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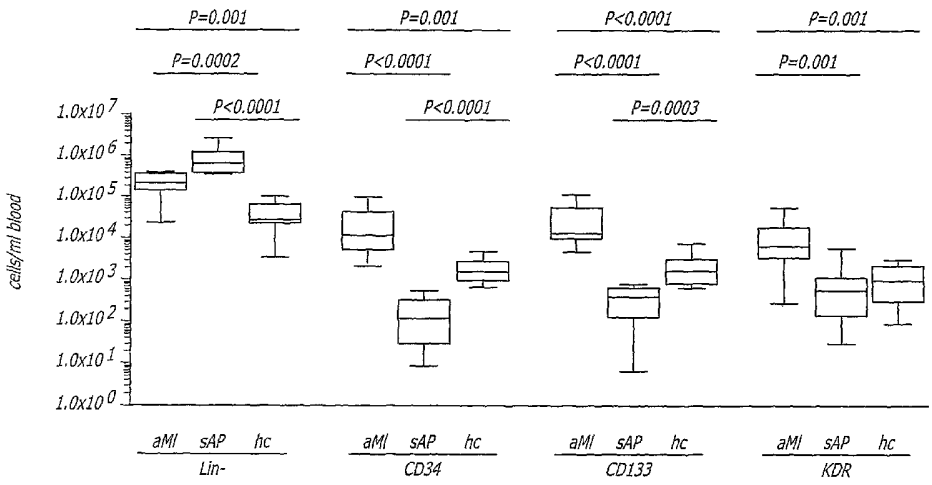
(54) Title: ISOLATION OF ENDOTHELIAL PROGENITOR CELL SUBSETS AND METHODS FOR THEIR USE




(57) Abstract: A method is provided for the isolation of endothelial progenitor cells from a source of progenitor cells by isolating a population of lineage-negative cells and further isolating $CD34^+$ cells from the lineage-negative population by fluorescence-activated cell sorting. Isolated populations of endothelial progenitor cells and therapeutic compositions containing $CD34^+$ cells for the induction of blood vessels, induction of angiogenic responses in surrounding blood vessels and the chemotaxis of inflammatory cells are also provided.

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# ISOLATION OF ENDOTHELIAL PROGENITOR CELL SUBSETS AND METHODS FOR THEIR USE

## RELATED APPLICATIONS

**[0001]** The application claims priority under 35 U.S.C. §119(e) to United States Provisional Patent Application 60/601,188 filed August 13, 2004.

## FIELD OF THE INVENTION

**[0002]** The present invention relates generally to methods of isolating endothelial progenitor cells for the treatment of cardiovascular disease.

## BACKGROUND OF THE INVENTION

**[0003]** The development of new blood vessels in response to tissue ischemia constitutes a natural host reaction intended to maintain tissue perfusion required for physiologic organ function. This natural angiogenesis is impaired in advanced age, diabetes and hypercholesterolemia. In each of these conditions, there is a reduction in endogenous expression of vascular endothelial growth factor (VEGF) and exogenous VEGF administration leads to enhanced neovascularization.

**[0004]** Ischemic tissue injury triggers a series of events, including mobilization and recruitment of circulating progenitor cells (CPC) to the injury site. In models of post-ischemic angiogenesis, a subpopulation of CPC, namely endothelial progenitor cells (EPC), incorporate into neovessels. Moreover, in animal models, as well as in clinical settings of acute myocardial infarction (aMI), systemic administration of EPC contributes to revascularization of the myocardium and is associated with improved myocardial function.

**[0005]** Since their original description, bone marrow-derived EPC have become a focal point in regenerative therapy following evolving vascular damage. Because numbers of CPC, which are normally low in peripheral blood, increase significantly after an ischemic event, a causal link between vascular damage and CPC-mediated repair has been postulated. In animal models of angiogenesis following ischemia, bone marrow-derived EPC incorporate into neovessels. Moreover, local and systemic levels of angiogenic growth factors, including VEGF, rise after ischemia and are associated with increased numbers of circulating CPC.

**[0006]** The obvious therapeutic potential of exogenous growth factor administration has been successfully assessed in animals and humans. In various animal models, mobilization of EPC after vascular damage by administration of VEGF, granulocyte macrophage colony

stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), fibroblast growth factor 1 (FGF-1), stromal derived factor 1 (SDF-1) or a statin drug, positively correlated with increased numbers of circulating EPC and improved therapeutic neovascularization. Direct evidence for the vasculogenic potential of EPC has been provided by studies in which EPC transplanted in mice with hind limb ischemia incorporated into newly formed blood vessels (Kalka C. *et al.*, "Transplantation of *ex vivo* expanded endothelial progenitor cells for therapeutic neovascularization," Proc. Natl. Acad. Sci. 97:3422-7, 2000). In a murine model of myocardial infarction (MI) intravenous injection of human $CD34^+$ CPC contributed to revascularization of the myocardium, and was associated with salvage of myocardial function (Kocher A.A. *et al.*, "Neovascularization of ischemic myocardium by human bone marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function," Nat. Med. 7:412-3, 2001). Moreover, intracoronary infusion of autologous EPC into the infarct artery in patients with aMI resulted in increased myocardial viability in the infarct area (Assmus B. *et al.*, "Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction [TOPCARE-AMI]," Circulation 106:3009-17, 2002).

**[0007]** Current progenitor cell research is focused on the clinical application of CPC in therapeutic neovascularization. Therefore, future large-scale therapeutic application of CPC will require an understanding of the phenotypic and functional properties of these cells. It has been demonstrated that phenotypically diverging subsets of CPC can be distinguished. The cell surface markers CD34, CD133 and VEGFR-2 (KDR, flk-1) have been used as CPC markers for single- and dual-parameter flow cytometric analysis of CPC, which leads to enrichment of $CD34^+$ progenitor cells (PC). The shortcomings of this approach are technical limitations and include restrictions in determining the identity and relationship between CPC subsets when they are defined by single and dual parameter detection.

**[0008]** Interestingly, the methods used for CPC detection and isolation also determine the outcome of CPC functional assays. When isolated by flow cytometry and cultured under angiogenic conditions, $CD34^+$ CPC form spindle-shaped cells, which, over time, organize in capillary-like structures. Moreover, these cells express markers specific for mature endothelial cells (EC) such as CD31, E-selectin and Tie-2.

**[0009]** Alternatively CPC have been isolated based on *in vitro* culture of mononuclear cells on fibronectin- or gelatin-coated plates in the presence of angiogenic growth factors. Isolated adherent cells that were low density lipoprotein (LDL) negative and exhibited lectin-binding ability were called CPC. Although these cells promote angiogenesis *in vivo*, they have monocytic features and their angiogenicity is actually caused by their production of angiogenic factors, such as VEGF, hepatocyte growth factor (HGF), G-CSF and GM-CSF.

Thus, while these $LDL^-$, lectin-binding cells do not directly form EC, they can modulate angiogenesis.

**[0010]** Based on the foregoing, both heterogenous and homogenous populations of endothelial progenitor cells present an opportunity for treatment of cardiovascular disease. Therefore, methods for sorting and isolating specific populations of cells suitable for use in regenerative therapy are needed.

## SUMMARY OF THE INVENTION

**[0011]** The present invention describes methods for the isolation of human peripheral blood endothelial progenitor cells yielding cells which can form blood vessels or induce angiogenesis and inflammation-mediating cells using four-parameter fluorescence activated cell sorting. Additionally, the present invention provides for biodegradable implants containing endothelial progenitor cells having the ability to induce angiogenesis and/or chemotaxis for inflammatory cells. In one embodiment of the present invention, a subset of human peripheral blood endothelial progenitor cells, $CD34^+$ CPC, is identified which gives rise to both blood vessel-forming/angiogenic cells and inflammation-mediating cells.

**[0012]** In one embodiment of the present invention, a method is provided for isolation of endothelial progenitor cells comprising identifying lineage-committed cells from a source of progenitor cells by contacting the progenitor cells with a plurality of fluorochrome-labeled antibodies specific for the cell markers selected from the group consisting of CD3, CD14, CD16/56, CD19 and CD31; depleting the lineage-committed cells by fluorescence activated cell sorting to form a population of lineage-negative cells; reacting the lineage-negative cells with a plurality of fluorochrome-labeled antibodies specific for the cell markers selected from the group consisting of CD34, CD133 and KDR wherein each antibody is labeled with a fluorochrome with a unique emission wavelength; and sorting the labeled lineage-negative cells by three-color fluorescence activated cell sorting to form a population of endothelial progenitor cells.

**[0013]** In an embodiment of the present invention, the source of progenitor cells is a mammalian source, including a human source such as peripheral blood.

**[0014]** In another embodiment of the present invention, the antibodies useful for identifying lineage-committed cells include antibodies specific for the cell markers CD3, CD14, CD16/56, CD19 and CD31.

**[0015]** In yet another embodiment of the present invention, the reacting step comprises reacting the lineage-negative cells with antibodies specific for the cell markers CD34, CD133 and KDR. In another embodiment of the present invention, the resulting endothelial

progenitor cells express CD34. In yet another embodiment of the present invention the resulting endothelial progenitor cells are $CD34^{+}CD133^{-}KDR^{-}$.

**[0016]** In one embodiment of the present invention, the endothelial progenitor cells are blood vessel generating cells, inflammation-mediating cells or both. In another embodiment of the present invention, the blood vessel-generating cells are $CD34^{+}CD133^{-}KDR^{-}$ endothelial progenitor cells. In yet another embodiment of the present invention, the endothelial progenitor cells are inflammation-mediating cells which can express interleukin-8.

**[0017]** In another embodiment of the present invention, the endothelial progenitor cells induce angiogenic responses in surrounding blood vessels.

**[0018]** In one embodiment of the present invention, a therapeutic composition for inducing angiogenesis at a treatment site is provided comprising a biodegradable matrix having $CD34^{+}$ endothelial progenitor cells disposed therein. In another embodiment of the present invention, the biodegradable biocompatible matrix contains $CD34^{+}CD133^{-}KDR^{-}$ endothelial progenitor cells.

**[0019]** In another embodiment of the present invention, a therapeutic composition having a chemotactic effect on inflammation-mediating cells at a treatment site is provided comprising a biodegradable biocompatible matrix having $CD34^{+}$ endothelial progenitor cells disposed therein. In another embodiment of the present invention, the biodegradable biocompatible matrix contains $CD34^{+}CD133^{-}KDR^{-}$ endothelial progenitor cells.

**[0020]** In yet another embodiment of the present invention, the biodegradable biocompatible matrix is selected from the group consisting of solubilized basement membrane, autologous platelet gel, collagen gels or collagenous substrates based on elastin, fibronectin, laminin, extracellular matrix and fibrillar proteins.

**[0021]** In one embodiment of the present invention, an isolated population of endothelial progenitor cells is provided wherein the isolated population is lineage-negative, $CD34^{+}CD133^{-}KDR^{-}$.

**[0022]** In an embodiment of the present invention, the isolated population of endothelial progenitor cells comprises blood vessel-generating cells. In another embodiment of the present invention, the isolated population of endothelial progenitor cells comprises inflammation-mediating cells which can express interleukin-8. In another embodiment of the present invention, the isolated population of endothelial progenitor cells induce angiogenic responses in surrounding blood vessels.

## BRIEF DESCRIPTION OF THE FIGURES

**[0023]** FIG. **1** depicts the numbers of circulating progenitor cells (CPC) subsets, determined by flow cytometry, in peripheral blood from patients with acute myocardial infarct (aMI, n=10), stable angina pectoris (sAP, n=10) and healthy controls (hc, n=9) defined as Lin-, CD34, CD133 or KDR according to the teachings of the present invention.

**[0024]** FIG. **2** depicts the *in vivo* behavior of human CPC subsets, either $CD34^+$ or $CD133^+$ sorted in Matrigel® pellets implanted in nude mice after 14 days according to the teachings of the present invention. (A) bare Matrigel®; (B) Matrigel® containing $CD34^+$ cells, (C) Matrigel® containing $CD133^+$ cells. Arrows indicate representative network structures formed by spindle-shaped cells, considered potential EPC. Objective magnification 40x.

**[0025]** FIG. **3** depicts morphological detection of human endothelial progenitor markers on isolated CPC enclosed in Matrigel® pellets implanted in nude mice according to the teachings of the present invention. In panels A-C human endothelial cells (EC) were detected with lectin *Ulex europeus-1* agglutinin (UEA-1 conjugated to TRITC), a human- and EC-specific lectin; (A) human umbilical vein endothelial cell (HUVEC) positive control; (B) murine EC cell line (H5V) negative control; (C) cells in Matrigel® seeded with human $CD34^+$ EPC bound UEA-1 lectin. UEA-1-binding cells were spindle shaped and made contacts (arrows). In panels D-E the EC phenotype was confirmed by (D) staining for human CD34 (EPC, EC), and (E) CD31 (EC). Positive endothelial cells are indicated by arrows. The inserts show additional examples of human blood vessels. Objective magnification 40x.

**[0026]** FIG. **4** depicts the murine angiogenic and inflammatory response to human CPC subsets, implanted in nude mice in Matrigel®, isolated according to the teachings of the present invention. The formation of murine blood vessels in Matrigel® were detected using monoclonal antibodies specific for murine CD31 (A-C). Murine monocytes/macrophages were detected with monoclonal antibodies specific to those cells (D-F). The insert in panel E shows the lack of human $CD68^+$ macrophages in the section. Objective magnification 40x.

**[0027]** FIG. **5** depicts detection of interleukin-8 (IL-8) in $CD34^+$ cells, isolated according to the teachings of the present invention, implanted in nude mice in Matrigel®. Interleukin-8 expression was determined immediately after sorting (A) and 14 days after implantation (B). Objective magnification 40x.

**[0028]** FIG. **6** depicts flow cytometric analysis of CPC subsets in peripheral blood from patients with aMI (n=10) and healthy controls (hc, n=9) according to the teachings of the present invention. The lineage-negative ($Lin^-$) (A) cell population were stained with antibodies to CD34, CD133 and KDR and cells expressing each of the three markers were

gated and analyzed for the expression of the remaining two markers. (B) CD133 analysis; (C) CD34 analysis; (D) KDR analysis.

**[0029]** FIG. **7** depicts the number of CPC in peripheral blood from aMI patients (n=10) and healthy controls (hc, n=9) as subsets defined as $Lin^-$, $CD34^+$, $CD133^+$ or $KDR^+$. Dots represent individuals; horizontal lines represent the median; fold increase based on means. P=p value.

**[0030]** FIG. **8** depicts cell numbers, determined by flow cytometric analysis, of the seven CPC subsets defined according to the teachings of the present invention in aMI patients (n=10) and healthy controls (hc, n=9). Horizontal lines represent the median; fold increase based on means.

**[0031]** FIG. **9** depicts flow cytometric sorting of four $CD34^+$ CPC subsets, or combinations of subsets according to the teachings of the present invention. (A) $CD34^+CD133^+KDR^-$; (B) $CD34^+CD133^+KDR^+$; (C) $CD34^+CD133^-KDR^-$; (D) $CD34^+CD133^-KDR^+$.

**[0032]** FIG. **10** depicts the presence of human CD31-positive cells in $CD34^+$ CPC-loaded Matrigel®, according to the teachings of the present invention, 14 days after implantation in nude mice. (A) clusters of human CD31-positive cells with an immature phenotype; (B) Human CD31-positive cells in vessel-like structures.

**[0033]** FIG. **11** depicts the presence of murine CD31-positive vasculature in $CD34^+CD133^-KDR^-$ CPC-loaded Matrigel®, according to the teachings of the present invention, 14 days after implantation in nude mice. Vessels of different sizes ranging from capillaries (arrows) to small (asterisk) and large (inset) vessels were detected. Both images (large and inset) were taken at the same magnification of the same section of the Matrigel® pellet.

**[0034]** FIG. **12** depicts the quantification of murine CD31-positive blood vessels/$nm^2$ in Matrigel® loaded with $CD34^+$ CPC from four $CD34^+$ subsets according to the teachings of the present invention, 14 days after implantation in nude mice. (A) capillaries, (B) small blood vessels and (C) large blood vessels. ++-, $CD34^+CD133^+KDR^-$; +--, $CD34^+CD133^-KDR^-$; +++, $CD34^+CD133^+KDR^+$; +-+, $CD34^+CD133^-KDR^+$; B, bare Matrigel®.

## DETAILED DESCRIPTION OF THE INVENTION

**[0035]** The present invention describes methods for the isolation of human peripheral blood endothelial progenitor cells yielding cells which can form blood vessels or induce angiogenesis and inflammation-mediating cells using four-parameter fluorescence activated cell sorting. Additionally, the present invention provides for biodegradable implants

containing endothelial progenitor cells having the ability to induce angiogenesis and/or chemotaxis for inflammatory cells. In one embodiment of the present invention, a subset of human peripheral blood endothelial progenitor cells, $CD34^+$ circulating progenitor cells (CPC), is identified which gives rise to both blood vessel-forming/angiogenic cells and inflammation-mediating cells. It is the non-binding hypothesis of the present inventors that a subset of $CD34^+$ CPC, $CD34^+CD133^-KDR^-$ cells, are responsible for these activities

**[0036]** Over the past several years CPC have become a focal point in cardiovascular regenerative therapy, especially since therapeutic mobilization of CPC by growth factor administration and transplantation of these cells into the infarcted region have proven beneficial for patients with ischemic conditions. Previously, a subset of CPCs, endothelial progenitor cells (EPC), have been designated as key players in neovascularization. However, there is accumulating evidence that EPC are phenotypically and functionally a heterogeneous population with endothelium-forming capacity. This heterogeneous population of CPC therefore provides a source of EPCs with different functionalities. For the purposes of describing the invention in this specification, circulating progenitor cells and endothelial progenitor cells refer to the same cell population.

**[0037]** The present inventors have unexpectedly discovered a subset of CPCs, which are lineage-negative and express CD34, but not CD133 and KDR, which are responsible for forming blood vessels. The CD designation refers to a "cluster of differentiation" antigen which systematically identifies antigens present on leukocyte cell surfaces. CD34 is a transmembrane glycoprotein constitutively expressed on endothelial cells and on hematopoietic stem cells. CD133 is a hematopoietic stem cell antigen also known as prominin. KDR is the precursor to the human vascular endothelial growth factor receptor 2 (VEGFR2) and is also known as Flk-1. It had been previously thought that this blood-vessel forming population of CPCs was $KDR^+$.

**[0038]** Stem and progenitor cells lack certain markers that are characteristic of more mature, lineage-committed ($Lin^+$) cells. Lineage-specific markers include, but are not limited to, CD3, CD8, CD10, CD14, CD16/56, CD19, CD20, CD31 and CD33. In an embodiment of the present invention, $Lin^+$ cells express CD3, CD14, CD16/56, CD19 and CD31. In another embodiment of the present invention, $Lin^-$ cells do not express CD3, CD14, CD16/56, CD19 and CD31.

**[0039]** The *in vivo* behavior of human CPC expressing the widely accepted CPC markers CD34, CD133 and KDR was studied by transcription profiling and fine dissection of EPC phenotypes based on the expression pattern of these markers. CPCs from three groups of patients were studied: (i) acute myocardial infarct (aMI) patients who had undergone successful reperfusion therapy, (ii) healthy volunteers and (iii) patients with stable

angina undergoing treatment with statin drugs. Statin therapy has been reported to increase the levels of CPCs as early as 7 days after the initiation of treatment and many aMI patients are on statin therapy.

**[0040]** Peripheral blood mononuclear cells from each group of patients, sorted for the lineage-negative population and expressing either of the CPC markers CD34, CD133 or KDR, can be subdivided into a total of seven discrete subsets based on a three-parameter assessment (three-color fluorescence activated cell sorting) of cell expression of CD34, CD133 and KDR. These seven subsets were present in the circulation of healthy subjects and in stable angina patients undergoing treatment with statin drugs and were all increased in cell number after aMI.

**[0041]** The mobilization of all three CPC subsets (i.e. $CD34^+$, $CD133^+$ and $KDR^+$) after aMI indicates non-preferential recruitment of progenitor cells (PC) (FIG. **6A**). Moreover, expression analysis of genes involved in endothelial cell differentiation and function revealed no major differences in gene expression within CPC of the same subset between aMI patients and healthy controls. These findings suggest that increased mobilization of CPC after aMI is not a consequence of altered expression makeup of these cells, but rather of external factors enhancing CPC detachment from the bone marrow. Additionally, vascular endothelial growth factor (VEGF), produced in the ischemic lesion, induces expression of matrix metalloproteinase-9 in the bone marrow. This process results in release of soluble Kit ligand, which drives the mobilization of $cKit^+$ stem and progenitor cells to the circulation.

**[0042]** To study the behavior of these three CPC subsets *in vivo,* the present inventors established a model in which human CPC, enclosed in a biodegradable matrix such as Matrigel® (BD Biosciences), are allowed to mature in a murine host. Matrigel® is a biodegradable and biocompatible solubilized basement membrane matrix. Other biodegradable matrices as are known to those persons skilled in the art can be used within the scope of the present invention. Other examples of biodegradable matrices include, but are not limited to, autologous platelet gel, collagen gels or collagenous substrates based on elastin, fibronectin, laminin, extracellular matrix and fibrillar proteins. The use of a biodegradable matrix has a number of advantages, one of which is local confinement of CPC, which makes it possible to observe relatively low numbers of target cells. Additionally, soluble factors produced by CPC can freely diffuse through the biodegradable matrix and reach the host environment. Therefore, not only can differentiation of human CPC be monitored, but the impact of these cells on host-derived angiogenesis (i.e. sprouting of surrounding murine blood vessels) is readily visible. Additionally, in an embodiment of the present invention, biodegradable matrices are useful in the implantation of CPC into mammals for the treatment of diseases that will benefit from the localized transplantation of

progenitor cells. Finally, the interplay between human CPC and murine inflammatory cells can be studied, thus providing indications regarding the role of EPC in inflammatory remodeling after ischemia.

**[0043]** In one embodiment of the present invention, $CD34^+$ cells, encapsulated in Matrigel® implants, but not $CD133^+$ cells or $KDR^+$ cells, formed mature endothelial cells (EC) (FIG. **2B**). Gene expression analysis revealed that CD31 transcripts were present in all three subsets, suggesting endothelium-forming potential. CD31 has served as a surrogate for endothelial cells with a monocytic phenotype, or monocytes with angiogenic potential, whereby the presence of CD31 transcripts as a proof for endothelial commitment is attenuated. Von Willebrand Factor transcripts were detectable in $CD34^+$ cells and $CD133^+$ cells, but not always in $KDR^+$ cells, indicating that the differentiation of the former two subsets may indeed be skewed towards the endothelial lineage.

**[0044]** In another embodiment of the present invention, the $CD34^+$ population which gives rise to mature endothelial cells also expresses Tie-2, a tyrosine kinase receptor. Gene transcripts for CD34, Tie-1, Tie-2, VEGF and KDR, which are characteristic of CPC, were present in $CD34^+$ cells and at low levels in $CD133^+$ cells but were absent in $KDR^+$ cells, indicating a stronger commitment of the former two subsets to the endothelial lineage. Tie-2 expression was found only in the $CD34^+$ subset, which was the only subset giving rise to endothelial cells *in vivo*. Since Tie-2 is essential for endothelial cell survival and capillary morphogenesis, the presence of this molecule may be instrumental for endothelial cell formation in this subset *in vivo*.

**[0045]** In order to distinguish discrete, phenotypically distinct CPC subsets of lineage-negative cells, three-parameter flow cytometry analysis of the CPC markers CD34, CD133 and KDR was established. The advantage of this strategy, as compared to previous techniques, consists of an unbiased inclusion of all CPC subtypes in the analysis. This unbiased inclusion is accomplished by the use of uncommitted progenitor cells, enriched for lineage-negative ($Lin^-$) cells rather than CD34 pre-selection, as the starting population for analysis. Because this approach allows the dissection of CPC populations implanted *in vivo* (i.e. $CD34^+$, $CD133^+$, $KDR^+$ cells), probable CPC subsets responsible for the observed *in vivo* effects are identified. Using this technique, seven phenotypically distinct CPC subsets within the major $CD34^+$, $CD133^+$ and $KDR^+$ cell populations were identified (FIGS. **6C,D**).

**[0046]** A recurrent CPC subset in all three major population is the CD34+CD133+KDR+ or triple-positive subset. These cells have been previously described, after preselection of $CD34^+$ cells, and were shown to harbor EPC. In the hands of the present inventors these cells did not contribute to EC formation *in vivo*, possibly due to their low frequency.

**[0047]** Within the $KDR^+$ population three more EPC subsets were identified: $CD34^+CD133^-KDR^+$ cells, $CD34^-CD133^-KDR^+$ cells and $CD34^-CD133^+KDR^+$ cells. $CD34^+KDR^+$ cells have been described as potential hematopoietic stem cells or adult hemangioblasts. CD133 is a marker of primitive progenitors but not of mature endothelial cells. Therefore, the $CD34^+CD133^-KDR^+$ cells detected may be more matured cells. The significance of $CD34^-CD133^-KDR^+$ cells is as yet difficult to determine, since KDR is expressed on a variety of progenitor cells. The potential identity of $CD34^-CD133^+KDR^+$ cells can be inferred from the observation that $CD133^+KDR^+$ cells are EPC recruited to the circulation upon vascular trauma. Moreover, this subset resembles – as far as expression of these three markers is concerned – a mesenchymal stem cell population from the bone marrow described by Reyes *et al.* (Reyes M., *et al.* Origin of endothelial progenitors in human postnatal bone marrow. J. Clin. Invest. 109:337-46, 2002), who have also demonstrated the endothelial cell forming capacity of these cells. Irrespective of their phenotype, however, the combination of these four subsets (i.e., $CD34^+/CD133^+/KDR^+$, $CD34^-/CD133^+/KDR^+$, $CD34^-/CD133^-/KDR^+$, and $CD34^+/CD133^-/KDR^+$) did not result in EC differentiation as demonstrated in Example 3 (FIGS. **2** and **3**).

**[0048]** Similarly to $KDR^+$ cells, $CD133^+$ cells alone do not form endothelial cells *in vivo*. The $CD133^+$ population shares with the $KDR^+$ population the $CD34^-CD133^+KDR^+$ subset, previously discussed. The remaining two subsets within the $CD133^+$ population are $CD34^-CD133^+KDR^-$ and $CD34^+CD133^+KDR^-$. $CD34^-CD133^+$ cells may be precursors of $CD34^+CD133^+$ cells, based on their capacity to give rise to $CD34^+$ hematopoietic progenitor cells. For this latter phenotype ($CD34^+CD133^+$), functions of hematopoietic progenitor cells, EPC and vascular lymphatic cell progenitors have been proposed. However, in the *in vivo* system described in Example 3 the combination of the four $CD133^+$ PC subsets did not give rise to endothelial cells, suggesting that the constellation of factors may not have been adequate to induce endothelial cell differentiation.

**[0049]** A summary of the phenotype and behavior of the seven CPC subsets is found below in Table 1.

Table 1

| Subset | | | aMI | *In vivo* behavior | Function |
|---|---|---|---|---|---|
| CD34 | CD133 | KDR | | | |
| + | + | + | ↑ | induce human EC, angiogenesis, inflammation | EC |
| + | + | - | ↑ | | EPC, HSC, HPC |
| + | - | - | ↑ | | undefined progenitor |
| + | - | + | ↑ | | HPC, hemangioblast |
| - | + | + | ↑ | no response | undefined progenitor |
| - | - | + | ↑ | | undefined progenitor |
| - | + | - | ↑ | | undefined progenitor |

Presence (+) or absence (-) of cell phenotype markers in the seven subsets.

**[0050]** In one embodiment of the present invention, the $CD34^+$ population was the only one of the seven identified CPC subpopulations to form mature EC *in vivo,* as demonstrated by binding to the lectin *Ulex europeus-1* agglutinin (UEA-1), expression of $CD34^+$ and $CD31^+$, spindle shape and organization in networks. The $CD34^+$ population shares with the $CD133^+$ population the $CD34^+CD133^+KDR^-$ subset and with the $KDR^+$ population the $CD34^+CD133^-KDR^+$ subset. Whereas these two subsets did not contribute to endothelial cell differentiation in the context of the $CD133^+$ and $KDR^+$ populations respectively, they did do so in combination with the $CD34^+CD133^-KDR^-$ subset, which is unique to the $CD34^+$ population. Expression of CD34 on peripheral blood mononuclear cells (MNC) was the criterion by which Asahara *et al.* (Asahara T. *et al.*, Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964-967, 1997) observed that cells with this phenotype, if grown on fibronectin and under angiogenic conditions, could give rise to endothelial cells.

**[0051]** In an embodiment of the present invention, the *in vivo* endothelial cell-forming capacity of $CD34^+$ cells is due to the presence of $CD34^+CD133^-KDR^-$ cells, and optionally, expression of Tie-2 within this subset. In another embodiment of the present invention, the combined presence of $CD34^+CD133^-KDR^+$ cells and $CD34^+CD133^+KDR^-$ cells, possibly in combination with the $CD34^+CD133^-KDR^-$ subset, may be required for endothelial differentiation.

**[0052]** Unexpectedly, $CD34^+$ CPC subsets, besides differentiating into EC, also stimulated ingrowth of murine blood vessels into Matrigel®. Although angiogenic by its composition of extracellular matrix components and growth factors, Matrigel® itself (bare Matrigel®) did not induce ingrowth of murine blood vessels during a 14 day observation period (Example 3 and Example 5).

**[0053]** The implantation of $CD34^+$ CPC results in neovascularization in two ways. First, human $CD34^+$ CPC differentiate into human endothelium. Secondly, the human $CD34^+$ CPC induce vascular ingrowth by the host. Not all the CD34+ CPC subsets induced host neovascularization equally. The $CD34^+CD133^-KDR^-$ subset and the $CD34^+CD133^+KDR^-$ subset were important for the induction of host neovascularization. Large vessels were primarily seen in the $CD34^+CD133^-KDR^-$ subset and to a lesser extent in the $CD34^+CD133^+KDR^-$ subset. Furthermore, a combination of these two subsets was not synergistic and did not lead to higher levels of neovascularization than either subset alone. Additionally, the combination of the $CD34^+CD133^-KDR^-$ and the $CD34^+CD133^-KDR^+$ subsets resulted in only the formation of capillaries and not in formation of small and large blood vessels. Induction of primarily capillaries may be useful in treating ischemic heart disease.

**[0054]** Thus far, CPC have been viewed as cells that could directly give rise to new blood vessels and thus to contribute to neovascularization after damage. The unexpected observation by the present inventors demonstrate that CPC can exert a modulatory function on local vasculature, enhancing sprouting angiogenesis. This finding provides new perspectives for improved therapeutic neovascularization.

**[0055]** In another embodiment of the present invention, $CD34^+$ CPCs isolated according the teachings of the present invention recruit inflammatory cells of the monocyte/macrophage lineage to the Matrigel® microenvironment. Bare Matrigel® exerted little attraction of murine macrophages, indicating that only a low-grade foreign body reaction against Matrigel® was mounted. Since the inflammatory responses to Matrigel® loaded with $CD133^+$ cells or $KDR^+$ cells did not exceed those of bare Matrigel®, these subsets did not modulate macrophage infiltration by themselves. In comparison, macrophage infiltration of Matrigel® loaded with $CD34^+$ cells was markedly higher, indicating that an additional macrophage-attracting effect of these cells was superimposed on the effect of Matrigel®. Therefore the function of CPC may stretch beyond that of differentiation to endothelial cells and may have additional therapeutic implications. In yet another embodiment of the present invention, progenitor cells recruited by damage signals from the ischemic myocardium may not only contribute to neovascularization by directly differentiating to endothelial cells and promoting sprouting of local blood vessels, but may also recruit inflammatory cells to the damaged area.

**[0056]** Pro-inflammatory chemoattractants are produced by the $CD34^+$ progenitor cells. While all three CPC subsets, $CD34^+$, $CD133^+$ and $KDR^+$, contained transcripts for the inflammation-associated cytokines/chemokines tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$), only the $CD34^+$ subpopulation responsible for recruiting inflammatory cells expressed high levels (3-fold increased over $KDR^+$ cells) of

human interleukin-8 (IL-8). Moreover, expression of human IL-8 by single cells persisted for 14 days after Matrigel® implantation.

**[0057]** The surprising observations by the present inventors demonstrates a need for revising the existing definition of CPC, which has previously been based solely on expression of markers such as CD34, CD133 and KDR, because various subsets with varying expression patterns of these molecules exist, which are not equally able to differentiate into endothelial cells. Acute MI leads to a mobilization of all detected progenitor cell subtypes, demonstrated by similar gene expression patterns in CPC subsets from healthy individuals and aMI patients, indicating that CPC do not respond adaptively to damage signals but rather are passively released from the bone marrow. Finally, $CD34^+$ progenitor cells harbor angiogenic as well as immunomodulatory potential, which may be exploited for the generation of new therapeutic strategies using the teachings of the present invention.

**[0058]** In an embodiment of the present invention, four-parameter fluorescence activated cell sorting is used to identify CPC which yield both blood-vessel forming cells and inflammation mediated cells. The four parameters of the present invention are lineage, CD34, CD133 and KDR.

**[0059]** In an embodiment of the present invention, a source of cells containing the desired cell population is separated into a desired population and an undesired population by exposing the cells to a cocktail, or mixture, of antibodies, either monoclonal or polyclonal, that define the desired cells. The antibodies are conjugated with fluorescent labels which allow a fluorescence-activated cell sorter to identify cells to which one or more of the antibodies have bound. Individual antibodies can be conjugated with a variety of fluorescent labels (fluorochromes) which are well known to those persons skilled in the art. In one embodiment of the present invention, the antibodies can be linked to one or more than one fluorochrome having the same or unique fluorescence emission wavelengths. Each fluorescence emission wavelength corresponds to a color. Exemplary fluorochromes include, but are not limited to, Texas Red® (Molecular Probes, Eugene, OR), allophycocyanin, phycoerythrin, fluorescein isothiocyanate, rhodamine, SpectralRed® (Southern Biotech, Birmingham, AL), Cy-Chrome, and others.

**[0060]** In an embodiment of the present invention, a source of endothelial progenitor cells or circulating progenitor cells are contacted with a cocktail of antibodies that define lineage-committed ($Lin^+$) cells. In an embodiment of the present invention, this cocktail of antibodies are all conjugated to the same fluorochrome. In another embodiment of the present invention, the lineage-committed markers include, but are not limited to, CD3, CD8, CD10, CD14, CD16/56, CD19, CD20, CD31 and CD33. In one embodiment of the present

invention, $Lin^+$ cells express CD3, CD14, CD16/56, CD19 and CD31. In another embodiment of the present invention, lineage-uncommitted (progenitor, $Lin^-$) cells do not express CD3, CD14, CD16/56, CD19 and CD31.

**[0061]** In an embodiment of the present invention, $Lin^-$ cells are isolated by contacting a source of CPC with a cocktail of fluorochrome-labeled antibodies and sorting the cells on a fluorescence activated cell sorter such that a sterile purified population of cells is obtained. Protocols and methods for fluorescence-activated cell sorting are readily available and well known to persons skilled in the art.

**[0062]** In another embodiment of the present invention, isolated progenitor cells are obtained by contacting $Lin^-$ cells with fluorochrome-labeled antibodies to CD34, CD133 and KDR and sorting the labeled cells to identify a population of $Lin^-$ cells expressing CD34 but not expressing CD133 or KDR. In one embodiment of the present invention this sorting step is conducted under sterile conditions.

**[0063]** In one embodiment of the present invention, the isolated progenitor cells are useful for inducing new blood vessel formation in a patient. New blood vessels can be formed by vasculogenesis (formation of blood vessels from embryonic precursors), angiogenesis (in-growth of blood vessels from the surrounding tissue) or the formation of neovascularization (formation of new blood vessels where they had not been previously) including forming blood vessels from endothelial progenitor cells linking to existing blood vessels. There are numerous conditions in which a mammal may be in need of forming new blood vessels such as injury due to trauma, surgery or acute or chronic diseases. In a non-limiting example, the mammal may have a wound that requires healing. In another non-limiting example, the patient may have undergone cardiovascular surgery, cardiovascular angioplasty, carotid angioplasty, or coronary angioplasty, which are all conditions requiring new blood vessel formation. In another non-limiting example, patients who have had a myocardial infarction, such as an aMI, are in need of new blood vessel formation. Other conditions which may require new blood vessel formation include sickle cell anemia and thalassemia.

**[0064]** In another embodiment of the present invention, the isolated progenitor cells can be administered to the mammal in need of forming new blood vessels by any route or method that allows the preferential migration of the cells to the site in need of new blood vessel formation. Exemplary routes of administration include, but are not limited to, systemic administration such as intravenous injection, localized implantation such as localized intramuscular or subcutaneous injection of the progenitor cells in biocompatible solutions or biodegradable biocompatible matrices. Biocompatible solutions are known to those skilled in the art. Examples of biodegradable biocompatible matrices include, but are not limited to,

solubilized basement membrane, autologous platelet gel, collagen gels or collagenous substrates based on elastin, fibronectin, laminin, extracellular matrix and fibrillar proteins.

**[0065]** These examples are meant to illustrate one or more embodiments of the present invention and are not meant to limit the invention to that which is described below.

Example 1

Identification of seven CPC subsets by four-parameter flow cytometry

**[0066]** The phenotypic heterogeneity of endothelial progenitor cells (EPC) based on patterns of combined expression of three circulating progenitor cells (CPC) markers, CD34, CD133 and KDR was analyzed using four-parameter (three-color) flow cytometric analysis.

**[0067]** Mononuclear cells were isolated from heparinized blood by lymphoprep density gradient centrifugation (Nycomed, Oslo, Norway). Because the number of circulating progenitor cells (PC), irrespective of phenotype, is low, lineage-negative ($Lin^-$) cells, i.e. uncommitted, potential PCs, were enriched from total peripheral blood mononuclear cells (MNC) by high-speed flow cytometry sorting, whereas Lin+ cells were discarded (FIG. **6A**). Total MNC were stained with a cocktail of phycoerythrin (PE)-labeled monoclonal antibodies (moAbs) against CD3 (T cells), CD14 (monocytes), CD19 (B cells), CD16/56 (NK cells) and CD31 (mature endothelial cells) (all from IQ Corp., Groningen, The Netherlands). $Lin^-$ cells were sorted in basal endothelial medium (Becton Dickinson, Erembodegem-Aalst, Belgium) by high speed flow cytometry using a MoFlo cell sorter (Cytomation, Fort Collins, CO). The obtained $Lin^-$ populations were typically 95-98% free of $Lin^+$ cells and accounted for an average 11.3 % of all MNC (range 0.6-22.4%), whereas in healthy controls (hc, n=9) an average 3.0% of the MNC were $Lin^-$ (range 1.0-5.5%; P=0.0003) (Figure **6A**).

**[0068]** To determine expression patterns of the CPC markers CD34, CD133 and KDR, sorted $Lin^-$ cells were subjected to three-color staining using CD34-allophycocyanin (APC) (clone 581, IQ Corp.), CD133-PE (Miltenyi Biotech, Germany) and rabbit polyclonal anti-KDR-fluorescein isothiocyanate (FITC) (Sigma Chemical Co.).

**[0069]** Within the $Lin^-$ population, cells expressing one of the three CPC markers CD34, CD133 and KDR were gated, followed by analysis of the expression of the remaining two markers (FIG **6B**: CD34, FIG **6C**: CD34, FIG **6D**: KDR). Using this approach, seven CPC subsets were detected based on combined expression of CD34, CD133 and KDR. Triple-negative cells were not considered EPC. All seven subsets were present in aMI patients and healthy controls.

**[0070]** The $CD34^+$ population consisted mainly of $CD34^+CD133^+KDR^-$ cells (aMI, mean 62% of all CD34+ cells, range 33-89%; healthy controls, mean 38%, range 24-50%) and $CD34^+CD133^-KDR^-$ cells (aMI, mean 37% of all $CD34^+$ cells, range 10-61%; healthy controls,

mean 60%, range 0.1-0.8%), whereas the triple-positive subset and $CD34^+CD133^-KDR^+$ subset accounted for less than 1% of this subpopulation (FIG. **6C**).

**[0071]** In the $CD133^+$ population in aMI patients, $CD34^+CD133^+KDR^-$ cells (mean 52% of all $CD133^+$ cells, range 33-89%) and $CD34^-CD133^+KDR^-$ cells (mean 28% of all $CD133^+$ cells, range 0.1-85%) dominated, whereas in healthy controls $CD34^+CD133^+KDR^-$ cells (mean 38%, range 13-76%, one outlier 1%) and $CD34^-CD133^+KDR^+$ cells (mean 58%, range 12-73%) were the dominating subsets (FIG. **6B**).

**[0072]** Differences between aMI patients and healthy controls were also present in the $KDR^+$ population, which in aMI patients consisted mainly of $CD34^-CD133^-KDR^+$ cells (mean 65%, range 1-95%) and $CD34^-CD133^+KDR^+$ cells (mean 33%, range 3-71%) (FIG. **6D**). In healthy controls $CD34^-CD133^+KDR^+$ cells dominated (mean 93%, range 52-99%), whereas the $CD34^-CD133^-KDR^+$ subset encompassed only about 5% of all $KDR^+$ cells (FIG. **6D**).

## Example 2

## CPC numbers in aMI patients and healthy controls

**[0073]** Ten aMI patients and nine healthy control volunteers were compared with regard to EPC numbers to determine whether the number of cells in the seven CPC subsets correlated with the event of aMI. A possible correlation between CPC numbers and aMI is reflected in the number of $Lin^-$ cells (within which CPC were detected). Numbers of $Lin^-$ cells were compared between the two subject groups. In aMI patients the number of $Lin^-$ cells averaged $2.6x10^5$ cells/mL blood (range $0.2\text{-}4.7x10^5$ cells/mL blood), which was significantly higher ($P=0.001$) than in healthy controls (mean $0.5x10^5$ cells/mL blood, range $0.04\text{-}1.4x10^5$ cells/mL blood) (FIG. **7**), equivalent with a 5.2-fold higher number of $Lin^-$ cells in aMI patients as compared to controls.

**[0074]** The numbers of CPC expressing CD34, CD133 or KDR were compared in aMI patients and healthy controls. CPC numbers in all three subsets were significantly higher in aMI patients than in healthy controls. In the CD34 subset an 8.6-fold higher cell number was found in aMI patients (mean $2.6x10^4$ cells/mL blood, range $2.1\text{-}11.0x10^4$ cells/mL blood, $P=0.005$) relative to healthy controls (mean $0.3x10^4$ cells/mL blood, range $0.06\text{-}1.2x10^4$ cells/mL blood). In the CD133 subset an 11.6-fold higher cell number was found in aMI patients (mean $3.5x10^4$ cells/mL blood, range $0.5\text{-}13.5x10^4$ cells/mL blood, $P<0.0001$) relative to healthy controls (mean $0.3x10^4$ cells/mL blood, range $0.07\text{-}0.7x10^4$ cells/mL blood). Finally, in the KDR subset a 6.2-fold higher cell number was found in aMI patients (mean $1.3x10^4$ cells/mL blood, range $0.03\text{-}5.8x10^4$ cells/mL blood, $P=0.005$) relative to healthy controls (mean $0.2x10^4$ cells/mL blood, range $0.009\text{-}0.8x10^4$ cells/mL blood).

**[0075]** To establish whether aMI triggered the mobilization of a specific CPC subset, possibly for repair of damaged myocardial blood vessels, the number of CPC within the seven subsets was determined. In all seven subsets, irrespective of their phenotype, significantly higher CPC numbers were present in aMI patients than in healthy controls (FIG. 8). At the patient level, outliers in CPC numbers in specific subsets were readily apparent, although in these patients CPC numbers were not consistently higher in all seven CPC subsets.

**[0076]** Because numbers of CPC, irrespective of their phenotype, were increased in aMI patients, suggesting a causal link between cardiovascular damage and CPC recruitment, correlations between CPC numbers and disease parameters were sought (Table 2). Fifteen aMI patients and 10 patients with stable angina pectoris were included in this analysis. Cardiovascular disease (CVD) history indicates previous episodes of CVD in patients. Number of aMI indicates the number of the current aMI episode. In addition to the risk factors for aMI listed in Table 2, age (>60 years) and male gender were considered risk factors for aMI, resulting in a total of six possible risk factors. The cumulative risk factors indicate the number of risk factors out of these six possible risk factors, present in a given patient.

**[0077]** There was no correlation between CPC numbers in various subsets and age, cumulative number of risk factors or ischemic time. Moreover, there was also no correlation between EPC numbers and serum lactate dehydrogenase (LDH), creatinine phsophokinase myocard band (CKMB) or troponin.

Table 2. Demographic and post aMI clinical characteristics of aMI patients

| Indic | age | gender | CVD history | aMI number | MI risk factors | | | | | ischemic time (h) | LDH (U/l) | CKMB U/l) | troponin Ug/l) | post aMI medication |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | smoking | CVD family history | hyper-tens. | hyper-cholest. | cum. risk factors | | | | | |
| aMI | 48 | m | 1 | 2 | yes | no | no | yes | 3 | 3.2 | 1600 | 165 | 2392 | B,AS,S,O |
| aMI | 45 | m | 0 | 1 | yes | yes | no | yes | 4 | 2.67 | 473 | 63 | 40 | B,AS,S |
| aMI | 41 | m | 1 | 1 | yes | yes | no | no | 3 | 3 | 991 | 168 | 733.7 | B,AS,S |
| aMI | 55 | m | 0 | 1 | yes | no | no | no | 2 | 5 | 573 | 54 | 346 | B,N,AS,S |
| aMI | 43 | m | 1 | 1 | yes | yes | yes | yes | 5 | 7 | 940 | 80 | 216.4 | B,AS,S,O |
| aMI | 69 | f | 0 | 1 | yes | yes | no | yes | 4 | 4 | 592 | 88 | 0.2 | B,AS,S,O |
| aMI | 63 | f | 1 | 1 | yes | yes | no | yes | 4 | 8.5 | 825 | 97 | 503.6 | B,AS,S |
| aMI | 52 | m | 0 | 1 | yes | no | no | no | 2 | 6.5 | 829 | 86 | 27.1 | B,AS |
| aMI | 59 | m | 1 | 1 | no | no | yes | no | 2 | 6.5 | 1975 | 264 | 1392 | B,AS |
| aMI | 56 | f | 1 | 1 | yes | no | yes | n | 2 | 6.5 | 716 | 46 | 83.6 | B,AS,S,O |
| aMI | 58 | m | 1 | 1 | no | yes | no | no | 2 | 2 | 924 | 125 | 1085.6 | B,AS,AI,S |
| aMI | 58 | f | 0 | 1 | yes | yes | no | yes | 3 | 2 | 427 | 48 | 176.4 | B,AS,S |
| aMI | 40 | m | 0 | 1 | yes | no | no | yes | 3 | 2 | 907 | 97 | 352.3 | B,AS,AI,S |
| aMI | 57 | m | 0 | 1 | no | yes | no | yes | 3 | 2 | 219 | 3 | 0 | AS,S |
| aMI | 44 | m | 0 | 1 | yes | yes | no | yes | 4 | 3 | 1144 | 114 | 1255.2 | B,AS,S |
| sAP | 51 | m | 1 | 1 | no | yes | no | yes | 2 | - | - | - | - | B,AS,S,C,AI,TI |
| sAP | 58 | m | 1 | 0 | no | yes | yes | yes | 4 | - | - | - | - | B,AS,S,C,AI |
| sAP | 55 | m | 1 | 1 | ? | ? | no | no | ? | - | - | - | - | AS,S |
| sAP | 64 | m | 1 | 1 | yes | yes | no | yes | 3 | - | - | - | - | B,AS,S,AI,O |
| sAP | 67 | m | 1 | 0 | no | no | no | no | 0 | - | - | - | - | B,S |
| sAP | 71 | f | 1 | 1 | no | yes | no | yes | 2 | - | - | - | - | B,AS,S,C,ARB,O |
| sAP | 62 | m | 1 | 0 | no | yes | no | no | 1 | - | - | - | - | AS,TI,S,C,AI |
| sAP | 53 | m | 1 | 1 | no | no | yes | yes | 2 | - | - | - | - | B,AS,S,AI |
| sAP | 63 | m | 1 | 0 | no | yes | yes | yes | 4 | - | - | - | - | B,S,C,O |
| sAP | 72 | m | 1 | 0 | no | yes | no | yes | 3 | - | - | - | - | B,S,O |

Post aMI medication: beta blockers (B), acetyl salicylate (AS), nitroglycerine (N), ace inhibitor (AI), statins (S), ticlopidin (TI), Ca antagonist (C), angiotensin receptor blocker (ARB) or others (O). Ischemic time = period between onset of chest pain and intervention.

Example 3

Blood vessel-forming activity of CPC subsets

**[0078]** This experiment investigated the behavior of CPC subsets *in vivo*, primarily with respect to differentiation into mature endothelial cells (EC). CPC expressing either CD34, CD133 or KDR were sorted in 200 $\mu$L Matrigel® and supplemented with 10 ng basic fibroblast growth factor (b-FGF, Chemicon, Temecula, CA) and 12 U heparin (Leo Pharma, Ballerup, Denmark) at 5,000 to 15,000 cells per implant and implanted subcutaneously in nude mice. Bare Matrigel® contains the b-FGF and heparin supplement. After 14 days, the Matrigel® pellets were explanted, partly snap-frozen in liquid nitrogen for immunohistochemistry, or fixed in 2% paraformaldehyde in 0.1 M sodium phosphate buffer, dehydrated and embedded in resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany). For overall morphologic evaluation, 2 $\mu$m sections of resin-embedded Matrigel® pellets were stained with toluidin blue. Morphologic analysis of the implants showed that by 14 days after implantation, high cellularity was present in Matrigel® seeded with $CD34^+$ cells (FIG. **2B**). Cellularity was markedly lower in $CD133^+$-loaded Matrigel® (FIG. **2C**) and minimal in Matrigel® seeded with $KDR^+$ cells or bare Matrigel® (FIG. 2A). Network structures composed of spindle shaped cells, strongly resembling capillary networks, were abundant in Matrigel® seeded with $CD34^+$ cells but scarce or virtually absent in Matrigel® seeded with $CD133^+$ cells, $KDR^+$ cells, or bare Matrigel®.

**[0079]** To determine whether these network structures were formed by human CPC, Matrigel® sections were stained with UEA-1, which binds specifically to human, but not to murine, EC (FIGS. **3A,B**). Human umbilical vein endothelial cells (HUVEC) were used as a positive control for UEA-1 staining (FIG. **3A**) and H5V cells (murine EC line) as a negative control (FIG. **3B**). Network structures in $CD34^+$ implants were positive for UEA-1 (FIG. **3C**); however, UEA-1-positive cells were not present in Matrigel® seeded with the other subsets or in bare Matrigel®.

**[0080]** Because the UEA-1 staining demonstrated the human origin of network structures in Matrigel® *in vivo*, but cannot differentiate between progenitor and mature EC, the explants were stained with the EPC/EC marker CD34 and the EC maturation marker CD31. In Matrigel® seeded with CD34+ EPC, spindle-shaped cells, occasionally arranged in network structures and similar to the cells observed after staining with UEA-1, were positive for human CD34 (FIG. **3D**). CD31 was present on cells with similar morphology in Matrigel® seeded with $CD34^+$ cells (FIG. **3E**). Neither CD34, nor CD31 was detectable in Matrigel® seeded with $CD133^+$ CPC, $KDR^+$ CPC or bare Matrigel®.

## Example 4

### Induction of angiogenic and inflammatory responses to CPC subsets *in vivo*

**[0081]** Because not all cells detected in Matrigel® seeded with $CD34^+$ cells stained for human EC markers, it was investigated whether they were murine EC. Using immunohistochemistry, murine blood vessels and inflammatory cells were detected using rat monoclonal antibodies directed against murine CD31 (Southern Biotech, Birmingham, AL) and monocytes/macrophages (MoMa, Serotech, Oxford, UK). Strong host-derived angiogenic responses were detected towards Matrigel® seeded with human $CD34^+$ cells (FIGS. **4B,E**). However, Matrigel® containing human $CD133^+$ cells triggered weaker murine angiogenic responses (FIGS. **4C,F**) whereas the angiogenic reaction upon implantation of Matrigel® seeded with $KDR^+$ cells or bare Matrigel® was marginal (FIGS. **4A,D**). A strong influx of murine $MoMa^+$ inflammatory cells towards human $CD34^+$ cells and weak responses against the other 2 subsets (FIGS. **4D-F**) were observed in all 5 tested subjects (2 aMI patients, 3 healthy controls) and did not differ between CPC from aMI patients and healthy controls. No differentiation towards human monocytes ($CD14^+$ cells) or macrophages ($CD68^+$, FIG. **4E** inset) was detected.

## Example 5

### Effects of $CD34^+$ subsets on Angiogenesis

**[0082]** $CD34^+$ CPC were isolated as described in Example 1 and propidium iodine staining was included to ensure that only living cells were isolated (FIG. **9**). Four $CD34^+$ subsets were further isolated from the $CD34^+$ population: $CD34^+CD133^-KDR^-$ (+--); $CD34^+CD133^+KDR^-$ (++-); $CD34^+CD133^-KDR^+$ (+-+); and $CD34^+CD133^+KDR^+$ (+++). The four CD34+ CPC subsets were resuspended in supplemented Matrigel® and implanted in nude mice as described in Example 3. Single subsets or mixed populations were implanted in mice according to the Experimental Setup in Table 3.

Table 3

| Experimental Group | Injected Subset(s) CD34/CD133/KDR | N |
|---|---|---|
| 1 | + + - | 3 |
| 2 | + - - | 3 |
| 3 | + + - / + - - | 3 |
| 4 | + + + / + - - | 3 |
| 5 | + - - / + - + | 3 |

**[0083]** For overall histologic evaluation, 2 $\mu$m sections were prepared from the resin-embedded Matrigel® pellets and stained with toluidin blue. The number of blood vessels were counted and corrected for the area of investigated tissue, resulting in the number of vessels per square micrometer. Blood vessels are defined as follows: large vessels containing erythrocytes, surrounded by smooth muscle; medium vessels consisting of erythrocytes and smooth muscle; and capillaries.

**[0084]** The specific binding of lectins to endothelium was used to detect the presence of both human and murine endothelial cells. The lectin UEA-1 binds specifically to human endothelium and BS-1 lectin (*Bandeiraea simplicifolia-1*, Sigma) selectively binds to murine endothelium. Additionally, antibodies directed specifically against human and murine CD31, a marker for endothelial cells, were used. Detection was achieved using the ABC kit (Vector labs) and amino-ethyl carbazole (AEC). The number of CD31-positive cells was counted and corrected for the area of tissue.

**[0085]** $CD34^+$ CPC-containing Matrigel® had increased vascularization when compared to bare Matrigel®. Staining these Matrigel® pellets for human CD31 demonstrated the presence of large CD31-positive cell clusters representing immature endothelial cells (FIG. **10A**). These clusters were present primarily in the $CD34^+CD133^-KDR^-$ and $CD34^+CD133^+KDR^-$ subsets. A small number of human CD31-positive cells associated with vessel-like structures were seen in the $CD34^+CD133^-KDR^-$ subset (FIG. **10B**).

**[0086]** While the human CPCs had not differentiated into human blood vessels, the $CD34^+$ human CPC subsets did provide and inductive effect of host murine neovascularization. There was an increased incidence of murine CD31-positive vasculature in the CD34+ CPC-loaded Matrigel® with vessels ranging in size from capillaries to large vessels (FIG. **11**).

**[0087]** The number of murine CD31-positive vessels/$mm^2$ (including capillaries, small and large vessels was determined for five experimental groups listed in Table 3 (FIG. **12**). The criteria for identification of blood vessels are: capillaries have 1-2 endothelial cells; small vessels have 3-5 endothelial cells; and large vessels have more than 5 endothelial cells and may also have vascular smooth muscle surrounding the vessel. The CD34+ CPC subsets induced higher levels of host murine vascularization over bare Matrigel® controls. Highest levels of vascularization were seen in the $CD34^+CD133^-KDR^-$ and $CD34^+CD133^+KDR^-$ subsets. Large vessels were primarily seen in the $CD34^+CD133^-KDR^-$ subset and to a lesser extent in the $CD34^+CD133^+KDR^-$ subset. Furthermore, a combination of these two subsets was not synergistic and did not lead to higher levels of neovascularization than either subset alone. Additionally, the combination of the

$CD34^+CD133^-KDR^-$ and the $CD34^+CD133^-KDR^+$ subsets resulted in only the formation of capillaries and not in formation of small and large blood vessels.

Example 6

CPC transcription profiles of aMI patients and healthy controls

**[0088]** Previous studies indicate that CPC can express a panel of markers related to their development and function. Although the above described four-parameter flow cytometric analysis allows simultaneous assessment of three CPC markers on single CPC, this approach does not cover all CPC markers known so far. Therefore quantitative RT-PCR was used to investigate the presence and expression level of a number of transcripts related to CPC development, maturation and function and to determine which factors mediated the observed pro-angiogenic and pro-inflammatory effects seen in $CD34^+$ CPC.

**[0089]** Total RNA was isolated from $10^3$-$10^4$ CPC from the desired phenotype ($CD34^+$, $CD133^+$ and $KDR^+$) and random hexamers and copy DNA was synthesized. Primer/probe sets (TaqMan, Applied Biosystems, Foster City, CA) for human GAPDK, beta-2-microglobulin (B2M), beta-actin, c-abl, CD34, CD133, Tie-1, Tie-2, flt-1, KDR, VEGF, CD31, VE-cadherin, von Willebrand factor (vWF), interleukin-8 (IL-8), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$), macrophage chemoattractant protein-1 (MCP-1), MCP-2 and MCP-3 were used for CPC transcript analysis. Triplicate RT-PCR reactions were performed on equal amounts of cDNA using the following parameters: 2 min 50°C, 10 min 95°C, and 45 cycles consisting of 15 sec denaturation (95°C) and 1 min annealing/extension (60°C). The variation (SD) of combined cDNA synthesis and PCR was less than 0.5 $C_T$ (cycle threshold) for the GAPDH housekeeping mRNA. Cycle threshold values were normalized to beta-2-microglobulin using the $\Delta C_T$ method and differences in expression levels between patients and controls or between subsets are expressed as fold variance of expression, calculated as $2^{-\Delta\Delta CT}$ (Livak KJ *et al.* Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$ Method. Methods. 25:402-8, 2001).

**[0090]** The results of RT-PCT to determine the presence of mRNA transcripts of 14 markers potentially involved in CPC maturation and function are presented in Table 4. Because there was inter-individual variance with respect to gene expression, the expression of a gene in a given CPC subset was defined as the presence of a PCR product (i.e. a $C_T$ value < 45) in at least 3/5 subjects. Based on this definition, gene expression profiles in aMI patients and healthy controls were similar.

Table 4. Gene expression profiling in CPC subsets

| | Healthy control | | | aMI | | | HUVEC |
|---|---|---|---|---|---|---|---|
| Marker | KDR | CD133 | CD34 | KDR | CD133 | CD34 | |
| GAPDH | + | + | + | + | + | + | + |
| B2M | + | + | + | + | + | + | + |
| B-Act | + | + | + | + | + | + | + |
| c-abl | n.d. | + | + | + | + | + | + |
| Tie-1 | n.d. | + | + | n.d. | + | + | + |
| Tie-2 | n.d. | n.d. | + | n.d. | n.d. | + | + |
| CD34 | n.d. | + | + | n.d. | + | + | + |
| CD133 | n.d. | + | + | n.d. | n.d. | + | n.d. |
| flt-1 | + | n.d. | + | + | n.d. | + | + |
| KDR | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | + |
| VEGF | + | + | + | + | + | + | + |
| CD31 | + | + | + | + | + | + | + |
| VE-cadh | n,d. | n.d. | n.d. | n.d. | n.d. | n.d. | + |
| vWF | n.d. | + | + | + | + | + | + |

$10^3$-$10^4$ CPC were sorted from 5 aMI patients and 5 healthy controls. When a PCR product was detected in at least 3/5 individuals, gene expression was considered to be present in the respective subset (+). n.d. = not detected. RNA isolated from 1000 HUVEC (human umbilical vein endothelial cells) was used as a positive control.

**[0091]** When comparing the three CPC subsets, gain of gene expression was apparent in the order KDR → CD133 → CD34. Tie-1, CD34 and, in healthy controls, CD133 and vWF transcripts were present in the CD133 subset, but not in the KDR subset. Flt-1 transcripts, which were detectable in the $KDR^+$ subset, were absent in $CD133^+$ cells. In the $CD34^+$ subset, transcripts of Tie-2, flt-1, CD133 and, in aMI patients, CD34 were gained in comparison to $KDR^+$ and $CD133^+$ cells.

**[0092]** Transcripts of the inflammation-associated molecules GM-CSF, MCP-1, CMP-2 and MCP-3 were below PCT detection limits in all subsets. TNF-$\alpha$ and MIP-1$\alpha$ transcripts were present in similar amounts in $CD34^+$ CPC and $KDR^+$ CPC. Interleukin-8 (IL-8) transcripts were present in $CD34^+$ transcripts from all included individuals. In $KDR^+$ CPC, however, IL-8 transcripts were found in only 2 of 5 individuals. Moreover, IL-8 transcript levels were 3-fold higher in the $CD34^+$ subsets than in the $KDR^+$ subset. Interleukin-8 transcripts were found in $CD34^+$ CPC both directly after sorting and after 14 day implantation in Matrigel® in nude mice (FIG. **5**).

**[0093]** Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in

the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

**[0094]** The terms "a" and "an" and "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

**[0095]** Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

**[0096]** Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and

equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

**[0097]** Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above cited references and printed publications are herein individually incorporated by reference in their entirety.

**[0098]** In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

What is claimed is:

1. A method for isolation of endothelial progenitor cells comprising:

identifying lineage-committed cells from a source of progenitor cells by contacting said progenitor cells with a plurality of fluorochrome-labeled antibodies specific for the cell markers selected from the group consisting of CD3, CD14, CD16/56, CD19 and CD31;

depleting said lineage-committed cells by fluorescence activated cell sorting to form a population of lineage-negative cells;

reacting said lineage-negative cells with a plurality of fluorochrome-labeled antibodies specific for the cell markers selected from the group consisting of CD34, CD133 and KDR wherein each antibody is labeled with a fluorochrome with a unique emission wavelength; and

sorting said labeled lineage-negative cells by three-color fluorescence activated cell sorting to form a population of endothelial progenitor cells.

2. The method of claim 1 wherein said source of progenitor cells is a mammalian source.

3. The method of claim 2 wherein said mammalian source is a human source.

4. The method of claim 1 wherein said source of progenitor cells is peripheral blood.

5. The method of claim 1 wherein said identifying step comprises contacting said progenitor cells with antibodies specific for the cell markers CD3, CD14, CD16/56, CD19 and CD31.

6. The method of claim 1 wherein said reacting step comprises reacting said lineage-negative cells with antibodies specific for the cell markers CD34, CD133 and KDR.

7. The method of claim 1 wherein said endothelial progenitor cells express CD34.

8. The method of claim 1 wherein said endothelial progenitor cells are blood vessel generating cells, inflammation-mediating cells or both.

9. The method of claim 8 wherein said blood vessel-generating cells are $CD34^+$ endothelial progenitor cells.

10. The method of claim 9 wherein said blood vessel-generating cells are $CD34^+CD133^-KDR^-$ endothelial progenitor cells.

11. The method of claim 8 wherein said inflammation-mediating cells are $CD34^+$ endothelial progenitor cells.

12. The method of claim 11 wherein said inflammation-mediating cells are $CD34^+CD133^-KDR^-$ endothelial progenitor cells.

13. The method of claim 11 wherein said inflammation mediating cells express interleukin-8.

14. The method of claim 1 wherein said endothelial progenitor cells induce angiogenic responses in surrounding blood vessels.

15. A therapeutic composition for inducing angiogenesis at a treatment site comprising:

a biodegradable matrix having $CD34^+$ endothelial progenitor cells disposed therein.

16. The therapeutic composition of claim 15 wherein said $CD34^+$ endothelial progenitor cells are $CD34^+CD133^-KDR^-$ endothelial progenitor cells.

17. The therapeutic composition of 15 wherein said biodegradable biocompatible matrix is selected from the group consisting of solubilized basement membrane, autologous platelet gel, collagen gels or collagenous substrates based on elastin, fibronectin, laminin, extracellular matrix and fibrillar proteins.

18. A therapeutic composition having a chemotactic effect on inflammation-mediating cells at a treatment site comprising :

a biodegradable biocompatible matrix having $CD34^+$ endothelial progenitor cells disposed therein.

19. The therapeutic composition of claim 18 wherein said $CD34^+$ endothelial progenitor cells are $CD34^+CD133^-KDR^-$ endothelial progenitor cells.

20. The therapeutic composition of 18 wherein said biodegradable biocompatible matrix is selected from the group consisting of solubilized basement membrane, autologous platelet gel, collagen gels or collagenous substrates based on elastin, fibronectin, laminin, extracellular matrix and fibrillar proteins.

21. An isolated population of endothelial progenitor cells wherein said isolated population is lineage-negative and $CD34^+$.

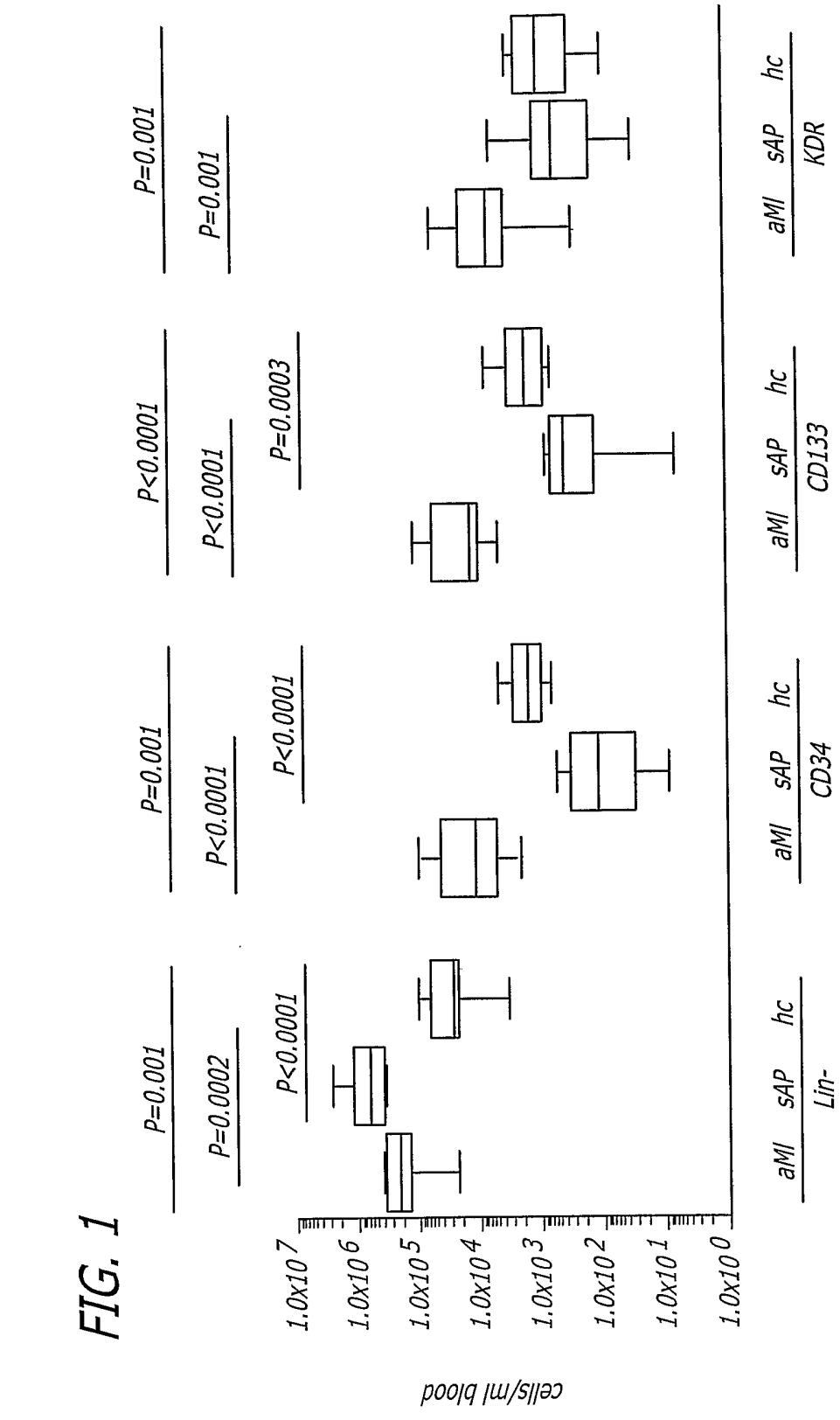
22. The isolated population of endothelial progenitor cells of claim 21 wherein said isolated population is lineage-negative and $CD34^+CD133^-KDR^-$.

23. The isolated population of endothelial progenitor cells of claim 21 wherein said isolated population comprises blood vessel-generating cells.

24. The isolated population of endothelial progenitor cells of claim 21 wherein said isolated population comprises inflammation-mediating cells.

25. The isolated population of endothelial progenitor cells of claim 21 wherein said isolated population induces angiogenic responses in surrounding blood vessels.

FIG. 1


P=0.001
P=0.0002
P<0.0001
P=0.001
P<0.0001
P<0.0001
P<0.0001
P<0.0001
P=0.0003
P=0.001
P=0.001
1.0x10 7
1.0x10 6
1.0x10 5
1.0x10 4
1.0x10 3
1.0x10 2
1.0x10 1
1.0x10 0
cells/ml blood
aMI sAP hc
Lin-
aMI sAP hc
CD34
aMI sAP hc
CD133
aMI sAP hc
KDR

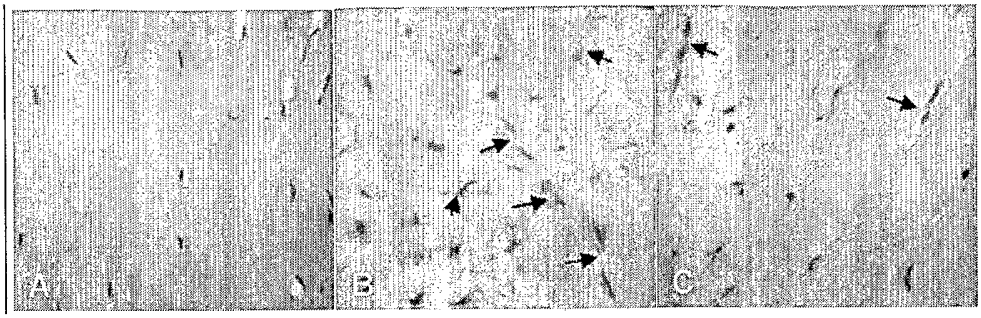



FIG. 2

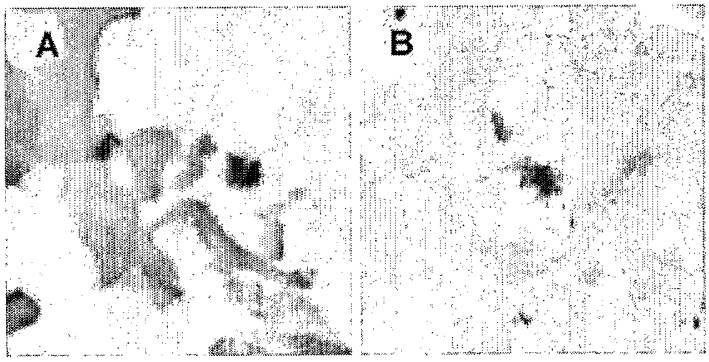



FIG. 5

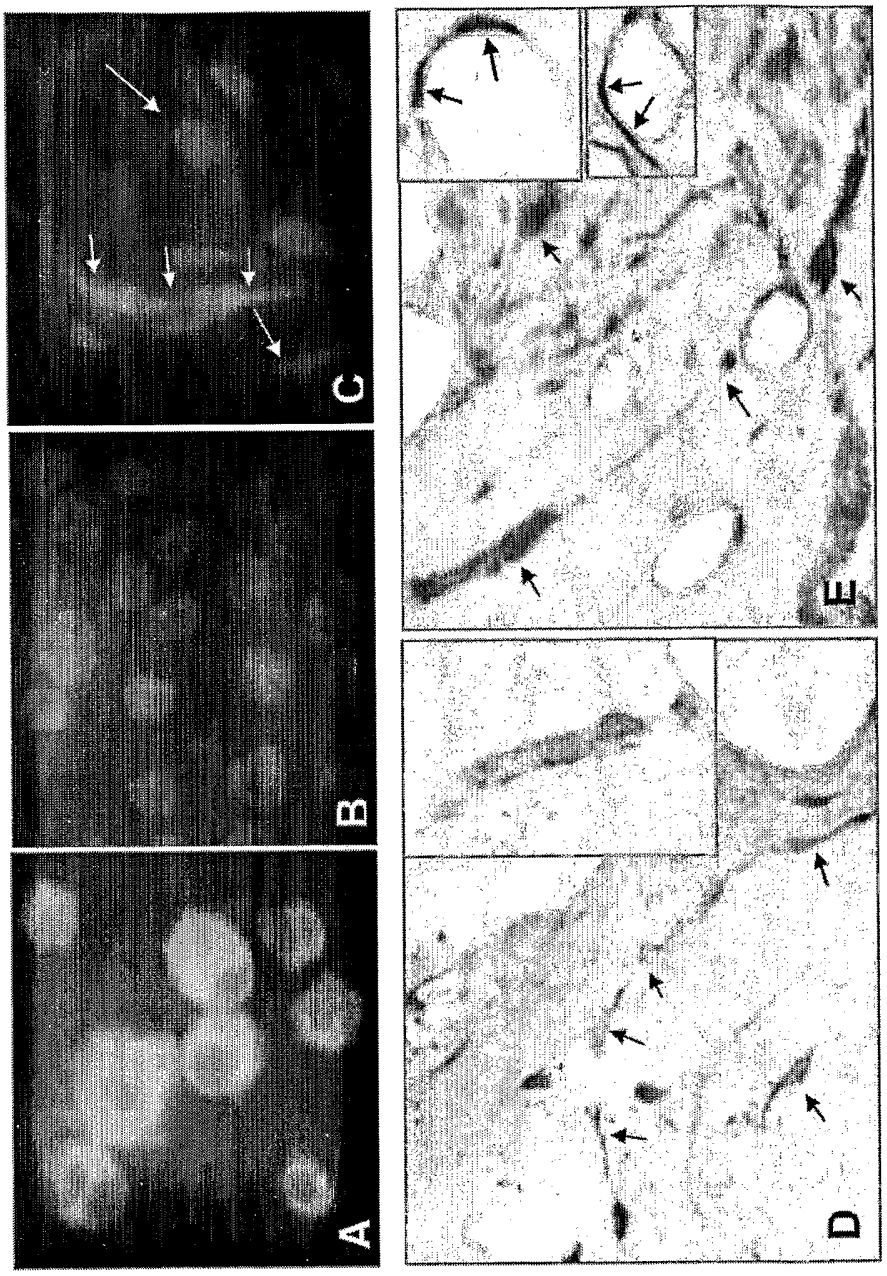



FIG. 3

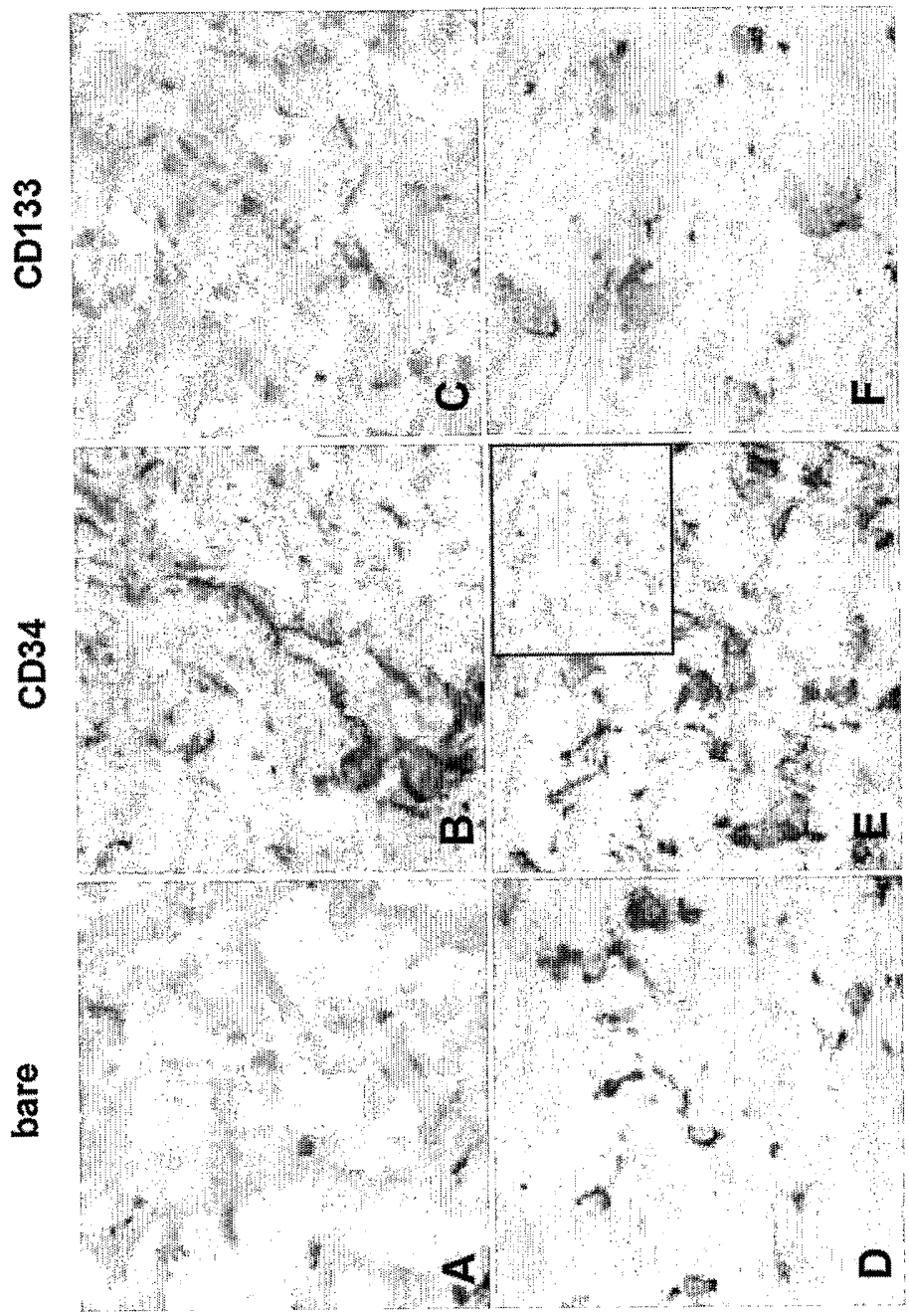



FIG. 4

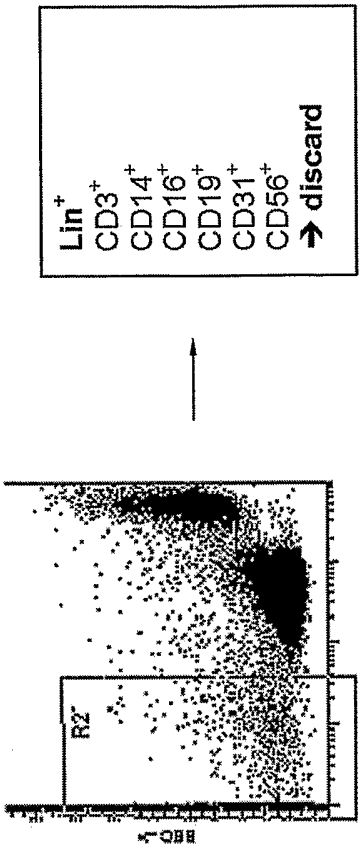

Lin-
→ sort
→ stain
CD34-APC
CD133-PE
KDR-FITC
Lin+
CD3+
CD14+
CD16+
CD19+
CD31+
CD56+
→ discard
R2
SSC
CD133

FIG. 6A

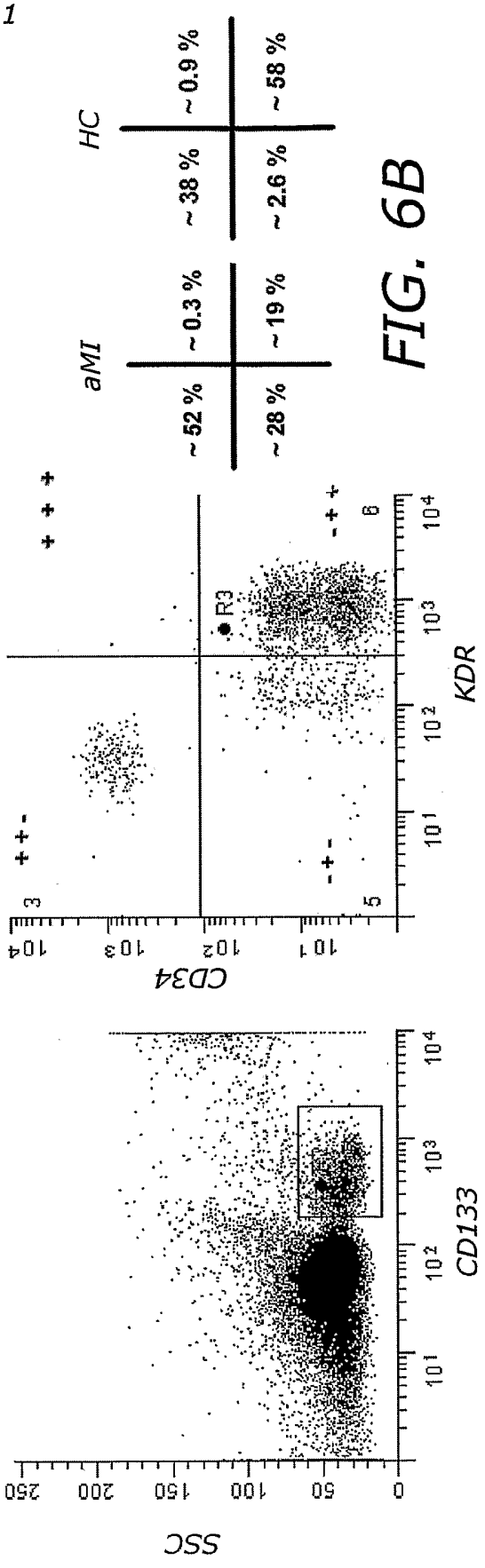
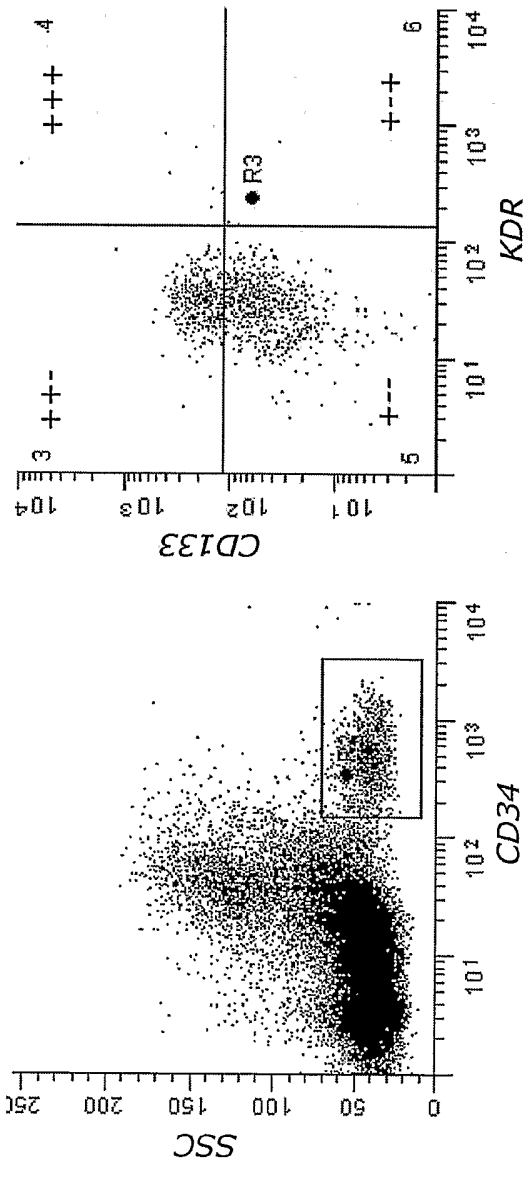
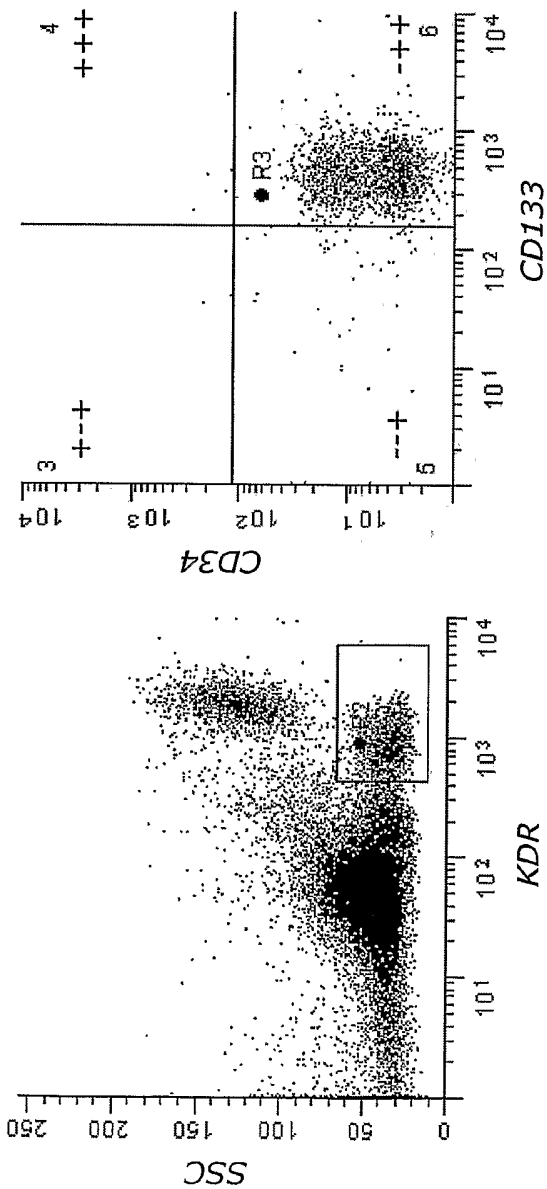

HC
aMI
~ 0.9 %
~ 58 %
~ 38 %
~ 2.6 %
~ 0.3 %
~ 19 %
~ 52 %
~ 28 %
R3
CD34
KDR
SSC
CD133

FIG. 6B




| aMI | | HC | |
|---|---|---|---|
| ~62% | ~0.3% | ~38% | ~0.9% |
| ~37% | ~0.5% | ~60% | ~0.2% |

FIG. 6C




| aMI | | HC | |
|---|---|---|---|
| ~0.7% | ~0.5% | ~0.4% | ~1.5% |
| ~65% | ~33% | ~4.5% | ~93% |

FIG. 6D

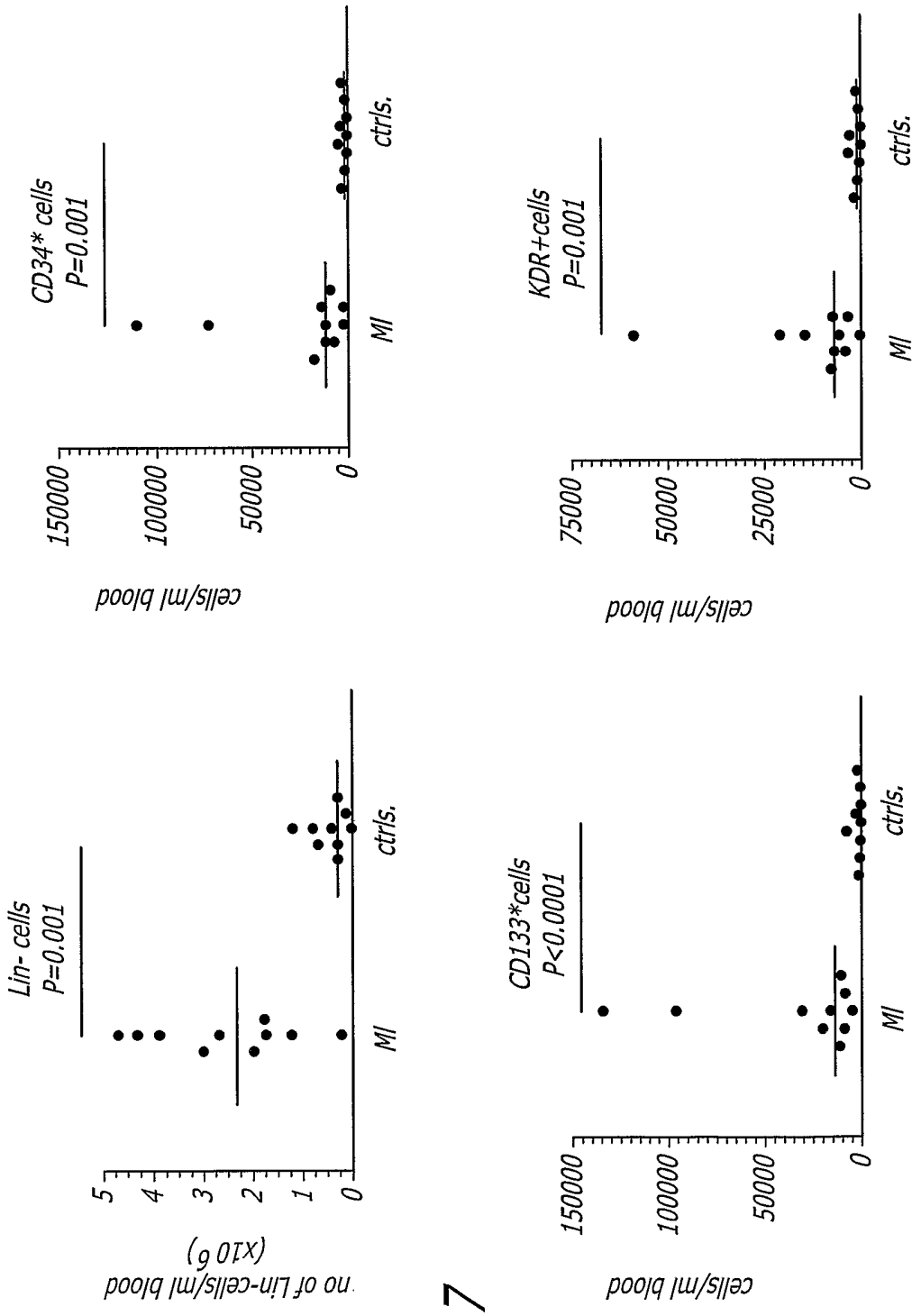
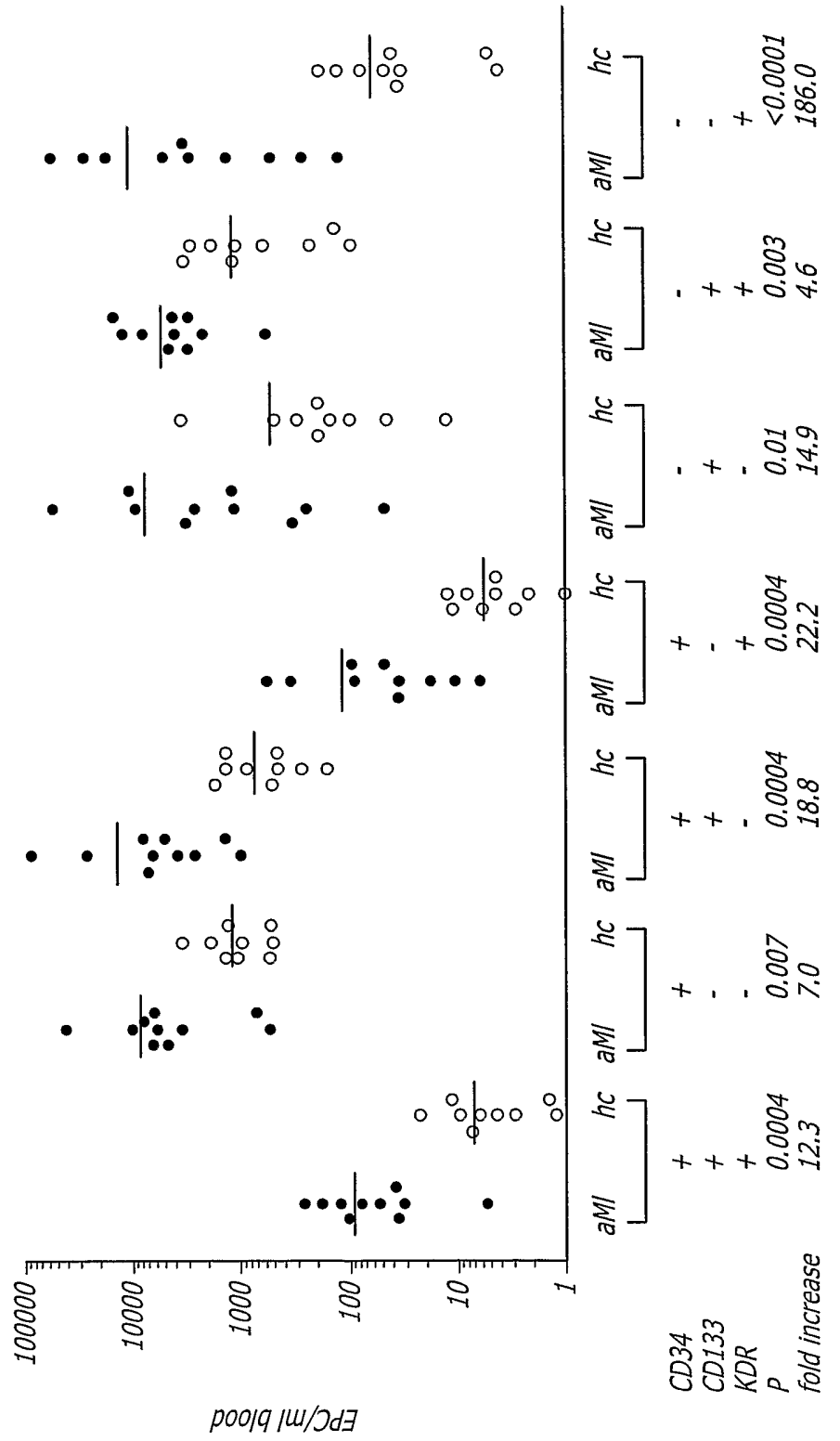

Lin- cells
P=0.001
no of Lin-cells/ml blood
(X10 6)
5
4
3
2
1
0
MI
ctrls.
CD34* cells
P=0.001
cells/ml blood
150000
100000
50000
0
MI
ctrls.
CD133*cells
P<0.0001
cells/ml blood
150000
100000
50000
0
MI
ctrls.
KDR+cells
P=0.001
cells/ml blood
75000
50000
25000
0
MI
ctrls.

FIG. 7

FIG. 8


EPC/ml blood
100000
10000
1000
100
10
1
aMI
hc
CD34
CD133
KDR
P
fold increase
+
-
0.0004
12.3
0.007
7.0
0.0004
18.8
0.0004
22.2
0.01
14.9
0.003
4.6
<0.0001
186.0

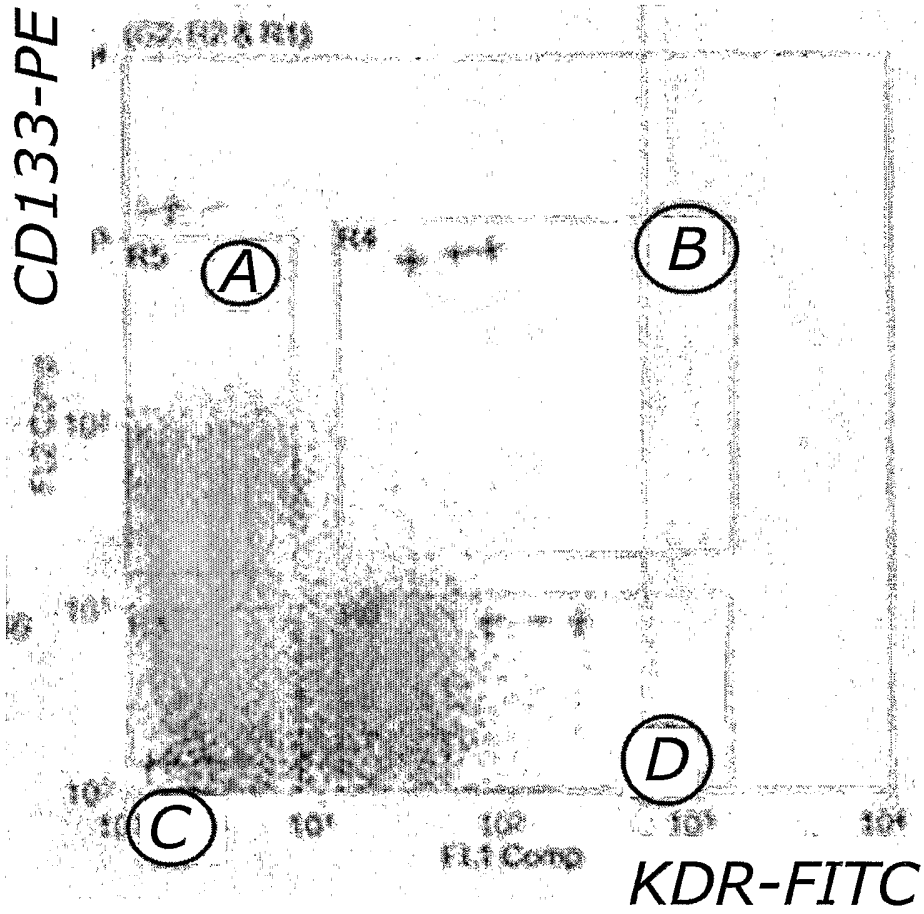



FIG. 9

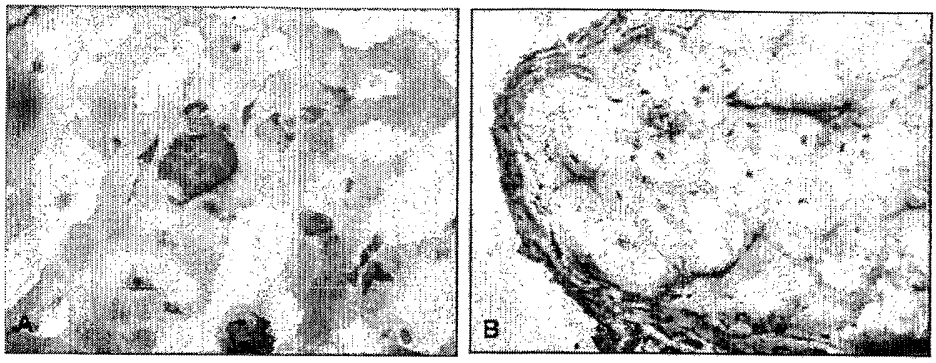



FIG. 10

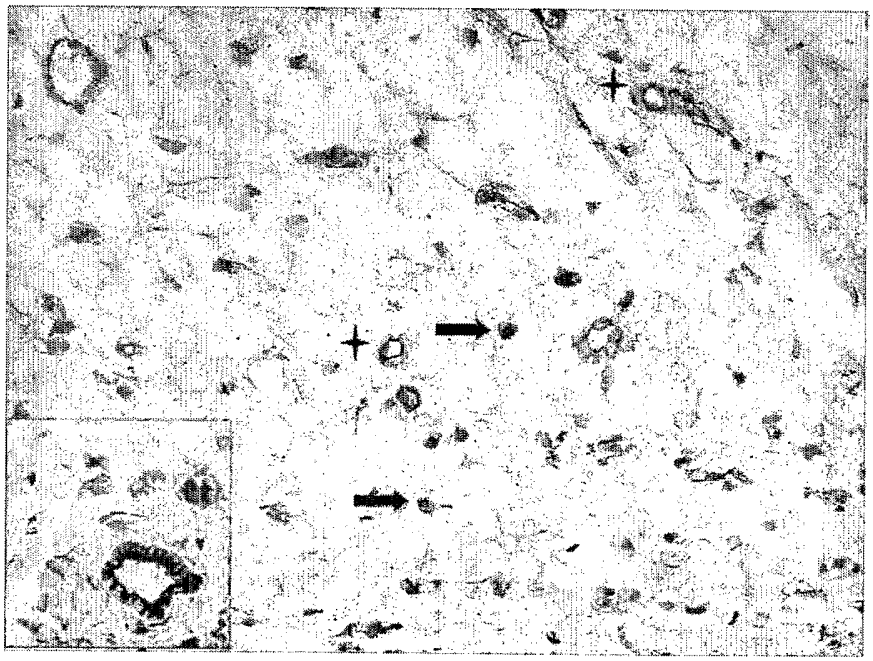
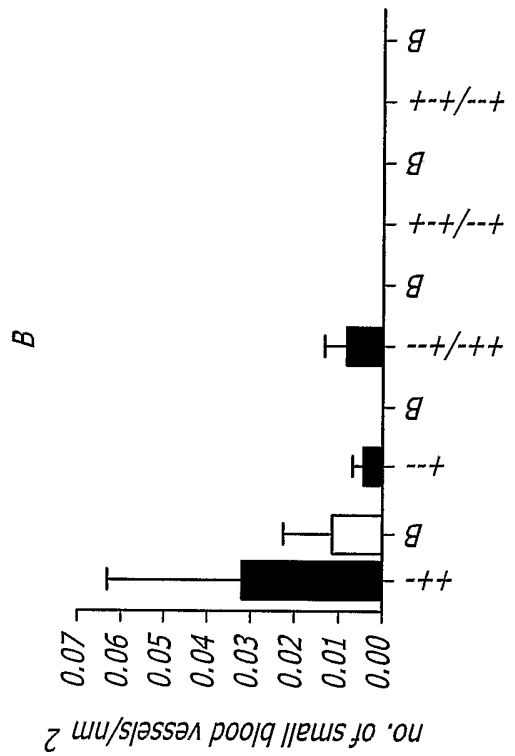
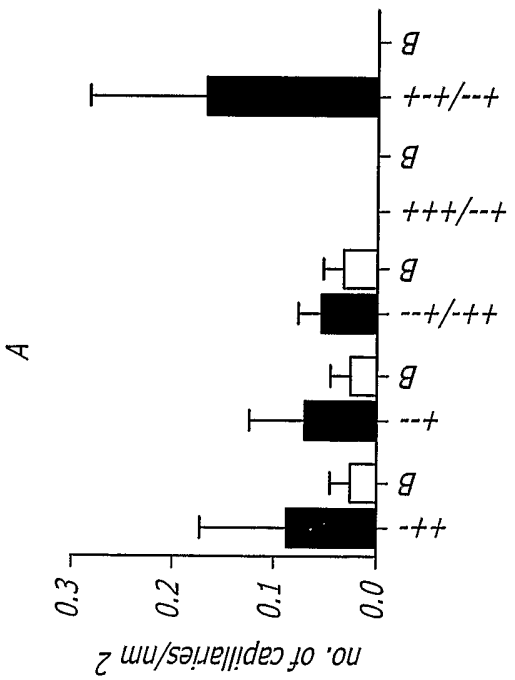


FIG. 11


B
no. of small blood vessels/nm 2
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
++-
B
+--
B
--+/++
B
+--/+-+
B
+--/+-+
B
++-
B


A
no. of capillaries/nm 2
0.0
0.1
0.2
0.3
++-
B
+--
B
--+/++
B
+--/+++
B
+--/+-+
B
++-
B

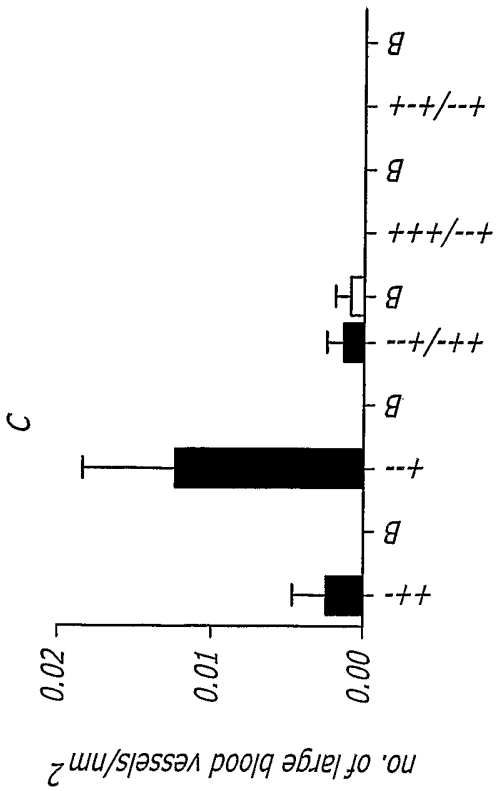

C
no. of large blood vessels/nm 2
0.00
0.01
0.02
++-
B
+--
B
--+/++
B
+--/+++
B
+--/+-+
B

FIG. 12

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/028923

A. CLASSIFICATION OF SUBJECT MATTER
C12N5/06

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)
C12N

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)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X<br>Y | WO 02/097066 A (MEDESTEA INTERNAZIONALES S.R.L; ALESSANDRI, GIULIO; CARUSO, ARNALDO; F) 5 December 2002 (2002-12-05)<br>the whole document | 21,23-25<br>1-14 |

-/--

[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 |
|---|---|
| 23 December 2005 | 12/01/2006 |

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

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y | the whole document | 1-14 |
| X | PEICHEV MM ET AL: "Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors" BLOOD, W.B.SAUNDERS COMPANY, ORLANDO, FL, US, vol. 95, no. 3, 1 February 2000 (2000-02-01), pages 952-958, XP002251073 ISSN: 0006-4971 | 21,23-25 |
| Y | the whole document | 1-14 |
| Y | MANCUSO P ET AL: "Resting and activated endothelial cells are increased in the peripheral blood of cancer patients" BLOOD, W.B.SAUNDERS COMPANY, ORLANDO, FL, US, vol. 97, no. 11, 1 June 2001 (2001-06-01), pages 3658-3661, XP002274898 ISSN: 0006-4971 the whole document | 1-14, 21-25 |
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Information on patent family members

International Application No
PCT/US2005/028923

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | EP 0863776 A1 | 16-09-1998 |
| | | ES 2189890 T3 | 16-07-2003 |
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| | | PT 863776 T | 30-06-2003 |
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| WO 2004098499 A | 18-11-2004 | NONE | |