; claim	1-10, 15-17, 23,31
Y	---	1-31
X	WO 95 06120 A (INST NAT SANTE RECH MED ;HADDADA HEDI (FR); LOPEZ MANUEL (FR); PER) 2 March 1995 (1995-03-02) claims	1,6-12, 15-17, 23-28
Y	---	1-31
Y	WO 98 44953 A (MAX PLANCK GESELLSCHAFT ;ITO WULF D (DE); SCHAPER WOLFGANG (DE)) 15 October 1998 (1998-10-15) claims	1-31
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2000	07.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Seegert, K

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 00/03087

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	ITO W D ET AL: "MONOCYTE CHEMOTACTIC PROTEIN-1 INCREASES COLLATERAL AND PERIPHERAL CONDUCTANCE AFTER FEMORAL ARTERY OCCLUSION" CIRCULATION RESEARCH,US,GRUNE AND STRATTON, BALTIMORE, vol. 80, no. 6, 1 June 1997 (1997-06-01), pages 829-837, XP002074060 ISSN: 0009-7330 abstract	1-31
Y	BUSCHMANN ET AL: "GM- CSF promotes collateral artery growth via prolongation of macrophage survival" JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY,XX,XX, vol. 30, no. 6, 1 June 1998 (1998-06-01), page A126 XP002094807 ISSN: 0022-2828 the whole document	1-31
P,Y	WO 99 17798 A (MAX PLANCK GESELLSCHAFT ;BUSCHMANN IVO R (DE); SCHAPER WOLFGANG (D) 15 April 1999 (1999-04-15) claims	1-31
Y	ARRAS, MARGARETE ET AL: "Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb" J. CLIN. INVEST. (1998), 101(1), 40-50 , XP000916766 abstract	1-31
Y	LEWIS, BASIL S. ET AL: "Angiogenesis by gene therapy: a new horizon for myocardial revascularization?" CARDIOVASC. RES. (1997), 35(3), 490-497 , XP000916748 abstract	1-31

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/03087

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: -
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1 - 10, 13-21, 23- 26, 29-31 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds, i.e. those mentioned in claims 11, 12, 22, 27, 28. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds mentioned in claims 11, 12, 22, 27, 28.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/EP 00/03087

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9913054 A	18-03-1999	AU 9441098 A	29-03-1999
		EP 1009806 A	21-06-2000
WO 9506120 A	02-03-1995	FR 2709309 A	03-03-1995
		AU 698043 B	22-10-1998
		AU 7539894 A	21-03-1995
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		JP 9501837 T	25-02-1997
		NO 960743 A	25-03-1996
		ZA 9406264 A	20-03-1995
WO 9844953 A	15-10-1998	EP 0969877 A	12-01-2000
WO 9917798 A	15-04-1999	EP 1019082 A	19-07-2000