Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

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Summary / discussion

An adagium to treat patients with critical limb ischemia in whom conventional revascularization therapy fails is therapeutic stimulation of collateral artery growth. Insight in molecular and cellular mechanisms of revascularization is necessary for development and refinement of therapeutic approaches for this category of patients. This thesis addresses several issues on molecular and cellular mechanisms, which comprise the involvement of leukocyte subsets in revascularization. It also addresses other factors that play a role in revascularization such as vascular growth factors, post-transcriptional regulation, and receptors involved in growth factor signaling.

In the past two decades knowledge on the involvement of the immune system in revascularization, especially arteriogenesis, has substantially increased. Different leukocyte subsets have been demonstrated to play a modulating role in arteriogenesis in experimental animal models. With the great variety of involved, leukocyte subsets a large 'toolbox' is at hand for the stimulation of arteriogenesis.

Chapter 2 provides an overview of the leukocyte subsets involved in arteriogenesis and illustrates a role for leukocytes from both the innate and adaptive immune system. Although the role of many of these cells have been studied individually [1-5], it is suggested that these cells are in fact team players. Cross talking has been reported between monocytes and T lymphocytes [6, 7] and it is most likely that this also occurs between other leukocyte subsets. Despite promising results from experimental studies, none of these leukocytes subsets have been applied in a clinical setting yet. Further insight in cellular and molecular mechanisms is necessary before these individual cells can be specifically applied in a clinical setting. *In vitro* and *in vivo* studies for more in depth assessment of the leukocyte involvement could provide this information.

To study the issues covered in this thesis, an experimental mouse model mimicking peripheral arterial obstructive disease was used. This so called mouse hind limb ischemia model has been used by many research groups in the field of arteriogenesis, and different surgical approaches to induce ischemia in the hind limb have been described. The effect of these different approaches and level of vascular occlusion was compared with respect to blood flow recovery, collateral artery formation and capillary formation in the ischemic hind limb in **chapter 3**. It was demonstrated that the extent of the arterial defect (single ligation of artery, total excision of artery or double ligation of artery) has consequences for the pattern of blood flow restoration, while the level of vascular occlusion (femoral or iliac) does not.

In this thesis the single femoral artery ligation approach was chosen because blood flow is effectively disrupted while all connections of the pre-existing collateral artery side branches are left intact. This allows assessment of collateral artery growth from pre-existing arterioles from a physiologic perspective. Variation in these collateral side branches have been reported between mouse strains, with significant impact on blood flow recovery [8, 9].

Besides this variation in collateral side branches, differences in immune bias were demonstrated to account for differences in blood flow recovery between C57BL/6 and BALB/c mice in **chapter 4**. This was supported by differences in lymphocyte accumulation around collateral arteries between these two strains. Subsequently, it was hypothesized that lymphocytes play a role in arteriogenesis, which was impaired in mice depleted or deficient for natural killer (NK) cells, but not in mice deficient for natural killer T (NKT) cells. Furthermore, arteriogenesis was impaired in mice lacking CD4+ T cells. Both NK and CD4+ T cells were demonstrated to accumulate around collateral arteries, and secrete a variety of inflammatory cytokines. Future studies should focus on the local effector function of these cells and their cytokines in arteriogenesis.

The different immune bias between C57BL/6 and BALB/c mice comprises a difference in T cell mediated immunity, associated with differences in vascular remodeling [10], and genetic differences in the Natural Killer gene complex (NKC), especially in the Ly49 NK cell receptor family [11, 12]. In C57BL/6xBALB/c F1 generation mice a dominant role for C57BL/6 genes in arteriogenesis was indicated, since poor collateral artery growth of BALB/c mice was completely abolished and similar to the rapid arteriogenesis of the C57BL/6 parent strain.

The modulating role of C57BL/6 NK cells in arteriogenesis and the immune genetic differences in NKC between C57BL/6 and BALB/c mice prompted us to study the effect of the strain dependent NKC differences on arteriogenesis in **chapter 5,** and on general vascular remodeling in **chapter 6**.

In **chapter 5** it was demonstrated that differences in NKC between C57BL/6 and BALB/c are linked to strain dependent differences in blood flow recovery. BALB/c mice congenic for the entire C57BL/6 NKC, BALB.B6-CMV1^{r} mice (CMV1^{r}) [13], displayed substantial improvement of their naturally poor arteriogenic phenotype towards the rapid arteriogenic response of C57BL/6 mice. Collateral artery diameters were significantly increased in these congenic mice. Subsequent NK cell depletion in CMV1^{r} mice resulted in impaired blood flow recovery and indicates that at least part of the improved arteriogenesis in these mice can be explained by a NK cell related factor that is present within the C57BL/6 NKC, but absent from the BALB/c NKC.

In addition, NKC differences between C57BL/6 and BALB/c mice could be translated into different NK cell functionality and responsiveness, since *in vitro* NK cell stimulation displayed significant greater responsiveness of NK cells from C57BL/6 and congenic CMV1^{r} mice compared to BALB/c derived NK cells. This observation provides a possible mechanism for how the NKC differences contribute to different blood flow recovery.

In **chapter 6** we wanted to demonstrate the role of NK cells in vascular remodeling in general. In particular, we wanted to study the effect of strain dependent differences in the Natural Killer gene complex on vascular remodeling in general. The depletion of

NK cells resulted in reduced intimal hyperplasia after non-constrictive cuff placement around the femoral artery in C57BL/6 mice, indicating that NK cells plays a modulating role in general vascular remodeling. Cuff placement and vein grafting in C57BL/6, BALB/c mice and in BALB/c mice congenic for the C57BL/6 NKC (CMV1^r mice) resulted in profound intimal hyperplasia in C57BL/6 mice, but also in the congenic CMV1^r mice, whereas this was significantly reduced in BALB/c mice. This indicates that strain dependent NKC differences result in different vascular remodeling. Assessment of leukocyte accumulation in vein graft segments revealed large numbers of leukocytes in both C57BL/6 and CMV1^r mice, but not in BALB/c mice. It suggests that strain dependent NKC differences induced a different inflammatory reaction and subsequently different vascular remodeling between these mice. This is supported by the differences in NK cell responsiveness as shown in the previous chapter. The availability of intra-NKC congenic mouse strains [14] allows further assessment of this gene locus to identify genes or key receptors, especially in the Ly49 NK cell receptor family, that are involved in modulating NK cell function during vascular remodeling.

To translate this into the human situation it should be realized that humans do not possess functional Ly49 receptor genes, but use instead the killer Ig-like receptor family (KIR) [15], which also has large genetic diversity [16]. Impact of this divergence on collateral formation in humans is not known yet, but may be suggested since a recent study linked several single nucleotide polymorphisms (SNPs) in inflammatory response related genes with the formation of coronary collateral arteries [17]. Assessment of the immune system functionality and immune responsiveness in the individual patient may enhance developing strategies for risk stratification and therapeutic stimulation of revascularization.

Besides inflammatory cells, growth factors are another potential category for therapeutic revascularization. The application of growth factors to stimulate revascularization has been studied in several animal models, pre-clinical and clinical trials [18]. Although the use of growth factors has resulted in a successful induction of arteriogenesis in many studies, the success rate in the clinical trials was not that obvious. Many studies do show effects, but often have been debated either because the effects were only seen on secondary endpoints, or due to effects that were also observed (partially) in the placebo groups. Therefore more studies are needed to augment the efficacy and safety of these growth factors in stimulating revascularization and to come to a good interpretation and understanding of these mixed results [19, 20].

One explanation for these mixed results might be the differential expression of angiogenic growth factors by ischemic tissue, which in patients seemed to depend on the duration of hypoxia. In particular concerning the expression of hypoxia inducible pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), stromal derived

factor-1 (SDF-1), and its chemokine receptor CXCR4. In **chapter 7** ischemic muscle tissue from patients with acute-on-chronic ischemia showed increased expression of VEGF, SDF-1 and CXCR4, whereas expression of these growth factors was seized to baseline levels in chronic ischemic muscle tissue. This may be explained by an inability of hypoxic tissues to sufficiently express the transcription factor hypoxia inducible factor 1α (HIF-1α) in chronic ischemia. Another important finding in this study was the pivotal role of SDF-1 in retention of bone-marrow-derived cells in hypoxic tissues.

The inability of growth factor production by chronic ischemic muscle tissue might indicate that in a chronic hypoxic state a new equilibrium is created, which might require a different approach when stimulating revascularization in patients suffering from chronic ischemia. As a suggestion for therapeutic optimization, growth factor treatment should always be combined with exercise training to mimic acute over chronic ischemia.

To gain more insight in complex signaling of vascular growth factors, research has recently focused on modulation of gene expression and translation by small interfering RNA molecules called microRNA (miR). These miRs are demonstrated to regulate angiogenic cell properties [21] by inhibiting translation of protein from messengerRNA (mRNA) or by promoting degradation of mRNA [22]. In **chapter 8** it is demonstrated that a highly endothelial specific miR, miR-126 [23], had pro-angiogenic properties by facilitating ischemia induced capillary angiogenesis. Specific silencing of miR-126 induced impaired capillary angiogenesis in the ischemic hind limb after femoral artery ligation in C57BL/6 mice. It is suggested that miR-126 facilitates angiogenesis by reducing repression of VEGF mediated signaling. The ability of miRs to regulate multiple targets [24, 25] provides a means for coordinated control of gene expression, while also making them especially attractive candidates for regulating both cell-type specific differentiation and modulation of cell function. The highly specific expression profiles for miRs provide a promising therapeutic approach with low risk for adverse side effects to occur. With this in mind, it might even be possible to augment impaired functionality of bone marrow, immune, or endothelial cells.

Endothelial cells play an important role in both arterio- and angiogenesis, for example by expressing target receptors through which cytokines and growth factors can modulate endothelial cell properties during revascularization. Transforming growth factor-beta (TGF-β) is such a cytokine with the ability to modulate endothelial cell properties during revascularization, and was shown to improve collateral artery growth [26]. TGF-β signals through several TGF-β receptors, such as activin receptor-like kinase 1 (ALK1) and the accessory receptor endoglin [27, 28]. For both these receptors an active role is suggested in shear stress driven vascular adaptation [29, 30]. **Chapter 9** elucidates a differential role for endoglin and ALK1 in shear-stress-induced collateral artery growth and ischemia-induced capillary angiogenesis, which was studied in mice haplo-insufficient for either

endoglin or ALK1. Haplo-insufficiency for endoglin revealed that endoglin modulates both shear-stress-induced collateral artery growth and ischemia-induced capillary angiogenesis, whereas haplo-insufficiency for ALK1 only resulted in disturbed ischemia-induced capillary angiogenesis by the formation of dysplastic capillaries. The differential role of endoglin and ALK1 in shear-stress-driven arteriogenesis is confirmed by increased endoglin mRNA levels, but not ALK1, in murine embryonic endothelial cells exposed to shear stress *in vitro*. Dysfunction of endothelial cells by for example haplo-insufficiency for these TGF-β receptors, may explain why growth factor stimulated revascularization was impaired in selected cases.

Conclusion

Revascularization is a multi-factorial process which provides a wide range of possible therapeutic targets to stimulate revascularization. By the rapid development in this research area many new therapeutic approaches are explored in a translational research setting. Because of the wide range of therapeutic approaches, stratification of the disease profile of each individual patient is recommended, which should include assessment of vascular risk factors and assessment of the functionality of endothelial cells, bone marrow, and of the immune system. By this assessment a tailor made treatment can be provided in the future, supporting the patient where it is necessary.

This thesis provides a role for multiple leukocytes in blood flow recovery, in particular Natural Killer cells and CD4+ T lymphocytes, and shows that genetic differences in the Natural Killer gene complex induce differences in vascular remodeling. It was also shown how growth factor expression, signaling and regulation of gene expression modulate revascularization.

Further research is required to gain more insight in these molecular and cellular mechanisms and interactions that play a role in revascularization. In particular, future research should focus on inter-individual differences in inflammatory response, and corresponding differences in the composition and condition of circulating leukocyte populations. Cooperation between clinicians and basic scientists is essential to finally bring new therapeutic approaches successfully from bench to bedside.

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