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Introduction
Scope of the thesis

Introduction

Since the discovery that tumour growth is dependent on the formation of a vasculature, the inhibition of angiogenesis in solid tumours has been an accepted therapeutic target[1-4]. In contrast, stimulation of neovascularization could be beneficial in the treatment of tissue damage after ischemia in e.g. myocardial infarction or peripheral vascular disease in diabetes and is the goal of several more recent therapeutic strategies. The gradual unravelling of the molecular and cellular mechanisms behind neovascularization will only stimulate this challenging field. This general introduction describes the mechanisms as well as the cells involved in neovascularization and the scope of this thesis.

I Neovascularization

In human development and tissue growth, three types of neovascularization can be recognized in the formation of the vascular network; vasculogenesis, angiogenesis and arteriogenesis. In each of these processes, different mechanisms, cells and growth factors are involved (fig 1) that will be described in more detail in the first part of this general introduction.

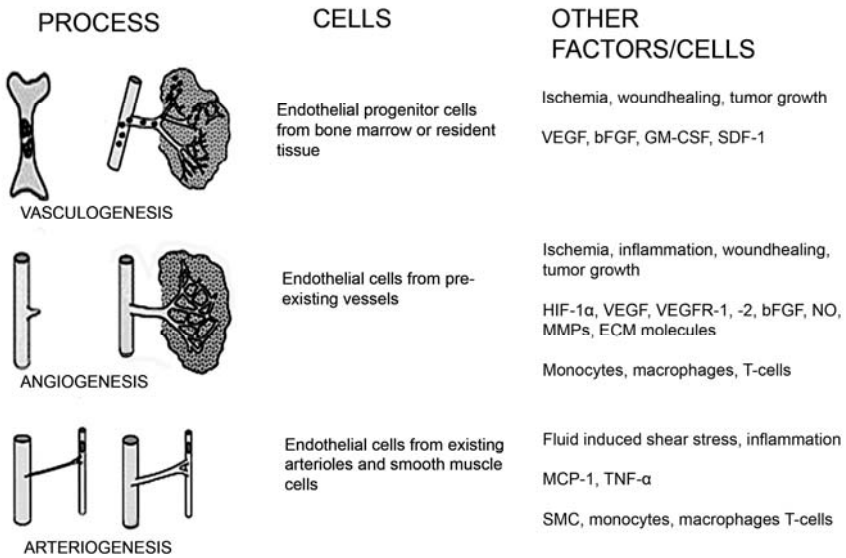

Figure 1: Types of neovascularization in the human adult
Vasculogenesis, angiogenesis and arteriogenesis are different processes in which different cells and factors are involved

Vasculogenesis

The participation of endothelial progenitors to new vessel formation is called vasculogenesis. During embryogenesis, the first endothelial and hematopoietic cells of the yolk sac arise from a common mesodermal progenitor, the "hemangioblast", located in the so-called blood islands[5,6]. The cells destined to generate hematopoietic cells are situated in the centre of the blood islands and are termed hematopoietic stem cells (HSC). Endothelial progenitor cells (EPC), are located at the periphery of the blood islands and participate in the formation of new blood vessels in the embryo. Once the embryonic heart has developed, the first primitive blood cells start to circulate from the yolk sac into the embryo[5,7]. VEGF and bFGF play an important role in these processes. Knockout of either the gene for VEGF or its receptors, VEGFR1 (flt-1) and VEGFR2 (KDR, Flk-1), leads in this respect to embryonic lethality due to defective vascular organisation and vasculogenesis[8-10]. It was only in the last decades that it was discovered that the adult blood circulation and tissues still contain small numbers of stem cells, other than the blood cell forming HSC. Among these stem cells are mesenchymal stem cells, that can give rise to mesenchymal tissues, like bone and cartilage, but also endothelial progenitor cells are found in the adult. Both, but especially the latter have gained much interest by their potential to contribute to new blood vessel formation, thus adult vasculogenesis[11-13].

The first step in this quest was the establishment of EPC cultures from CD34-enriched peripheral blood mononuclear cells (PBMC) in a landmark study by Asahara and colleagues in 1997[12]. In this study, cell colonies with endothelial markers (E-selectin, CD31, eNOS etc) were raised from peripheral blood cells, enriched with $CD34^+$ cells (15%), which were suggested to be the origin of these cultures, while $CD34^-$ cells were shown to facilitate these cultures[12]. Since this publication, much research has been directed to identify, isolate or enrich the EPC that are responsible for the described cultures.

A returning problem in this research, however, is that the methods used to quantify EPC numbers by flow cytometry or culture lack standardization. This is caused by the fact that the immuno phenotype of the EPC is still not completely known, and that there is a definite overlap with other cell types, like HSC and circulating endothelial cells. Most common markers for flow cytometry in humans are CD34 and CD133 for stem cells and

KDR/VEGFR2 as endothelial antigen[14]. CD34 is the prototypical stem cell antigen, it might function as an adhesion molecule for the interactions between endothelial cells and hematopoietic precursors and is also expressed on freshly isolated umbilical vein endothelial cells and micro vascular endothelial cells[15]. The function of CD133 (also known as AC133), is still unknown but its expression is helpful for discerning true immature hematopoietic $CD34^+$ cells, from $CD34^+$ endothelial cells[16]. Some groups have shown that EPC can be cultured from $CD133^+$ cells and have proposed that the $CD133^+/KDR^+/CD34^+$ cells form a distinct population of EPC[17]. However, a recent publication showed that these $CD133^+/KDR^+/CD34^+$ positive cells are not EPC, but (still) a population of very immature hematopoietic progenitors[18]. As a result, a fourth antigen, CD45 is often taken along. CD45 is the common leukocyte antigen and it has been suggested that EPC reside in the CD45 negative/low fraction[19]. In summary, even with the use of a combination of markers, one can only enrich for EPC in stead of selecting a pure population of EPC.

Notwithstanding the still imperfect methods to identify and quantify EPC, it became possible to study the physiologic and pathophysiologic regulation of EPC levels *in vivo* and subsequently gain more insight in their natural function. Using different quantification methods, many groups have shown that numbers of EPC reflect or correlate with disease state. For example, more circulating EPC are observed when patients suffer from severe idiopathic pulmonary hypertension or systemic sclerosis[20,21]. In contrast, decreased levels of circulating EPC were found in patients with coronary artery disease or diabetes[22-24]. Overall, it seems that EPC levels negatively correlate with (risk for) cardio-vascular disease and increase with ischemic pathophysiologies. Growth factors like GM-CSF, stromal cell derived factor-1 (SDF-1), erythropoietin and vascular endothelial growth factor (VEGF) have been found to correlate with EPC numbers and therefore been described as mobilizers of EPC[24,25]. Remarkably, similar correlations were found in the different assays, while it is not clear whether the quantified cells, either by flow cytometry or culture, are representative for the same EPC population.

The culture assays in this respect, enables us to confirm if the measured cells are true endothelial 'progenitor cells' which semantically should at least show an ability to proliferate and differentiate into mature endothelial cells. Furthermore, the EPC derived endothelial cells should have appropriate antigenic characteristics but also a functional

in vivo behavior, e.g. vessel formation and incorporation. In this respect, it now becomes clear that the term EPC includes a spectrum of cells with immature, i.e. more stem cell like features, as well as more mature cells with both endothelial and/ or monocytic markers. These cells all share one common feature: they contribute to neovascularization *in vivo* and *in vitro*[26-28]. The different types of EPC will be discussed in more detail in part IV of this introduction.

Angiogenesis

The formation of new capillaries from endothelial cells that sprout from pre-existing blood vessels, angiogenesis, is of high importance in a number of pathological and physiological processes. Not only the growth of normal tissue, but also wound healing after damage or solid tumour growth, is dependent on new vessel formation for the supply of nutrients and oxygen, as well as the removal of waste products.

A trigger for angiogenesis is most often hypoxia or ischemia. Hypoxia inducible factor-1α (HIF-1α) is one of the main regulators of angiogenesis. Under hypoxic conditions, HIF-1α, which is normally continuously degraded, escapes from degradation and is able to bind together with HIF-1β to hypoxia responsive elements in the promoter regions of target genes. VEGF is the most important angiogenesis modulator from the HIF induced genes[29]. This protein is a mitogen for vascular endothelial cells and induces a strong angiogenic response in a variety of *in vivo* models. In addition to VEGF, bFGF is also an important angiogenic factor, bFGF binds to FGF receptors on endothelial cells, and increases their motility, proliferation and proteinase activity, bFGF can also induce VEGF expression via HIF-1α activation[30].

When pro-angiogenic signals are provided, the first step is vasodilatation and an increase in vascular permeability, induced by nitric oxide and VEGF from the surrounding cells[31-33]. Plasma proteins extravasate and form a fibrin rich matrix, which provides a scaffold for the endothelial cells to migrate towards sites with high concentrations of (chemo attracting) growth factors. VEGF, interleukin (Il)-8 and bFGF are examples of such factors and are produced by tumour cells, T-cells, monocytes and/or macrophages[34-36]. After vasodilatation, vascular junctions loosen and the endothelial cells detach from their neighbouring cells[37]. The endothelial cells, but also surrounding inflammatory cells and fibroblasts produce matrix metallo proteinases

(MMP) that degrade the basement membrane. Endothelial migration and budding out of their original lumen is followed by the formation of primitive vascular-tube like structures with a new lumen. Finally, the newly formed vessels become part of the circulation and are shaped, in which shear stress is a critical factor[38,39]. Additionally, cell-cell, cell-extra cellular matrix interactions, vascular wall maturation and basal lamina modification[32,40,41], but also inflammatory mechanisms play a large role in angiogenesis. Inflammatory leukocytes (neutrophils, macrophages, and others) release a number of factors that are of influence on the behaviour of endothelial cells, like VEGF, hepatocyte growth factor (HGF), TNF-α and Il-8[34,42]. In addition, in an inflammatory disease like rheumatoid arthritis, angiogenesis plays a very important role in the pathophysiology[42].

Arteriogenesis

The increase in luminal size and maturation of immature pre-existing capillaries to mature collaterals is named arteriogenesis. The formation of just new capillaries (via either vasculogenesis or angiogenesis) is not enough for blood supply after complete occlusion Only muscular collateral arteries are able to support enough blood flow from outside the risk region to prevent the consequence of severe ischemia[43]. Other than for angiogenesis, arteriogenesis is not induced by hypoxia and ischemia related activities, such as VEGF production[31]. For example, the expression level of hypoxia induced genes, which play a prominent role in angiogenesis was unchanged during collateral formation in an animal model[44,45]. Studies showed that application of VEGF in a very high concentration had only minor effects on the diameter of pre-formed collaterals and on collateral conductance. In addition, VEGF blocking did not affect arteriogenesis negatively[44].

The trigger for arteriogenesis comes predominantly from the mechanic stimuli derived from the differences in flow and with it the vascular shear stress that is induced in the vascular bed upstream from an obstruction. When occlusion of a vessel occurs, surrounding vessels experience an increased flow due to the redirected blood flow leading to fluid shear stress and circumferential wall stress. Endothelial cells from the vessel wall can sense this stress, become activated and produce growth factors, like monocyte chemo attractant protein-1 (MCP-1) that attract inflammatory cells of which monocytes have been shown to be the most important for arteriogenesis[46-48]. In

addition, adhesion molecules on the surface of the EC are upregulated, in particular intercellular adhesion molecule (ICAM)-1, that mediates interaction with MAC-1 (CD11a/CD18) on the leukocyte surface[49]. Inflammatory cells then migrate from the lumen through the activated endothelium, but also from the surrounding perivascular region to the collaterals to produce growth factors, cytokines and proteases that support tissue remodelling and the collateral growth[50].

Studies by the group of Schaper showed that monocyte depletion in the rabbit hind limb model, induced decreased collateral formation[51]. Arras *et al* showed that monocytes accumulate in the perivascular region of newly formed collateral and that monocyte activation is crucial in arteriogenesis[52]. In addition, in knock out mice for the MCP-1 receptor CCR2, collateral artery growth was prevented after femoral artery growth[53].

In addition to monocytes, T-cells have been shown to play a major role in arteriogenesis. Stabile *et al* showed that CD4 knockout mice have an impaired arteriogenic response after ligation of the femoral artery in the hind limb[54]. These results were confirmed by Van Weel *et al*, who described a role for NK-cells and $CD4^+$ T-cells in arteriogenesis in knockout mice. They show that both NK-cells and $CD4^+$ T-cells modulate arteriogenesis and they suggest that promoting lymphocyte activation may represent a promising method to treat ischemic disease[55].

After the first acute response to increased shear, the arteriogenesis process is further finished by extra maturation of the vessel. MMP in this respect participate in the digestion of the extra cellular matrix to create extra space for the growing collateral vessel. The shear induced upregulation of factors as platelet derived growth factor (PDGF), additionally attracts smooth muscle cells (SMC)[56]. Growth and differentiation of the collaterals involve the proliferation of these smooth muscle cells and adventitial fibroblasts. Finally, the increase of collateral vessel diameter reduces the fluid shear stress and thereby the signal for maturation, resulting in stable conductive vessels.

II The ischemic hind limb model to study neovascularization *in vivo*

To study neovascularization *in vivo*, most groups use the ischemic hind limb model in mice, rats or rabbits. In this model, the femoral artery is ligated unilaterally above the knee, inducing an ischemic hind limb. The recovery of blood flow can be measured by various techniques, of which Laser Doppler Imaging (LDI) is most often used. Laser Doppler gives a direct measure of microcirculatory flow in the paw of the animal. A laser beam scans the paw, and while most of the light is reflected back by static structures, part of the light is reflected back by moving blood cells. This signal is processed and the flux of blood flow can be calculated. The ratio of perfusion of the ischemic limb and the control other limb is calculated and followed during time. In addition to LDI, end-point quantification methods like the use of micro spheres and angiograms are also often used to quantify collateral growth[57].

Due to the occlusion, increased flow in the upstream collaterals is induced and triggers *arteriogenesis* in the upper leg[58]. Moreover, the ischemia caused by depression of blood flow results in *angiogenesis* in the paw. By transplantation and/or labelling of endothelial progenitor cells, also *vasculogenesis* or EPC dependent facilitation of arteriogenesis and angiogenesis can be studied in this model. To test human cells in a mouse model, athymic nude mice, are a well suitable host for human cell transplantation. These mice lack the thymus, and thus show no immune response against xeno-grafts[59].

III Cell therapy for neovascularization

After ischemia, repair of the existing vascular bed is necessary to reverse the deprivation of nutrients and oxygen and to prevent permanent damage. Arteriogenesis in this respect seems most capable to react rapidly to vascular occlusion and probably has also the largest capacity to redirect and bypass blood to endangered sites. However,

new vessel formation should start with de novo tube formation by cells sprouting from pre-existing vessels (angiogenesis) or from circulating or resident EPC (vasculogenesis). Neovascularization requires the presence of growth factors and mobilization of circulating cells that can support the outgrowth of the new blood vessels. These compensatory processes occur naturally in healthy persons, but when this 'natural rescue team' is insufficient, defective, or when the damage is too large, extra therapy can be useful. Several types of therapy for the repair of ischemic tissue can be used. Numerous studies have tried the delivery of proangiogenic factors both systemically as well as locally within ischemic sites. First studies with local delivery of VEGF caused the formation of blood vessel tumours, haemangiomas, in the hearts of mice[60]. Later studies were able to circumvent this and it was shown that administration of VEGF or FGF protein improves blood flow in the ischemic myocardium. E.g. Tio *et al* showed that intramyocardial injection of the phVEGF165 gene improved blood flow and augmented function of the ischemic myocardium in pigs[61]. Similar approaches by others make an overall conclusion possible that the injection of an adenovirus coded with the VEGF121, VEGF1 or VEGF2 genes into stunned myocardium, increased collateral vessel flow and improved cardiac function[62,63]. For FGF similar results were obtained in e.g. myocardial infarction in dog and pig models.

However, in patients with cardiovascular disease, these proteins had no effect[64-67]. Results from several studies suggest that many of the traditional cardiovascular risk factors—such as age and hypercholesterolemia—may influence the response to treatment[68]. Moreover, in light of the complex processes as described above, frequent administration of a single agent seems a too simplistic approach. Additionally, the time and spatially adequate and specific targeting of growth factors creates also a problem. Cell therapy might circumvent some of these limitations, as cells can actively and possibly specifically home to required sites. Furthermore, cells can participate actively in new vessel formation and secrete on local cues the cytokines and growth factors that are known for its vasculogenic/ angiogenic/ arteriogenic potential.

IV Candidate cells for therapy

Cells to be used in cell therapy require a number of characteristics. The transplanted/infused cell fraction must be safe and harmless when infused, either locally or systemically. Although an autologous cell preparation seems to be the most preferable, most patients suffering from damage after ischemia, already have a impaired self-repair mechanism[69,70] Therefore, if autologous cells are being used, an ex vivo modulation or enrichment step might be needed. In addition, it should be possible to isolate active cells in sufficient therapeutic quantities from blood, bone marrow or other tissues. If not, ex vivo expansion and/or predifferentiation with GMP grade and preferably robust methods should be possible.

Cord blood, peripheral blood and bone marrow are the most evident sources to isolate (an autologous) cell suspension. These sources all contain high numbers of inflammatory cells, of which we know that they are important in most above mentioned neovascularization processes but which also have various other effects. On the contrary, progenitor cells, although they form only a very small percentage of the mononuclear cells (MNC) in the mentioned sources, are essential for vasculogenesis while at the same time their differentiation and proliferation capacity makes them attractive for use in cellular therapy. In this light, particularly cord blood might be an interesting source, since CB, besides their high percentage of HSC (0.05-0.1%), is known for its relatively high number of different types of EPC[71]. Finally, bone marrow is a main source of progenitor cells, of which the MSC is most easily culture expanded. In the next part of this introduction, the cells that are the focus of this thesis, namely monocytes, EPC and MSC will be described.

Monocytes

As mentioned above, angiogenesis and arteriogenesis share a lot of common players with inflammation; like cells, cytokines and growth factors. Monocytes are often mentioned as one of the key players in both types of neovascularization. They can be easily isolated with GMP grade methods, are abundantly present in the peripheral blood (10-20 % of MNC) and, more importantly: monocytes possess many characteristics that make them the perfect cells for the support and improvement of blood flow, either by

angio- or arteriogenesis. Already 30 years ago, Polverini *et al*[72,73] showed that monocytes and macrophages are potent stimulators of neovascularization in solid tumours and endothelial cell proliferation and that depletion of macrophages from tumour cell suspensions significantly decreased their angiogenic potential. Later studies showed that paracrine factors produced by the cells mediated these effects[72]. Macrophages in tumours differentiate from circulating monocytes and migrate in the tumour towards low oxygenated sites, attracted by macrophage-colony stimulating factor (M-CSF)[74]. Low oxygen leads to high levels of HIF-1, and expression of many pro-angiogenic factors. These tumour associated macrophages (TAM), can not only produce inflammatory cytokines like TNF-α[75] and IL-8, but also pro-angiogenic proteins, like EGF, FGF family members and VEGF[76].

Monocyte/macrophage released cytokines, growth factors and enzymes were also described to mediate angiogenesis in a number of mainly inflammatory pathologies. Neovascularization is a crucial process in a number of diseases, including rheumatoid arthritis (RA) and other systemic inflammatory conditions[42]. In RA, the macrophage plays a central role in the formation of new vessels. Besides their release of pro-angiogenic agents, monocytes and macrophages themselves adapt their phenotype to these agents by expressing receptors for most important angiogenic factors. For example, macrophages express VEGFR-1, which enables them to migrate towards VEGF and become activated upon VEGF release[77]. In addition, VEGFR-1 deficient arthritic mice showed diminished clinical symptoms compared to wild type mice[78]. Furthermore, macrophages express CXCR2, which recognizes a number of pro-angiogenic CXC chemokines[79]. Although both tumour growth and inflammatory diseases indicate that monocyte-facilitated angiogenesis can be a pathological, unwanted situation, these findings clearly indicate that if these mechanisms can be harnessed, the monocyte can be a suitable candidate for vascular regenerating therapies.

In addition to their pro-angiogenic side, the role of monocytes in arteriogenesis is crucial, as mentioned above. As the group Herold *et al*. already described and the group of Schaper has already shown in various ways: transplantation of monocytes might be a novel strategy for enhancement of collateral vessel growth[80].

An important and certainly not to be forgotten other feature of the monocyte is its detrimental effect in the development of atherosclerosis. After the initial endothelial cell

activation, probably mediated by atherogenic lipoproteins, monocytes are able to be recruited and attached to the endothelial layer of the blood vessel wall, via the upregulated endothelial cell adhesion molecules, ICAM and VCAM[81,82]. Sequential rolling and adhesion eventually leads to transmigration of the monocytes through the endothelial monolayer to the extra vascular compartment. This passage involves differentiation of the cells into macrophages. In this stage, the cell is able to participate actively in inflammatory and immune responses[81,83]. By taking up modified LDL, the cells are turned into fat-loaded macrophages that reside in the vessel wall and form the major residents of the fatty streaks just underneath the endothelium that eventually lead to the atherosclerotic, vessel disrupting plaques[84,85].

In order to use monocytes as a pro-angiogenic cell therapeutic product, it must be ensured that the negative side effects, namely the pro-atherogenesis effects, are diminished. To obtain pro-angiogenic monocytes, an *ex vivo* step is probably required.

Endothelial progenitor cells

Since the discovery of the endothelial progenitor cells in the late nineties of the last century, especially the culture assays have indicated that two cell types named EPC exist in the adult.

First, there is the more mature and less proliferative adherent EPC, also called attaching cell or early EPC. These cells are reported to appear 'early' in relatively short (5-7 days) cultures of peripheral blood mononuclear cells (PBMC)[24,27,86,87]. These cells acquire endothelial characteristics, like AcLDL-uptake, E-NOS and VWF-expression, and binding of Ulex-Lectin during culture. However, these EPC show no expansion or differentiation into mature endothelium. In addition, monocytic/macrophage characteristics have been described as well[87-89]. Pujol *et al* showed that endothelial like cells (ELC) can develop from $CD14^+$ mononuclear cells and dendritic cells in the presence of angiogenic growth factors[90,91]. Endothelial features were described as expression of vWF, VE-cadherin, CD105, CD36, both the VEGFR1 and 2, and endothelial adhesion molecules ICAM-1 and VCAM-1. In addition, tube formation in matrigel and endothelial-colony forming was also described[26,92]. The cells do not express dendritic cell markers, but expression of some macrophage markers, like CD68 and CD86, was still there[26].

In 2003 Hill *et al* published that the number of such early colony forming unit-endothelial cells (CFU-EC) after a short culture, represented EPC numbers and could be a surrogate biologic marker for vascular function and cumulative cardiovascular risk[22]. We and others found that CFU-EC are still formed after depletion of stem cells ($CD34^+$) or cells from the endothelial (progenitor) lineage($CD34^+/KDR^+/CD146^+$), but that depletion of monocytes completely abrogated the colony formation, indicating a myeloid origin[93-95].

The second kind of EPC, first described by Lin and colleagues, is called blood outgrowth endothelial cells (BOEC) or 'late' EPC[96], due to their late appearance in mononuclear cell cultures. BOEC can be cultured from cord blood and adult peripheral blood. They uniformly express endothelial, but no hematopoietic cell–specific surface antigens, and possess an extensive proliferative potential consistent with the definition of a progenitor cell[22,27,97,98]. The origin of these cells is still under debate. Ingram *et al* showed that they are part of an endothelial hierarchy in the vessel wall, while others have shown that they are derived from circulating $CD34^+$ cells that are mobilized from the bone marrow[98,99]. Recent studies also showed that EPC reside in the stromal zone or Wharton's jelly of human umbilical veins[100]. One wonders if semantically, only these high proliferative endothelial cells should be referred to as endothelial *progenitor* cells[89]. Whether the EPC that is quantified by flow cytometric analysis (as mentioned before), is representative for the EPC that is expanded in culture, is still unclear, although some groups have shown by isolation that the late outgrowth cells are derived from a subpopulation of $CD34^+$ cells[99].

In vivo studies showed that the BOEC perform de novo tube formation in a matrigel plug assay. In this assay, matrigels containing a suspension of BOEC are implanted subcutaneously in the mouse[101]. BOEC were shown to form long lasting functional vessels. In addition, the perfusion of ischemic hind limbs was improved after intravenous injection of BOEC in the ischemic hind limb model[102]. Although numbers are low in peripheral blood, it is possible to obtain BOEC from peripheral blood mononuclear cells. Moreover, their high proliferative capacity makes it possible to expand them to cell numbers that are sufficient for cellular therapy. Cord blood even contains a higher number of $CD34^+$ endothelial/hematopoietic progenitors[103], in

addition, CB BOEC appear faster in culture, are more proliferative and last longer than the BOEC from peripheral blood[98].

Mesenchymal stem cells

A possible source for vascular supporting cells are mesenchymal stem cells (MSC), which are also referred to as stromal progenitor cells or mesenchymal stromal cells. These cells can be isolated from bone marrow (BM), adipose tissue or foetal tissue. MSC are expandable stem cells that can differentiate into adipocytes, osteoblasts and chondrocytes[104]. Allogeneic BM-MSC have been successfully employed in graft versus host disease (GvHD) treatment, cartilage and bone repair, skin wounds healing, neuronal regeneration and osteogenesis imperfecta (OI)[105,106]. In addition, cell infusions of bone marrow or isolated MSC have led to an increase in repair after e.g. ischemia in pig models of myocardial infarction[105,107-109]. MSC have mainly been used for to their ability to 1) physical participate by the formation of a supportive microenvironment of the bone marrow or 2) by differentiation into different cell types that are needed to repair or replace damaged tissue.

The walls of larger vessels consist of three specialized layers: an intima composed of endothelial cells, a media of SMC and an adventitia of fibroblasts, together with matrix and elastic laminae[110]. SMC and elastic laminae contribute to the vessel tone and mediate the control of vessel diameter and blood flow. So not only endothelium replacement therapy, but also other vessel supporting cells might be a target to improve blood flow after ischemia. Recent reports also suggest that the observed and potential therapeutic effects of MSC are (partly) mediated by paracrine factors secreted by these cells[111-115]. MSC released factors could well play a role in inhibiting fibrosis (scar formation), apoptosis, and enhance angiogenesis[116,117]. These for tissue (re)generation very important roles are summarized as ''the trophic effect of MSC" [117]. A positive role of paracrine factors from MSC on neovascularization has been described by a number of groups. E.g. it was shown that stromal cells from adipose tissue can produce factors, like VEGF and HGF, that induce pro-angiogenic and anti-apoptotic effects of endothelial cells (EC) *in vitro*[111,118]. Furthermore, it was shown that transplantation of human MSC from adipose tissue in an *in vivo* hind limb model in nude mice gives improved neovascularization[119,120].

Combining all features of MSC, cell transplantation with MSC either separately or as a supportive cell fraction, could be considered for neovascularization improving therapy.

Scope of the thesis

The research in this thesis aims to describe which cells contribute to or reflect natural neovascularization capacity and thus might be the best candidates for neovascularization inducing cellular therapy.

Several assays have been developed to measure cells of which the number correlate with vascular diseases. One of which is the so called **colony forming unit-endothelial cells** (CFU-EC)-assay. Numbers of colonies cultured from peripheral blood mononuclear cells correlate with the risk of cardiovascular disease. The characteristics of the clonogenic cell in the **CFU-EC,** however, were not known and in **Chapter 2**, the cells responsible for colony formation are identified. To further investigate the *in vivo* role of these *in vitro* colony forming cells, we first validate the ischemic hind limb model for neovascularization in different mouse strains in **Chapter 3.** Since mouse strains are known to differ in their vascular regeneration capacity, different immune competent mouse strains are tested for the improvement of blood flow after ischemia. In the second part of Chapter 3, we show the *in vivo* effect of the CFU-EC forming cell in neovascularization.

Another relatively newly discovered cell, that has a profound *in vivo* effect on neovascularization, is the **blood outgrowth endothelial cell** (BOEC). Although, it was suggested that the cord blood (CB) BOEC is superior to the peripheral blood (PB) BOEC, systematic comparisons of these two sources were lacking. **Chapter 4** describes *in vitro* studies on the BOEC, generated from mononuclear cells from both cord blood and peripheral blood.

To gain more insight if cells with a an **endothelial progenitor cell** (EPC) phenotype are indeed differentially present in a disease state with chronic as well as acute ischemia, we investigated circulating EPC in the blood of sickle cell disease patients. Sickle cell disease (SCD) is an excellent model, since it is known for its pro-angiogenic state and recurrent vaso-occlusive and thus ischemic events. In **Chapter 5**, therefore, levels of $CD34^+/KDR^+$ circulating endothelial progenitor cells are measured by flow cytometric analysis in SCD patient blood.

Finally, a cell that also has been described to be of influence on vascular growth is the **mesenchymal stem cell**. This cell is known for its regenerating effects in many

disorders although the mechanisms behind these effects are not clearly identified yet. Since the cell is known for its production of many cytokines and growth factors, the paracrine effect of this cell type on endothelium in an angiogenesis setting is reported in **chapter 6**.

Despite the fact that the exact mechanisms behind the neovascularization processes are still unknown, clinical trials involving cell therapy after myocardial infarction are being conducted. In most of these trials **mononuclear cell** fractions are being infused intracoronary after myocardial infarction. **Chapter 7** involves a methodological study that describes the effect of different MNC isolation methods on the quality of the cell transplant, to recognize the best method for a maximal *in vivo* efficacy.

In **Chapter 8,** the findings in this thesis are summarized and future directions for neovascularization inducing cell therapies are given.

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