Conclusions,
General Discussion
and
Future Directions.

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8.1 Conclusions, general discussion

As described in chapter 1, EPC are bone-marrow derived cells that participate in postnatal neovascularization. Once they are mobilized into the periphery they home towards injured or hypoxic areas to facilitate angiogenesis and vasculogenesis. Postnatally, EPC are needed to maintain the integrity of the endothelium (reendothelialization) and to augment wound healing or to vascularize hypoxic areas (neovascularization). In patients with classical risk factors for ischemic vascular disease, EPC numbers have been shown to be reduced and their function has been shown to be impaired. The work presented in this thesis explored EPC in healthy subjects and focused on their altered properties under hyperglycemic conditions using cells from patients with diabetes mellitus and cells of STZ-induced diabetes in mice.

The nature of EPC

Although various EPC containing cell preparations have already been explored for therapeutic application, to date, EPC are poorly characterized and the nature of the optimal cells for therapy is unknown. As many groups use different methods and surface markers to isolate these cells the studies are hard to compare. Especially for short-term cultured EPC the different protocols use different growth factors and adhesive surfaces. To my opinion, to date there are no distinct markers to identify true EPC, that rule out mature EC and or other myeloid lineage cells like DC and Mph in both human or wild-type mouse. This makes it hard to distinguish and study endothelial progenitors and their sources. For that reason, the data described in chapter 5 are important as they introduce a transgenic mouse model, in which GFP is placed before an EC-specific human eNOS promoter. Although, some groups have described eNOS protein in alveolar lung-derived Mph, we could not show any eNOS expression in mature monocytes, Mph or DC derived from bone marrow cells cultured under various conditions. Therefore, this transgenic mouse model gives us and other researchers a valuable tool to further explore origin and function of BM-derived EPC cultures under different experimental conditions. In chapter 5, mostly BM-derived EPC cultures were studied but when we used the spleen as another source of EPC, we saw that progenitor cells from the different origins reacted different to various growth stimuli suggesting a “different” nature of the EPC-like progenitors in the periphery when compared to those cultured from the progenitors in the BM. This intriguing finding is important and
needs to be taken in consideration when determining which progenitor fraction is best to
use for cell therapies.

Another aspect of EPC that has not been described and understood yet is the differen-
tiation lineage of EPC. The study in chapter 5 shows us that short-term cultured EPC are mainly
derived from a specific myeloid precursor fraction of the BM, with an abundant expression
of both CD31 and Ly6C on their surface. This is in line with several studies that show
monocytic features on the progenitors of EPC \(^4\text{-}^7\). Taken together our data for short-term
mouse bone marrow derived EPC, supports a myeloid transition phase in the differen-
tiation of EPC toward the mature endothelial cell. However, if EPC also can differentiate from
“full” monocytes is not known. In chapter 5 we describe that we failed to culture EPC from
a more mature monocyte population (Ly6C\(^{+}\), CD31\(^{+}\)) of murine BM suggesting that there is
a less mature myeloid progenitor cell that still has the potential to develop into the EC-
lineage. This is somehow unexpected as other groups have shown that circulating human
EPC can be derived from “mature” circulating CD14\(^{+}\) monocytes.

It has to be taken into consideration that Mph and DC can be cultured from the same
myeloid progenitor fraction and similar to EPC, osteoclasts can be solely cultured from this
same fraction (data Leenen \textit{et al}, not published). It is not completely sure if all these
myeloid derived cells are truly derived from the same fraction or if the CD31\(^{-}\)/Ly6C\(^{-}\)
fraction contains different subfractions of progenitors. Mph and DC likely do have the same
progenitor as switching different growth factors \textit{in vitro} can completely shift the fate of the
total population to the desired population of cells\(^8\). Recently, other studies described a
common progenitor for adipocytes and endothelial cells\(^8\), showing an even wider diversity
and plasticity of the differentiation lineage of EPC. These data indicate a tight regulation of
the differentiation of myeloid progenitor cells, a topic that needs to be further explored. In
particular, the use of clonal assays may give answers to the relation of these the myeloid
lineage derived phenotypes.

Using the eNOS-GFP transgenic mouse model, we were able to look at the heterogeneity
between different mouse strains and a marked difference was observed in the capacity of
BM-progenitors from C57BL/6J and FVB mice to differentiate into EPC. In particular in
FVB mice a 40\% increase in the total number of EPC derived after 7 days culture was
shown. Given the fact that FVB mice are much less susceptible for atherosclerosis
compared to C57BL/6J mice, one could speculate about the importance of the capacity to
form EPC in a relative short time. This heterogeneity may also be observed in humans,
however because of a lack of good distinct EPC markers it is hard to reveal this difference.
Function of EPC

EPC have been shown to enhance neovascularization in many preclinical and in vitro studies. In this thesis, a couple of additional insights in the function of EPC are presented. Chapter 2 describes a model that can dissect different mechanisms involved in neovascularization like migration, proliferation, differentiation, stimulation of, and integration in capillary-like structures. With an in vitro, 3-dimensional angiogenesis model we were able to study the effect of different subsets of EPC on neovasculogenesis. Early HSC CD34+ cells were used and compared to cultured EPC, previously shown to be mainly derived from myeloid CD34- fractions. CD34+ cells were shown to home to sites of neovasculogenesis and proliferate at site. Furthermore, a small part of the CD34+ cells did differentiate and incorporate in the mature EC monolayer and these cells were shown to enhance neovasculogenesis. However, CD34+ cells could enhance this neovasculogenesis even further and to a similar extend as cultured EPC indicating that interactions between CD34+ cells and CD34- cells can contribute to stimulation of capillary growth. Many studies have suggested an angiogenic paracrine effect of CD34- cells. Another study describes that incorporation of CD34+CD14+ cells in to capillary-like structures requires co-injection with CD34+ cells, indicating that direct leukocyte-leukocyte interactions may play a critical role in in vivo neovascularization. Studies and models as presented in chapter 2 can give more insight in cell-cell interactions, diverse mechanisms of neovasculogenesis and different functions and capacities of the various subsets of EPC in future experiments.

Data presented in chapter 7 suggest that EPC may also function as cells involved in immune surveillance as gene expression data reveal that early cultured EPC (that express many myeloid cells markers) highly express immunoregulatory genes like C1q when compared to monocytes or mature EC. C1q is a component of the complement system which is designed to allow rapid and efficient activation and clearance of either foreign targets or altered/apoptotic/necrotic cells. Furthermore, C1q has been implicated in preventing autoimmunity and maintaining tolerance by modulating professional phagocytes by for instance regulating IL-12p40 production and reducing NFκB activity. The latter has been studied in immature dendritic cells derived from BM. If the same mechanisms play a part in BM-derived EPC still needs to be further explored. The finding that EPC do produce IL-12p40 (chapter 5) further supports the idea that these cells could regulate immune responses. These responses could imply another important role for EPC in process like wound healing.
**EPC in hyperglycemia**

At the onset of the studies described in this thesis no such thing as EPC dysfunction had been reported. Our hypothesis that EPC might be dysfunctional under hyperglycemic conditions arose from the finding that diabetic conditions could severely impair endothelial function and induce ischemic vascular events. If EPC are really so important in vascular maintenance and repair there could be either less or dysfunctional EPC in Diabetes Mellitus, contributing to the pathogenesis of vascular disease in these patients.

**Effect of hyperglycemia on the number of EPC**

In chapter 3 it is described that we indeed observed a 40% reduction in the circulating number of short-term cultured EPC in patients with type 1 diabetes. This finding was directly related to the severity of the disease as we demonstrated a significant inverse correlation between the number of EPC and glycemic control (HbA1C). Similar results were also found in a hyperglycemic mouse model (chapter 6), further supporting the hypothesis that indeed hyperglycemia could affect EPC numbers. Likewise, Tepper *et al* found a reduced number of EPC in type 2 DM patients, however these patients, next to hyperglycemia, could also have other prevalent risk factors for EPC dysfunction. Many pathophysiological mechanisms could account for this reduction in the number of EPC including impaired mobilization, impaired differentiation, increased apoptosis or cell senescence, reduced adherence capacities or exhausted pools of progenitor cells. Impaired mobilization due to a lack of eNOS has for instance been described. Although, no reduction in the total number of circulating CD34+ cells in diabetes patients was observed, a closer look at the distribution of different subsets of CD34+ cells (KDR+) might provide more insight in this matter. Altered differentiation could well be a mechanism involved in lower numbers of EPC as chapter 6 describes a possible skew of BM myeloid progenitors towards the macrophage lineage. Next to the reduced number of EPC, we observed an increased number of Mph that also related to the glycemic control of the mice. We speculate that the altered milieu in diabetes not only directs differentiation of progenitor cells towards Mph but that it does so at the cost of the generation of EPC.

**Effect of hyperglycemia on the function of EPC**

Chapter 3 not only describes a reduced number of EPC in type-1 DM patients but also that EPC isolated from the diabetes patients have an impaired angiogenic capacity compared to healthy control subjects. A similar observation in STZ-induced diabetic mice is described in
chapter 6 also revealing an impaired paracrine angiogenic function of hyperglycemic EPC. In addition, these dysfunctional EPC also display higher pro-inflammatory capacities (chapter 6). By using gene expression profiles (chapter 4), we show that human EPC from type 1 DM patients upregulate numerous pro-inflammatory genes known to be associated with hyperglycemia and oxidative stress. The pro-inflammatory nature of EPC in type 1 DM patients is a concern as they may contribute to an adverse (immune) response that may be pro-atherogenic and contribute to progressive ischemic vascular disease. This may be particularly relevant when using autologous EPC for therapeutic purposes. EPC are thought to function in sites of ischemia or reperfused tissue, which can be characterized as an inflammatory site. Inflammatory sites are known to be a high oxidative stress environment, which could be another mechanism for EPC dysfunction as described in chapter 420. Given the central role of oxidative stress in type 1 diabetes, oxidative stress or altered redox signaling may also directly affect the survival, differentiation and function of EPC. As described in chapter 4, another potential mechanism for altered EPC function is the hyperglycemia associated formation of intra- and extra-cellular advanced glycation endproducts (AGE). Differential gene expression profiles support these hypotheses as we observed a striking number of genes upregulated in the EPC from type 1 DM patients that have been reported to be associated with diabetes mellitus in general, with hyperglycemia, oxidative stress or AGE formation. It is somehow remarkable that these different gene profiles, and consequently altered functional behavior, of EPC derived from either diabetic patients or hyperglycemic mice are found as these EPC are cultured for either 4 (chapter 3, 4 and 7) or 7 days (chapter 6) under normoglycemic conditions. So somehow EPC are “biosensors” that translate metabolic cues into altered gene expression partially imprinted for at least a week.

EPC in therapeutic applications.

Since the discovery of EPC, the cells have been of great interest as therapeutic entities. As mobilization of progenitor cells after injury, an important natural response to vascular injuries21-23, did correlate with the long term outcome of cardiovascular disease24 autologous cell transplantations became relevant. Over the last years, many promising preclinical studies have shown evidence that transplantation of different angiogenic cell populations did indeed contribute to repair of vascular injuries25,26. A series of clinical pilot studies, using cell transplantation in vascular diseases yield promising data27-29, however some larger trails and longer follow-up studies did not reveal any benefits30 or some very
minor benefits as discussed in chapter 1. It needs to be pointed out that treatments of myocardial infarction were less promising than treatments of in critical ischemia of extremities\textsuperscript{28,31}. So far, most studies have mainly focused on unselected mononuclear cell fractions of bone marrow or peripheral blood. A recent clinical study showed that intracoronary administration of early hematopoietic CD133\textsuperscript{+} BM derived cells in patients with recent myocardial infarction was associated with increased incidence of coronary events\textsuperscript{32}. So although cell transplantation was associated with improved heart function there were unexpected side effects when using these early progenitor cells. These and other discouraging outcomes sharpen the field of progenitor cell use for vascular therapy a bit and make researchers think about major questions like: what subset of cells should we transplant, an early progenitor or a further differentiated and maybe more dedicated cell? And what are the exact mechanisms these cells work by? To my opinion these questions have to be addressed first before further clinical trials can be initiated.

The work presented in this thesis gives insight into some of these questions. Chapter 5 shows a BM specific fraction (CD31\textsuperscript{+}/Ly6C\textsuperscript{+}) from which EPC could be derived but it also reveals a very high plasticity in this precursor fraction. This high plasticity of early progenitor cells could be a problem in cell transplantation therapies in general as precursors could differentiate to unwanted phenotypes depending on local differentiation/growth factors and inflict unwanted side-effects.

Chapters 3 and 6 show EPC dysfunction in type 1 diabetes patients and in hyperglycemic mice respectively. These EPC dispose dysfunctional properties in their angiogenic capacities (chapter 3 and 6) but they also reveal a higher inflammatory phenotype (chapter 6). Furthermore, our data implicate a possible skew of myeloid progenitors towards macrophage-like phenotypes. It can not be excluded that transplantation of these autologous myeloid progenitor cells would inflict adverse side effects. Chapter 6 and 7 suggest possible mechanisms to (partially) overcome these effects with statin interventions (Chapter 6, \textit{in vitro}) or with PPAR\textgamma agonist interventions (chapter 7, \textit{in vivo}). HMG-CoA reductase inhibitors have been shown to increase the number of circulating EPC\textsuperscript{19,33,34} and they have been shown to generate potent anti-inflammatory actions\textsuperscript{35} and can improve properties of dysfunctional EPC populations \textit{in vitro}\textsuperscript{19,34,36}. In addition, statins were shown to reduce the \textit{in vitro} differentiation of monocytes to Mph\textsuperscript{37}. Indeed we find that \textit{in vitro} addition of atorvastatin (chapter 6) increases the number of EPC and conversely decreases the number of macrophages (\textit{in vitro}). Statins could also recover the altered angiogenic properties of the EPC cell population derived from hyperglycemic mice.
Short treatment of diabetic patients with PPARγ agonists do show transrepression of many genes involved in inflammation, which might be another option to decrease the inflammatory phenotype of EPC.

Optimal treatment of diabetic patients to overcome EPC dysfunction and its side effects would still first be controlling the HbA1C levels of the patients and secondly find adjunctive therapy like statin/PPARγ agonist interventions to improve EPC functions. Recently an interesting paper appeared discussing the potential to augment experimentally-induced ischemia of peripheral CD34+ - and monocytic-like CD14+ endothelial cell progenitors in diabetes. The same group had found before that CD34+ cells were dysfunctional in diabetic conditions and they claim in that CD14+ cells are less affected by diabetes when compared to more primitive CD34+ cells. The group suggests that CD14+ cells could provide a therapeutic option for people with diabetes, as these cells improve wound healing and vascular growth. This thesis shows that CD14+ cells are definitely affected in patients with diabetes and that autologous cells could only be used for transplantation if these progenitors could somehow be converted from pro-inflammatory phenotype to a pro-angiogenic phenotype.

8.2 Future directions

A couple of suggestions for future directions have already been made including better characterization of true EPC and their progenitors. Clonal assays will be needed to answer questions about precursor subsets. The proposed mouse model (chapter 5) could help exploring these fields. Another interesting concept is skew of progenitor cells under adverse metabolic conditions. It would be very interesting to see if this observation can be confirmed in patients?

Recently microRNAs have been implicated in the regulation of hematopoietic lineage development. MicroRNAs might be candidates to direct EPC and macrophage differentiation. It would be very interesting to elucidate whether microRNAs are indeed involved in these processes and to see if these microRNAs are altered under hyperglycemic conditions. If so, microRNA profiling of circulating progenitor populations could provide a potent means to monitor the susceptibility of patients to hemodynamic and metabolic risk
factors. Finally, future research on the reversal of EPC dysfunction needs to be directed towards interventions that convert pro-inflammatory phenotypes towards pro-angiogenic phenotypes.
References


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