Supporting cells in neovascularization: study on candidates for cellular therapy
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General discussion
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Monocytes
As was already shown by many studies, monocytes and macrophages that differentiate from monocytes, are very potent in stimulating angiogenesis\textsuperscript{5-8}. This role for monocytes has become evident from models for tumour growth or in inflammatory settings, like rheumatoid arthritis\textsuperscript{9}. Factors like VEGF and IL-8 produced and released by monocytes or the macrophages are mainly responsible for these effects\textsuperscript{5}. In addition, the group of Schaper showed that monocyte depletion in the rabbit hind limb model, decreased collateral formation\textsuperscript{10}. Arras \textit{et al} showed that monocytes accumulate in the perivascular region of newly formed collateral and that monocyte activation is crucial in arteriogenesis\textsuperscript{8}. Monocytes are very easy to isolate, and present in high numbers in peripheral blood. At first sight, monocytes thus seem the ideal candidate for neovascularization therapy. However, one major disadvantage of monocytes must be considered: macrophages, differentiated from monocytes, play a crucial role in the formation of plaques in atherosclerosis\textsuperscript{11}. Therefore, it must be ensured that cell therapy for the treatment of ischemia due to cardiovascular disease should not induce or augment atherosclerosis.

A possible way to circumvent this effect is to ‘skew’ the monocyte away from a pro-atherogenic towards a pro-angiogenic phenotype. Monocytes, in this respect can differentiate in culture towards cells with endothelial like features that improve neovascularization \textit{in vivo}\textsuperscript{12,13}. Most of these studies involve monocytes or adherent peripheral blood mononuclear cells that are cultured for 5-7 days in medium containing serum and high levels of angiogenic growth factors, like VEGF and bFGF\textsuperscript{12-14}. These cells are confusingly called ‘early endothelial progenitor cell (EPC)’ and will be discussed in the next section. Chapter 2 of this thesis describes another monocytic culture assay, that was initially described to be indicative of representing EPC numbers in blood\textsuperscript{4}. In this so called CFU-EC assay, peripheral blood mononuclear cells are cultured for 2 days after which the non-adherent cells are replated and form colonies. We and others found that these colonies have only modest expression of endothelial features, like KDR and CD146 (unpublished data), and are not derived from endothelial progenitor cells, but from hematopoietic cells, and in particular monocytes\textsuperscript{2,15-17}. We also showed that cytokines
derived from activated T-cells induce this colony formation by monocytes. In the stimulating T-cell supernatant, no VEGF was found, while TNF-α, GM-CSF and MCP-1 were present. This supernatant could clearly stimulate monocytes towards a CFU-EC forming phenotype. Interestingly, this T-cell supernatant was derived from T-cells activated via CD3 and CD28, mimicking an inflammatory stimulus via the antigen presentation pathway. In vivo, this pathway is induced by antigens presentation by MHC class II on monocytes to the TCR/CD3 complex on T-cells. Gene expression analysis of the CFU-EC monocytes showed upregulation of genes involved in inflammation (TNF-α, IFNγ), angiogenesis (MMP9, VEGF) and arteriogenesis (MCP-1, PDGF). The fact that numbers of these colonies correlate with disease state in patients with vascular disease suggests that there is a relation between the levels of this CFU-EC, the inflammatory mechanism that is measured in the culture and their suggested role in neovascularization. To investigate this, monocytes stimulated with T-cell supernatant were tested in chapter 3 in an in vivo experiment for neovascularization: the ischemic hind limb model. We observed that ex vivo stimulated monocytes with T-cell supernatant are able to reduce the time necessary for recovery after the induction of ischemia. An additional interesting observation is the fact that monocytes cultured without T-cell cytokines might inhibit (although this did not reach significance) the recovery after ischemia. This underscores the fact that, also detrimental effects of cellular therapy must be thoroughly investigated. In order to optimize CFU like monocyte cultures towards neovascularizing cells, it must be established which cytokines and/or growth factors produced by the T-cells are critical. Preliminary experiments with the TNF-α antibody Remicade, showed that this cytokine plays an important role in the CFU-EC formation. However, addition of TNF-α alone did not induce colony formation, indicating that multiple cytokines and growth factors are involved. A well defined cocktail should stimulate monocyte facilitated neovascularization in patients, but it must be excluded that the infused monocytes will accelerate atherogenesis as well. Preferably, a favourable phenotype needs to be fixed and reversion towards malign characteristics should be prevented. Since only a small number (0.05%) of the cultured monocytes forms a CFU-EC colony, it is a plausible explanation that the colony forming or pro-angiogenic monocytes are derived from a subset of monocytes. Recent papers have introduced the presence of
subpopulations within the circulating monocyte fraction, based on the expression of CD16 and CD1420-22. In preliminary experiments, we have tried to investigate colony formation of this subpopulation. Depletion of CD16+ cells from PBMC did not show any effect on the outgrowth of colonies. In addition, when CD14+/CD16+ cells were sorted and compared to fractions with either CD14+/CD16- cells or the complete CD14+ fraction, no difference was observed. The subpopulation of monocytes that probably forms the CFU-EC, does not reside specifically in the CD16 positive or negative fraction. The nature of the small subsets of monocytes with neovascularization capacity is therefore still elusive.

*Endothelial progenitor cells*

During the period the research in this thesis was performed, the name endothelial progenitor cell has been applied for several cell types in the adult, depicted in figure 1. First, the ‘early EPC’ or ‘endothelial like cell'; this cell appears in short cultures of PB-derived mononuclear cells and is known for its paracrine effect in in vivo neovascularization models23,24. Although it is still under debate, monocyctic/macrophage features have been ascribed to these cells13,14,25. Some overlap is seen between the CFU-EC culture and the early-EPC, the low frequencies of both cells are comparable and they share a common myelo-monocytic origin. However, the culture methods differ substantially. In the early EPC culture, the adherent cells are used, while in the CFU-EC culture the non-adherent cells are used that in the end form strongly adhering colonies. In addition, so far it has never been shown whether the early EPC is able to form CFU-EC, and vice versa.

The other EPC in the adult that can be cultured is the ‘late EPC’ or ‘blood outgrowth endothelial cell (BOEC)’. This cell type has features of real endothelial progenitor cells, namely, it is highly proliferative and can last for a high number of passages in culture. More importantly, the phenotype of these cells strongly resembles mature endothelial cells. This cell is most often cultured from peripheral blood (PB) and cord blood (CB). To obtain these cells, high numbers of freshly isolated mononuclear cells need to be cultured in small volumes. This is an indication that the originating cell of the BOEC comprises a very small percentage of mononuclear cells. E.g. from 160*10^6 MNC 1-2 colonies appear after 3 weeks. For cord blood this frequency is higher and the colonies
appear earlier in culture\textsuperscript{26}. This culture is also highly dependent on culture conditions; e.g. the successful outgrowth of BOEC colonies declines with the time after blood withdrawal (unpublished observation). Thus it remains hard to draw firm conclusions about the quantities of BOEC in the circulation.

BOEC from both CB and PB have been shown to be able to participate in in vivo neovascularization. In order to estimate which BOEC should be used for cellular therapy, a number of assays that test adhesion, migration and homing characteristics were applied on CB and PB- derived BOEC. Although BOEC from CB are shown to be superior in proliferation and the formation of long-lasting vessels\textsuperscript{3}, we found no important significant differences between BOEC from both sources. Moreover, while autologous cord blood is not yet available and will need long term cryopreservation, PB BOEC seem to be a suitable candidate for the induction of vascular repair after ischemia as well.

Cultures of EPC provide information about the proliferation and differentiation capacities of EPC; however, to quantify EPC, flow cytometric analyses would be more useful. Flow cytometric studies have shown correlations for EPC and disease state in patients with vascular diseases\textsuperscript{27-29}. EPC and hematopoietic stem cells share a lot of surface markers like CD34, but EPC express the endothelial marker KDR/VEGFR2. A consensus about the true EPC phenotype is still under debate, but the lack of the more universal and more mature hematopoietic marker CD45 is recently included for EPC. In chapter 6, KDR\textsuperscript{+}/CD34\textsuperscript{+}/CD45\textsuperscript{dim} EPC are measured in patients with sickle cell disease (SCD). Patients that have SCD undergo vaso-occlusive events that are characterized by an increase in angiogenic growth factors, like VEGF and EPO, which in turn are known EPC mobilizers. EPC, in this study defined as hematopoietic stem cells (CD34\textsuperscript{+}/CD45\textsuperscript{dim}) with expression of KDR/VEGFR-2, were found to be elevated during vaso-occlusive events. Although both VEGF and SDF-1 were elevated in patients during vaso-occlusive events, only SDF-1 levels correlated significantly with EPC numbers in these patients. In agreement, SDF-1 has not only been described to be involved in the mobilization, but also in the recruitment and incorporation of endothelial progenitor cells into ischemic sites\textsuperscript{30,31}.

Whether the identity of the cells quantified by flow cytometry is the same as the cells in the BOEC culture should be investigated by cultures of CD34\textsuperscript{-}KDR\textsuperscript{+} cells. In this respect, technical difficulties will remain, since the cell counts are very low (0-14 EPC/ml
peripheral blood in healthy donors), making cell sorting complicated. Timmermans et al were able to sort subpopulations of CD45+/CD34+ cells from bone marrow and cord blood and culture BOEC from these small cell fractions. They concluded that BOEC indeed are derived from CD45−CD34+-cells, and not from CD34+CD133+ cells or CD34+CD45+ cells. We were also able to culture BOEC from CD34 sorted cord blood cells, indicating that the cell, of which the BOEC is originating from, exists within the CD34+ fraction. This is in concordance with the observed correlation between the number of CD34+ cells in the source and the subsequent number of BOEC that can be obtained.

![Diagram of cell types and relationships]

**Figure 1: Endothelial progenitor cells: different cells share the same name**

*Mesenchymal stromal cells*

For cell therapy after myocardial ischemia, also MSC transplantation has been applied several times after myocardial infarction in humans. The exact mechanism is not clear yet; either the tissue regenerating differentiation of MSC into cardio myocytes or the paracrine effect of MSC might contribute to increased tissue repair and cardiac function.
function. Results from several groups have shown that when MSC are transplanted into ischemic hearts, an expression of cardio myocyte specific genes in these cells is observed. Also in vitro, differentiation of MSC towards cardio myocytes has been shown. However, it remains unclear whether the in vivo effects are really dependent on differentiation of the MSC or on cell fusion of the MSC with resident cardio myocytes. In chapter 6 we show that paracrine factors of foetal lung-derived MSC are inducing a pro-angiogenic phenotype of micro vascular endothelial cells. The pro-angiogenic effect from adipose tissue derived MSC and BM derived MSC has been shown by others and is mainly due to the production of VEGF and HGF. Our experiments showed that blocking antibodies against VEGF and HGF inhibited only 50% of the effect of MSC-CM on MVEC proliferation. This indicates that other, still unknown factor(s) are responsible for the effect of MSC CM on endothelial cells.

**Cellular therapy**

Applied medicine has in a way bypassed basic science in the field of regenerative medicine since multiple clinical trials with cellular therapy after myocardial infarction have been started. Recent publications on these trials have described reduced infarction size and improved functional capacities, such as an increased left ventricular ejection fraction, after treatment with mononuclear cells, mostly derived from bone marrow. In these studies, crude preparations of freshly isolated bone marrow mononuclear cells are infus ed intracoronary or within the infarction site. Although only some of the published studies are randomized, controlled trials, the available evidence given in a recently published meta-analysis suggests that BMC transplantation in general is associated with modest improvement in myocardial function. The fact that these cell products contain many different cells such as (hematopoietic) stem cells, EPC, mesenchymal stromal cells and lineage positive cells, make specific conclusions, difficult. In addition to cell composition, even the method used for isolation of the mononuclear cells for cellular therapy is also of importance. The recently finished HEBE trial compares the effects of intracoronary infusion of mononuclear cells from bone marrow
and peripheral blood with controls after myocardial infarction. In chapter 7 it is shown that the recovery and function of cells can be influenced by simple differences in standard isolation methods. Another drawback for firm conclusions is that the reported effects of the already performed trials are only short term and the lack of clear data on the final fate of the administrated cells. It is thus unclear whether the cells actively participate in the neovascularization or that they merely contribute to the process by e.g. production of paracrine factors or induction of an inflammatory or angiogenic response. Because of the unknown mechanisms behind the positive effects, one also has to reckon with possible detrimental effects like fibrosis and other (unknown) complications. Therefore, only when the responsible mechanisms are delineated, regenerative cell therapies can be optimized.

Which cell to use?
As mentioned before, neovascularization is not a simple process. In fact, three types of neovascularization: vasculogenesis, angiogenesis and arteriogenesis with each their own mechanisms, cells and soluble factors need to be considered. To be able to intervene in neovascularization with cell therapy, knowledge concerning all facets from neovascularization is required.
First, endothelial cell growth and proliferation should be stimulated to induce angiogenesis. Since the perfusion of just capillaries is not sufficient, the growth and maturation of vessels (arteriogenesis) should also be induced. For both, monocytes appear to be a suitable candidate since their high numbers in circulation. However, their proatherogenic potential should be kept in mind. Further studies concerning monocyte subsets, their phenotype after culture and long term in vivo effect should be performed. Concerning our approach to use T-cell cytokines in the modulation of monocytes, this could well be optimized by identifying the most optimal (combination of) cytokines. Earlier studies applying only one protein or the gene encoding it, showed no or unwanted systemic side effects51-54.
On the other hand cells just delivering paracrine factors might not be enough while most likely not one single cell type will be able to perform all features of neovascularization. In this respect, cells that are able to facilitate resident vessel growth via paracrine effect and additionally support the host vasculature mechanically might
even be more useful. BOEC and MSC in this respect are structural ‘building’ cells that can be expanded to sufficient numbers. In chapter 4 we showed that BOEC have excellent endothelial features and their expansion rate makes them attractive for use in e.g. tissue engineering or cell transplantation in ischemic tissue. Although PB-BOEC only proliferate extensively up to 12-15 passages, already within 6 passages, enough cells can be generated from 50 ml of peripheral blood to cover sufficient surfaces of vessel wall. Preliminary experiments with BOEC from higher passages showed equal migration capacities and expression of receptors and adhesion molecules. This indicates that a cell, once it starts to proliferate in vivo, can still function for at least a few passages as a normal resident endothelial cell.

In contrast to BOEC, MSC are not suitable for the replacement of endothelial cells, however, they can differentiate into fibroblasts and SMC and participate as mural cells that support the vessel mechanically. Moreover, they produce growth factors (as showed in chapter 6) that stimulate the angiogenic endothelial cell.

A disadvantage of these cells is the need to expand them in long cultures that should be GMP-grade for application in patients. Moreover, the long culture times will limit acute autologous availability.

Although most trials have used crude preparations containing HSC, MSC, EPC some trials have studied expanded MSC or EPC proving their efficacy in some degree. Trials with single cell populations not only advance our knowledge on the cell specific mechanisms but enable also imaging and thus homing studies with prior to infusion labelled cells. Non-invasive imaging has been performed in small studies and overall, the uptake and survival of cells appears to be low, independent on the used cell type or the route of delivery. Again, this demonstrates that the beneficial effect might be mainly due to temporal and paracrine actions of the delivered cells. Ensuring better homing and survival of these cells in vivo are further challenges that are needed for optimization of these therapies.

From bench to bedside?

Autologous cellular therapy appears to be most preferable, circumventing the host defence. A drawback for this approach is that neovascularizing therapy is most often needed for older patients with cardiovascular (related) disease. Moreover, many studies,
showed that in particularly these patients have lower numbers of EPC/ MSC which are also inferior in their function^{1,29,55,56,57}. Thus, when using autologous cells, augmentation of function and cell numbers is the goal of many present studies. A number of novel concepts for cell-enhancement strategies have been described by the group of Dimmeler\textsuperscript{58}. One option could be gene therapy or pre-treatment of e.g. EPC with small molecules. Gene therapy with genes that encode factors that enhance proliferation, like VEGF or telomerase reverse transcriptase, and function like eNOS\textsuperscript{59} has been shown to be possible. Genes encoding for specific pathways important in survival, like the AKT pathway are also interesting targets for cell enhancement\textsuperscript{60,61}. Alternatively, cells for therapy can also be ex vivo pre-treated with small molecules, like statins\textsuperscript{62,63} that activate specific pathways that promote function and survival\textsuperscript{58}. In addition, Zemani \textit{et al} showed that ex vivo pre-treating or “priming” EPC with SDF-1 increased the adhesion to HUVEC, have a greater resistance to shear stress and an enhanced capacity to form tubes in Matrigel. \textit{In vivo} experiments with these cells showed a better ability to incorporate into new vessels in a mouse ischemic hind limb model\textsuperscript{64}. Since EPC can be activated by other factors, such as VEGF, angiopoietin, G-CSF and EPO there are many potential variants of this approach.

Except for the mentioned risk of neovascularizing cells to induce the atherogenic process itself, the potential growth induction of (subclinical) malignancies by these therapies should be evaluated as well.

In summary, cellular therapy is a promising tool for tissue repair after ischemia and damage. After the first promising proof of concept results \textit{in vivo}, thorough (\textit{in vitro}) studies should be performed to design a cell therapy composed of the appropriate cells with the correct phenotype.
References


