Inflammatory Processes during Arteriogenesis

Daphne de Groot

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Inflammatory Processes during Arteriogenesis

The Contribution of the Innate Immune System to Collateral Artery Growth

Inflammatoire processen tijdens arteriogenese

De bijdrage van het aangeboren immuunsysteem aan collaterale vaatgroei

(met samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 21 juni 2011 des middags te 2.30 uur

door

Daphne de Groot, geboren op 29 augustus 1982 te Hoorn

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Introduction and Outline

INTRODUCTION

From 2010 on, it is predicted that cardiovascular disease is the leading cause of death worldwide and kills over 16 million people each year (source WHO). Most cardiovascular events are secondary to atherosclerosis. This inflammatory disease of the arterial wall leads to plaque formation in middle and large arteries [1]. Clinical manifestation of the disease occurs when the plaque destabilizes and causes a lumen reduction to such extent that insufficient blood can pass [2]. This can either be due to a narrowing of the artery at the height of the plaque itself, or secondary to an embolus traveling distally from the affected vessel. The resulting reduction in oxygen and nutrient supply will first lead to a loss of function and ultimately result in tissue death. Clinically, the latter is known as an infarct.

In the treatment of atherosclerosis, there are two main goals. First of all, to slow down the progression of the disease, which is achieved by medication (e.g. statins, β-blockers, ACE-inhibitors) and lifestyle modifications (e.g. stop smoking, weight loss). The second focus is on the prevention of complication of the already present disease. Although the latter also makes use of medication (e.g. anti-coagulant treatment), the primary goal here is to restore the perfusion in tissues acutely endangered by ischemia. Revascularization can be achieved by invasive techniques like percutaneous transluminal angioplasty (PTCA, with or without stent placement) or bypass surgery. These interventions relieve problems instantly, but they are not without risks and complications. Furthermore, it is estimated that one out of five patients does not qualify for these invasive treatment modalities [3]. Therefore, less invasive, alternative options are an important object of research.

Collateral arteries, the natural bypasses

The human body also has a rescue mechanism to protect itself from ischemia. It is capable of developing new blood vessels, thereby maintaining tissue perfusion. In the adult, there are two important forms of blood vessel growth: angiogenesis and arteriogenesis [4, 5]. Angiogenesis comprises of the *de novo* sprouting of blood vessels, which most often do not grow beyond capillary size. Arteriogenesis, on the other hand, describes the growth of larger arteries [6]. These arteries can only develop from pre-existing arteriolar connections (anastomoses), which were already constructed during the embryonic phase. Their presence in the coronary circulation was demonstrated for the first time by Fulton in 1963 [7]. They can, however, be found in all vascular territories in the body and exhibit the same morphology irrespective of their location. They remain dormant, until they are recruited and remodeled into functional collateral arteries. This process is therefore also called 'collateral artery growth'.

Although capillary sprouting may deliver some relieve to an underperfused region, the growth of the collateral arteries has been postulated as the most important mechanism to compensate for bulk perfusion loss. This is due to their large size. Following the Hagen-Poiseuille law of fluid dynamics, defining blood flow as a function of the vessel diameter to the 4th power, increasing the size of a blood vessel is far more efficient in enhancing blood flow, than increasing the number.

Since collateral arteries seem to have the ability to function as natural bypasses, they are a potential candidate for the treatment of patients suffering of vascular occlusive disease and more knowledge is needed on how they develop.

Driving forces in arteriogenesis

Although angiogenesis is ischemia-driven, for the initiation of arteriogenesis hypoxia is not required [8]. It can take place in normoxic environments, distant from the ischemic regions. This was nicely shown in rabbit experiments, in which the femoral artery was occluded. The occlusion resulted in transient ischemia only in the lower leg, where angiogenesis took place. Arteriogenesis, however, was observed in the upper thigh, where no signs of ischemia could be found [9, 10].

Early on, it has been postulated that biomechanical rather than biochemical changes are the initiating force in collateral growth [11]. In all healthy arteries, an increase in blood flow results in an enlargement of the arterial diameter by outward remodeling, while in absence of flow a regression is seen [12]. This also applies to the pre-existing anastomoses, which run between arterial territories. Normally, there is no flow through them, because the blood follows the path of least resistance. Progressive lumen narrowing of an original artery leads to a drop in its distal blood pressure, generating a pressure gradient over the pre-existing anastomoses. When this gradient is steep enough, the high vascular resistance of the anastomoses is overcome and blood starts flowing through them. The changes in mechanical forces acting on the endothelial cells lining the premature arteries will give rise to the inflammatory signals needed for the start of the collateral growth [13].

Arteriogenesis and inflammation

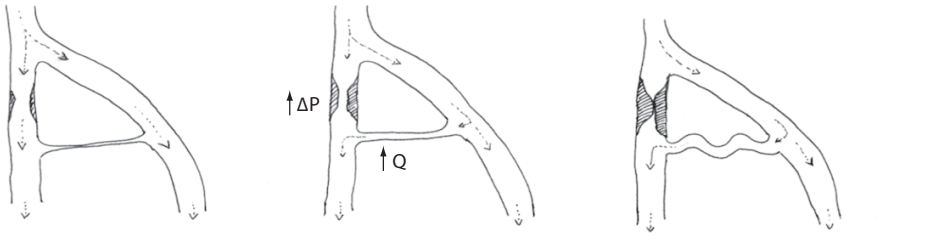
In response to the alteration in shear stress (a dragging force perpendicular to the vascular wall) and cyclic stretch (an outward directed force), the endothelial cells change their gene expression. This results in an upregulation of adhesion molecules on their surface [14], facilitating the entrapment of inflammatory cells from the blood stream. Simultaneous excretion of chemokines leads to the recruitment of leukocytes to the site of collateral growth.

Although lymphocytes play a role in this process [15], the monocyte deserves special attention. Already in 1976, they were identified in developing collateral arteries [11]. Over the years, their importance in arteriogenesis was emphasized by numerous papers. These studies provided evidence that peripheral blood monocyte counts [16-18], increased monocyte survival (e.g. granulocyte monocyte- colony stimulating factor [19, 20]), as well as enhanced attraction or adhesion (e.g. monocyte chemoattractant protein-1 [21], tumor necrosis factor–α [22], intracellular adhesion molecule-1 [23]) of these cells correlate directly with arteriogenic responses to restore perfusion.

$$\frac{8\mu L\boldsymbol{Q}}{\pi\boldsymbol{r^4}} = \Delta\boldsymbol{P} \quad or \quad \frac{\pi\boldsymbol{r^4}\Delta\boldsymbol{P}}{8\mu} = \boldsymbol{Q}$$

The Hagen-Poiseuille law of fluid dynamics

*From this law, fluid dynamics can be calculated. μ is the viscosity of the fluid, L is the length of the tube, **Q** is the flow, π is a mathematical constant, r is the radius of the tube and **Δ P** is the pressure difference. For a blood vessel, μ, L and π are constant, so only the blood flow (Q), the pressure drop (Δ P) and the radius of the vessel (r4) influence each other. The diameter of a blood vessel therefore has a large impact on tissue perfusion, because a 2x bigger artery transports 16 times more blood and a 4x bigger vessel even 256 times more! This is why collateral arteries give rise to large improvements in perfusion, while capillaries do not.*



Arteriogenesis takes place in response to flow and pressure changes
Blood flow (Q) always chooses the path of least resistance. Normally, the vascular anastomoses lay dormant, since their resistance is higher than the naïve blood vessel. The moment a significant stenosis develops, the blood pressure drops distally to the occlusion. Following this pressure gradient (ΔP), blood starts flowing through the pre-existing anastomosis, activating the endothelial cells lining them. The collaterals actively remodel to increase their size. The flow increases, lowering the pressure gradient, till this reaches an equilibrium. When a collateral artery is fully developed, it can de capable of taking over the perfusion of a entire arterial bed.

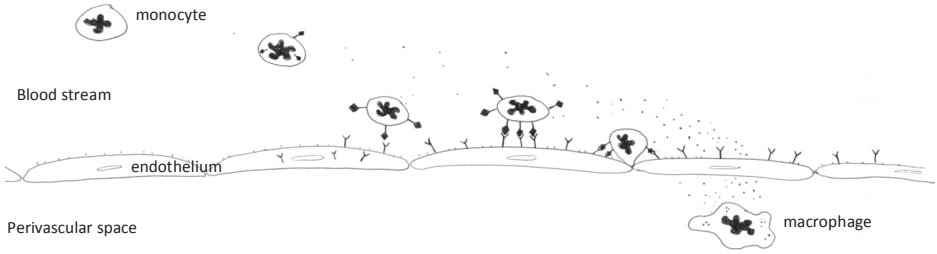
When the monocytes reach the premature collateral, they adhere to the activated endothelium and migrate into the perivascular space. Here, the monocytes mature into macrophages. These cells function as small factories, producing large amounts of cytokines, chemokines and growth factors, that, in an autocrine fashion, attract even more monocytes to the location. Furthermore, they locally promote active proliferation of endothelial and smooth muscle cells and provide the necessary space by extracellular matrix degradation.

Stimulation of arteriogenesis

The benefits of a well developed collateral circulation are widely acknowledged. A functional collateral network is associated with a reduced long-term mortality in coronary artery disease patients [24]. In an experimental setup it was shown that, in about one third of the coronary artery disease patients and in on fifth of the healthy controls, functional collaterals were already present in the heart. These collaterals were sufficient to prevent ischemic changes in the myocardium during brief balloon occlusions [25]. The remainder of the patients, however, will need collateral growth to protect them from ischemic complications.

The sheer number of patients still suffering from atherosclerosis related symptoms, such as angina pectoris and intermittent claudication, indicate that there are frequent shortcomings in the adaptive remodeling of the collateral circulation. In these patients, enhancement of collateral growth by therapeutic stimulation would be beneficial. It has been demonstrated in experimental models, that the stimulation of collateral artery growth is feasible and several factors influencing monocyte and endothelial function yielded positive results.

One of the most potent factors to promote arteriogenesis is monocyte chemoattractant protein-1 (MCP-1). Local infusion of this chemokine in developing collaterals of rabbits resulted in a significant enhancement of collateral growth 26 and similar results were obtained in a larger animal model [27-29]. The success depended on the attraction of more monocytes to the collateral arteries. MCP-1 has the potential to destabilize atherosclerotic plaques [30], but the effect of very



Monocyte migration from the blood stream into the perivascular space
During collateral artery growth, the migration of monocytes form the blood to the tissue surrounding the developing collateral arteries is extremely important. After activation of the endothelial cells lining the anastomoses and the circulating monocytes, adhesion receptors are upregulated on both their cellular surfaces. These adhesion receptors facilitate the entrapment of the monocytes on the blood vessel wall. Only after firm adhesion, the cells will migrates through the endothelium. Ones in the tissue the monocytes develop into macrophages and start producing various factors, which are beneficial for collateral growth.

local application on plaque progression elsewhere in the body was still unknown. Van Royen et al tested this in ApoE-/- mice, which are mice who easily develop atherosclerotic plaques. In these animals, systemic activation of the monocytes did occur and increased neo-intima formation as well as negative plaque changes were seen [31]. Therefore MCP-1 was considered not a good candidate for therapeutic stimulation.
Other factors did, however, show more promising data. In a pre-clinical setting was shown that local infusion of transforming growth factor- beta 1 (TGF-ß1) strongly stimulated collateral development [32]. This pluripotent cytokine is highly expressed in areas of collateral growth. It has an effect on endothelial and smooth muscle cells, but also attracts and activates monocytes. Interestingly, it is suggested that TGF-ß also has atheroprotective properties [33, 34], although this is still controversially discussed in literature.
Colony stimulation factors, like granulocyte- monocyte colony stimulation factor (GM-CSF) and granulocyte stimulating factor (G-CSF) also exerted positive effects on collateral artery growth, in both mouse, rat and pig models [19, 35-37]. Although their mode of action in stimulating arteriogenesis was not completely clear, their successes were appointed to the enhanced mobilization of (inflammatory) progenitor cells from the bone marrow and the prolongation of monocyte survival. The administration of G-CSF not only resulted in more arteriogenesis, it was also found to reduce the damage after myocardial infarction [38-40]. Recombinant forms of GM-CFS were already available and listed for clinical application in other types of disease [37, 41]. This facilitates an easy transition from animals to clinical studies.

Current perspectives

Clinical studies were initiated after the promising results obtained from intervention studies in the animals models. Initially, the smaller, non-randomized, trials supported the animal experimental data [42, 43]. However, larger randomized trials failed to show any effect on collateral growth [44, 45]. Some of these trails even had to be stopped due to serious safety concerns [46, 47].

Stimulation of arteriogenesis is prone to suffer from unwanted side effects. Both arteriogenesis and atherosclerosis are accelerated by endothelial activation, the upregulation of adhesion molecules on endothelial and circulating cells and the infiltration of monocytes into the vascular wall. Although beneficial in arteriogenesis, these effects are considered detrimental and destabilizing in atherosclerotic disease. Since most compounds tested for the stimulation of arteriogenesis influence one of these parameters, an aggravation of the atherosclerosis is frequently observed. Thus, therepeutical strategies to stimulate arteriogenesis are in fact a double edged sword, also referred as a "Janus phenomenon", after the two faced roman god [48]. However, although sharing many pathogenetic features, arteriogenesis and atherosclerosis are not identical processes. The responses of endothelial and inflammatory cells proceed in a temporal pattern, with specific chemokine and cytokine profiles. Insight into the subtle differences between the two pathogenetic mechanisms over time is necessary in the discovery of new therapeutic strategies to stimulate arteriogenesis without unwanted side effects like plaque destabilisation To fully understand both processes in depth, more exploratory research is required. In addition, significant efforts should be made to test the potential candidates for intervention in appropriate models that resemble the human situation best. Most models of arteriogenesis include acute ischemia in hind limbs while in the clinical situation gradual occlusion takes place within other organ systems that require more aggressive clinical treatment, e.g. the coronary circulation.

OUTLINE OF THIS THESIS

The central theme of the work presented in this thesis is restoration of tissue perfusion by collateral artery growth. The thesis starts with a short review of the current knowledge on the effects of classic cardiovascular risk factors on collateral artery growth (**chapter 2**).
In chapters 3- 5, we explore the role of the innate immune system in collateral artery growth. **Chapter 3** describes the involvement of TLR 2 and TLR 4 in arteriogenesis. In **chapter 4**, we focus on the regulation of collateral artery growth by NF-κB. In **chapter 5**, we look at one of the known endogenous ligand of TLR 2 and 4, Extra Domain A of fibronectin (EDA) and its role in collateral artery growth.
Chapters 6 and 7 focus on improving the translation of experimental data from bench to bedside: In **chapter 6**, we investigate the effects of aging on arteriogenesis. In **chapter 7**, we present a new porcine model for coronary collateral artery growth, which closely reflects the human situation. In **chapter 8**, we use this model to characterize the effects of Celecoxib, a clinically available selective COX-2 inhibitor, on coronary collateral artery growth.

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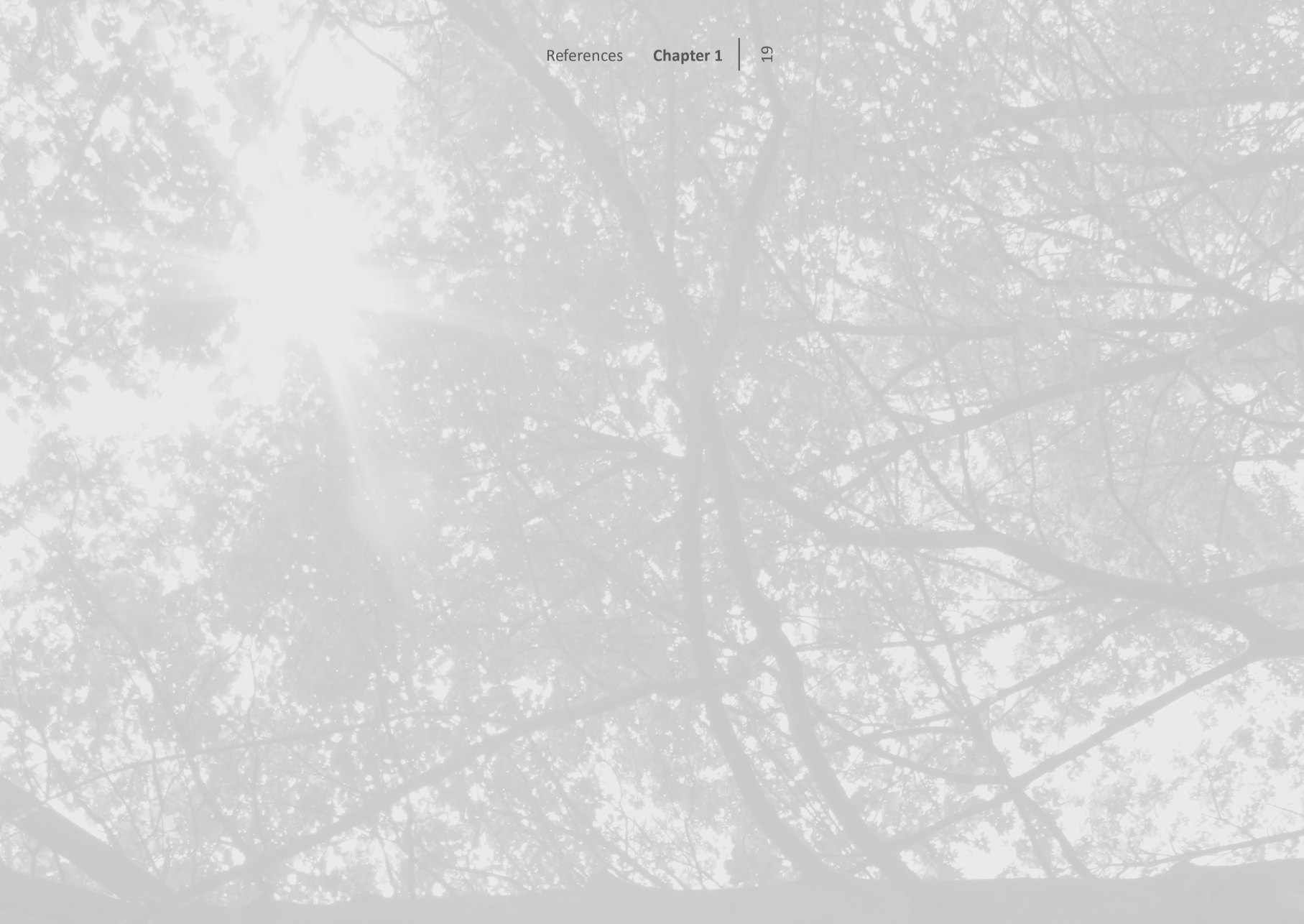

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Cardiovascular risk factors and collateral artery formation

ABSTRACT

Arterial lumen narrowing and vascular occlusion is the actual cause of morbidity and mortality in atherosclerotic disease. Collateral artery formation (arteriogenesis) refers to an active remodeling of non-functional vascular anastomoses to functional collateral arteries, capable to bypass the site of obstruction and preserve the tissue that is jeopardized by ischemia. Hemodynamic forces such as shear stress and wall stress play a pivotal role in collateral artery formation, accompanied by the expression of various cytokines and invasion of circulating leukocytes. Arteriogenesis hence represents an important compensatory mechanism for atherosclerotic vessel occlusion.

Since arteriogenesis mostly occurs when lumen narrowing by atherosclerotic plaques takes place, presence of cardiovascular risk factors (e.g. hypertension, hypercholesterolemia, diabetes) is highly likely. Risk factors for atherosclerotic disease affect collateral artery growth directly and indirectly by altering hemodynamic forces or influencing cellular function and proliferation. Adequate collateralization varies significantly among atherosclerotic patients, some profit from the presence of extensive collateral networks, whereas others do not. Cardiovascular risk factors could increase the risk of adverse cardiovascular events in certain patients because of the reduced protection through an alternative vascular network. Likewise, drugs primarily thought to control cardiovascular risk factors might contribute or counteract collateral artery growth.

This review summarizes current knowledge on the influence of cardiovascular risk factors and the effects of cardiovascular medication on the development of collateral vessels in experimental and clinical studies.

INTRODUCTION

Atherosclerotic lumen narrowing, leading to a reduction in oxygen and nutrient supply, loss of function and ultimately tissue necrosis is recognized as an inflammatory disease of the arterial vessel wall. In Western countries, it is one of the most common causes of death and represents an important socio-economic factor.

As a natural principle, each organism tries to maintain its integrity and homeostasis. In the case of lumen obstruction, the body has its means to protect tissue and organs from the doom of ischemia. When necessary, it is capable of developing new blood vessels to overcome hypoperfusion of certain areas. In the adult, there are two main mechanisms of blood vessel growth: a) sprouting and growth of new capillaries in hypoxic regions (angiogenesis) and b) the active remodeling of pre-existing vascular anastomoses toward larger functional arteries (arteriogenesis). Following the laws of nature defining blood flow as a function of the vessel diameter to the fourth power, arteriogenesis has been postulated as the most important mechanism to compensate for bulk perfusion loss. Hence, the presence of functional well-developed collateral arteries is a significant prognostic factor in cardiovascular disease [1, 2].

Once an atherosclerotic lesion becomes hemodynamically significant (~75% lumen narrowing) it causes a pressure gradient across the arterial network including the pre-existent anastomoses. The blood flow follows the gradient (Δp) and takes the line of the least resistance, in this case the collateral anastomoses, bypassing the obstruction. This change in flow pattern results in increased shear stress acting on the vascular wall of the undeveloped collateral anastomoses, thereby activating inflammatory pathways that allow the dormant micro vessel to develop into a full grown artery. This process is triggered and facilitated by several cell types, whose proper functionality is of crucial importance [3]. The endothelium, lining the vascular anastomosis, senses the change in flow pattern and the altered shear stress. Nitric oxide is produced, leading to vasodilatation and activation of the endothelial cells resulting in the expression of surface adhesion molecules, facilitating the entrapment of inflammatory cells. Cytokine and chemokine release (e.g. tumor necrosis factor–alpha (TNF-alpha) [4], granulocyte monocyte-colony stimulating factor (GM-CSF) [5], monocyte chemoattractant protein -1 (MCP-1) [6] attracts circulating leukocytes, with a leading role for monocytes and lymphocytes [7]. After entering the perivascular tissue, these cells promote active proliferation of endothelial and smooth muscle cells when provided the necessary space by extracellular matrix degradation. This remodeling process enables the now large functional arteries to redirect fair amounts of blood, perfusing the tissue downstream.

Unfortunately, this rescue mechanism is everything but flawless. Despite the lives and limbs saved by this process, the sheer number of patients suffering from atherosclerosis related symptoms such as angina pectoris and intermittent claudication indicate the frequent shortcomings in the adaptive remodeling of the collateral circulation.

Collateral artery growth shows large inter-individual differences regarding angiographic presence and functionality [8]. Irrespective of the increasing knowledge of the cellular and molecular mechanisms of arteriogenesis, the differences in the capability to adequately remodel collaterals between patients yet remain largely unexplained. Numerous factors could be held responsible for these variations, from fundamental disparities in genetic profiles to lifestyle differences (both contributing to the presence of risk factors).

In most patients suffering from atherosclerotic disease, one or more cardiovascular risk factors

are present. The risk factors unanimously alter molecular and cellular processes in the vascular system. Endothelial dysfunction indicated by a defective nitric oxide (NO) synthesis, a reduced number of circulating endothelial progenitor cells (EPCs), modifications of inflammatory responses or changes in wall stress are features associated with the presence of risk factors and atherosclerosis. Furthermore, these processes are also involved in and intervening with arteriogenesis.

Risk factors are not equally distributed among atherosclerotic patients. It remains the question whether variations in the presence of risk factors or the combination of certain risk factors in patients are responsible for the inhomogeneity in collateral artery development and if risk factor management could be essential for successful vascular remodeling in the clinical setting. In this review we try to identify the factors leading to inter-patient variability and the effects of cardiovascular risk factors on collateral artery formation.

GENETIC BACKGROUND

Collateral arteries develop from recruitable but not yet functional pre-existing arteriolar anastomoses. These connections are built during the embryonic phase (vasculogenesis) and the extent to which this happens could be a limiting factor for the number of collaterals that can be recruited later on. This hypothesis is supported by studies in mice, where large strain differences in the number of pre-constructed blood vessels can be observed [9], resulting in significant perfusion differences after acute arterial occlusion [10, 11]. Whether this also holds true for humans needs to be clarified. Interestingly, such differences not only exist between strains but even, though to a lesser extent, within inbred mouse strains, where animals are supposed to be genetically almost identical. This results in marked inter-animal variability in collateral flow and hindlimb function [12]. Considering the much larger genetic diversity amongst humans, varying numbers of pre-existent connecting vessels seems likely. However, to the best of our knowledge this has not yet been adequately documented.

Basically all cellular processes are directly or indirectly influenced by genetic predisposition [13]. This also applies to the cellular responses to exogenous and endogenous stimuli, accounting for another possibility of innate variation in patients with seemingly similar clinical characteristics. For instance, discrepancies in the ability to remodel pre-existing collaterals into fully functional arteries may be caused by variations in cell adhesion capacity and reaction of inflammatory cells to chemokines. These processes are the driving force in collateral development and molecular differences lead to diversity in arteriogenic capacity [14]. As recently shown, the genetic profile of monocytes from patients with poor and good collaterals may differ significantly [15]. The differences can be directly related to monocyte proliferation, intracellular transport and apoptosis [16]. This is supported by the observation that despite the fact that baseline patient characteristics were comparable, isolated monocytes from patients with good collateralization produced significantly more VEGF under hypoxic conditions [17]. These variations in monocyte function could be predictive of to what extent a patient could profit from angiogenic or arteriogenic therapies [18].

Certain risk factors show a clear familial cumulation, indicating a genetic component [19]. While some of these gene profiles have been identified, no profile to predict the individual's arteriogenic capacity has been found yet.

Genetic differences may account for the embryonic development of the pre-existing anastomoses, meaning that some patients may have a head start. However, it is unlikely that this serves as the sole explanation for the differences in collateralization between patients. Evidence is accumulating that certain cardiovascular risk factors affect vascular growth processes. This is particularly important as basically in each patient in need for arteriogenesis one or more of these factors is likely to be present. Considering the pathophysiological changes that underlie certain risk factors, it is presumable that these may interfere with collateral artery growth during one or more steps of this multistep process. Although risk factors are present in most patients, their combinations differ and they are not equally distributed among the atherosclerotic patient population. Hence, they may explain the different arteriogenic response between patients. To make things even more complicated, it might turn out that it is a certain combination of factors that predicts outcome, even though single factors may have significant impact. Data on risk combinations is scarce; the current knowledge about cardiovascular risk factors in relation to arteriogenesis is therefore discussed for each factor individually below.

Hypercholesterolemia and hyperlipidemia

Elevated cholesterol levels maintain a well established relation with the occurrence of cardiovascular disease and their related events [20]. Strong evidence in animal models suggests that hyperlipidemia may retard collateral remodeling. Murine models of both dietary and genetic hypercholesterolemia have demonstrated reduced collateralization in several ways[21-23]. The presence of elevated blood cholesterol levels impairs monocyte chemotaxis in coronary artery disease patients [24] and thereby results in reduced early monocyte/macrophage influx. Secondly, it leads to a delayed and impaired arterial growth response to growth factor therapy [25].

Statins (3-hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors) are widely prescribed to lower blood cholesterol levels. Their success in preventing the progression of cardiovascular disease was initially attributed to their cholesterol lowering properties [26, 27]. Nowadays, their so-called pleiotropic effects become more and more interesting as many of the statins' effects do not directly depend on cholesterol lowering. Statins display significant anti-inflammatory and anti-thrombotic properties, improve endothelial dysfunction and promote smooth muscle cell migration and proliferation [28-30]. Could their success possibly partly base on stimulation of collateral artery growth? The first indications of a positive link were reported by Kureishi [31] and Sata [32], who showed that in response to ischemia, normocholesterolemic animals (rabbits and mice) show an enhanced arteriogenic response when given a HMG-CoA reductase inhibitor. Patient studies provide supporting evidence that collateralization benefits from statin use [33-35]. Dose, drug of choice and duration of therapy and delivery method are of importance [36-40], however all HMG-CoA reductase inhibitors seem to be positively related to collateral formation. Statin dosage deserves special attention as there seems to be an important dose-dependency and positive effects can only be expected above certain levels [41].

When stimulating blood vessel growth, there is always the possibility that the therapy could backfire in serious complications such as cancer, diabetic retinopathy and atherosclerosis. Statin treatment successfully enhances angiogenesis as well as arteriogenesis, even when there

is no underlying hypercholesterolemia and thereby shows to be a potent vessel stimulator [42]. However, it is probably one of the safest drugs known to enhance neovascularization. When arteriogenesis is stimulated by HMG CoA inhibition in a tumor model no progression of the cancer is found [43], indicating safe use in oncological patients.

Hypertension

Despite the link between arteriogenesis and hemodynamic forces, evidence on the connection between collateral growth and arterial hypertension is low. Hypertension could affect arteriogenesis in many ways. Elevated blood pressure increases shear stress. The stiffening of the arterial wall as a result of longstanding elevation of blood pressure modulates the tangential stress on the blood vessel wall [44]. As shear stress is one of the main driving forces of arteriogenesis, hypertension might actually promote collateral growth. When shear stress increases, the activated endothelial cells phosphorylate endothelial nitric oxide synthase (eNOS) and produce nitric oxide (NO). NO is a versatile product importantly involved in arteriogenesis by altering gene expression/ activity, matrix metalloproteinase activation and cell recruitment [45-49].

On the other hand, hypertension is widely associated with vascular dysfunction, likely reducing arterial remodeling. In the hypertensive patient, the renin angiotensin system is activated leading to elevated levels of circulating angiotensin II (Ang II). Ang II, has pro- as well as anti-angiogenic capacities [50-52] modulating the vasculature in several ways. It enhances NAD(P)H oxidase activity, which is the major contributor to reactive oxygen species (ROS) and superoxide levels in the vessel wall. The elevated oxidative stress leads to the uncoupling of eNOS, reducing the bioavailability of NO, ultimately resulting in endothelial dysfunction [53]. Dysfunctional endothelium contributes to the inhibition of collateral artery growth. ROS on the other hand are not per se negatively associated with arteriogenesis; a certain level of ROS has been shown to be actually required for effective collateral growth [54].

Besides its effect on vascular tone, angiotensin modulates the inflammatory response and could hence promote an inflammation-driven process like arteriogenesis. Angiotensin induces the production of various angiogenic and arteriogenic growth factors such as vascular endothelial growth factor (VEGF) [55, 56], platelet derived growth factor (PDGF) [57] and fibroblast growth factor (FGF) [58-62]. Hypertension might therefore be a double edged sword having both positive and negative effects on arteriogenesis. Two observational studies have been executed over the past two decades and both pointing into a different direction. A positive relation between high blood pressure and collateral formation was described by Kyriakides in 1991, but Koerselman et al stated exactly the opposite in 2005 [63, 64]. Focusing on anti-hypertensive medication might help to further elucidate the role of hypertension in collateral growth.

Diuretics could potentially affect fluid shear stress by altering blood viscosity. β-blockers could interfere with wall stress by changing blood pressure and stroke volume. However, none of them have been linked to arteriogenesis. The opposite applies to ACE inhibitors and angiotensin II receptor blockers (ARBs).

ACE (angiotensin-converting enzyme) converts angiotensin I into angiotensin II. Besides decreasing angiotensin II formation, ACE inhibitors reduce bradykinin inactivation, leading to a local increase of nitric oxide production and hypotension. ACE inhibitor have furthermore been shown to have a favorable effect on endothelial dysfunction [65]. This translates into an improved collateral formation capacity in animal as well as human studies [66-69].

Following the promising reports on the effects of ACE-inhibitors on arteriogenesis, blocking of the angiotensin II receptor itself came into focus. Despite the fact that a plausible mechanism by which ACE-inhibitors promote collateralization is a reduction of Ang II signaling, angiotensin II receptor blockers (ARBs) did not lead to comparable results [70]. These differences might be due to their lack of any effect on bradykinin and hence NO metabolism.

Smoking

Smoking impacts the cardiovascular system in several ways, of which most are still not fully understood. Tobacco smoke contains over 4000 chemical compounds and all of them may influence physiological processes. As indicated above, a proper endothelial function is of crucial importance in arteriogenesis. One of the earliest pathological effects of smoking on the cardiovascular system is a change in endothelial function, by affecting several signaling pathways. Moreover, EPC (endothelial progenitor cell) numbers and function are reduced by cigarette smoke, contributing to this effect [71].

Besides the changes in the endothelial (progenitor) cells, cigarette smoke exposure also alters inflammatory responses. A decline in VEGF biological availability in macrophages [72] leads to impaired VEGF actions on endothelial cells [73]. Circulating monocytes become less susceptible to chemoattractants in the presence of ROS and super oxides in particular, inhibiting their migratory capacities. As previously described, anti-oxidant therapy (e.g. vitamin C supplementation) may recover monocyte function to normal levels [74].

The nicotine component of cigarette smoke is known for its pro- angiogenic and arteriogenic effect [75-77]. Nicotine is thought to signal via the acetylcholine receptors (AchR) present on e.g. monocytes, macrophages and endothelial cells [78]. It promotes *in vitro* angiogenesis via the induction of growth factor dependent endothelial cell migration to the same extent as VEGF and bFGF [79]. However, this *in vitro* effect of purified nicotine might differ *in vivo* in the presence of other factors contained in cigarette smoke.

Chronic extensive smoking may furthermore lead chronic hypoxia (e.g. through increased carbonmonoxide binding to hemoglobin), potentially influencing vascular growth processes. Although collateral artery growth does not depend on the presence of ischemia/hypoxia [80], such effects cannot be excluded. However, the exposure to cigarette smoke under hypoxic conditions impairs rather than stimulates vessel growth by inhibiting VEGF expression via reduced hypoxia inducible factor -1alpha (HIF-1alpha) expression [81]. Therefore, smoking induced hypoxia unlikely plays a direct role in changes in the arteriogenic response.

So far, clinical studies do not show a consistent relation between collateralization and smoking [82-85]. Recently, Koerselman et al. described smoking to be positively correlated to the presence of collateral arteries. The amount of pack years did not contribute to this observation, only current smoking status did, making a direct nicotine effect unlikely. Occasional smoking might induce an ischemic stimulus by local vasoconstriction without chronically disturbing endothelial function and could thus function via the same mechanism as preconditioning angina. However, in chronic smokers, the endothelium becomes dysfunctional and the observed positive effect disappears [86].

Hyperglycemia and diabetes mellitus (DM)

Diabetes mellitus (DM) is considered an independent risk factor in cardiovascular disease, predicting short and long term mortality [87]. The poorer prognosis in diabetics has been ascribed

to an attenuated development of collaterals [88]. However, the question whether hyperglycemia and/or hyperinsulinemia affect arteriogenesis in any way is controversially discussed. Most of the data available has come from clinical studies, without making the distinction between DM type I (no hyperinsulinemia) or DM type II (with hyperinsulinemia). Therefore, no such distinction will be made in the following paragraph.

Sufficient evidence indicates that high fasting blood glucose levels are strongly related to the presence of blood vessel wall abnormalities [89]. Endothelial function, vascular smooth muscle cell proliferation and the interaction between vessel wall and circulating cells change under the influence of hyperglycemia. Since these processes are involved in collateral artery formation, an effect of DM seems plausible.

The first clinical study focusing on the link between diabetes and collateral artery growth was published by Abaci et al almost 10 years ago and consisted of 205 diabetic patients with significant coronary stenosis and matching controls. In diabetics, coronary collaterals showed to be significantly reduced [90]. The retrospective design of the study certainly has several limitations, but the awareness for the subject was raised, leading to a number of subsequent studies with conflicting results varying from positive [91], no [92] to negative observations [93-99]. A recent study with a prospectively selected patient cohort (n=387) showed a clear inverse relationship between the presence of DM and the extent of collaterals formed [100].

When trying to explain the variations in the outcome among the studies, the means of measuring collateralization catches the eye. When assessed by invasive measuring techniques, no link between DM and collaterals can be found, whereas scoring based on analyzing angiographic movies mostly implies inhibition of collateral growth by hyperglycemia. This may be due to the fact that angiography fails to show smaller collateral anastomoses [101, 102] and especially in DM, where microangiopathy is part of the disease [103] this could influence outcome. Theoretically, hyperglycemia may mainly affect the formation of larger conducting arteries, but does not hamper the increase in remodeling in smaller segments [104] even though this is hemodynamically disadvantageous.

Animal studies executed on this subject could provide more insight into the effects of DM on collateral formation. Van Weel et al. examined arteriogenesis after femoral artery occlusion in hyperglycemic (STZ treated C57BL/6 and NOD)and insulin resistant (Ob/Ob)mice [105]. Both the STZ treated (DM type I) and Ob/Ob (DM type II) mice showed no gross impairment in perfusion restoration. While the NOD mice did not show any difference between mild and high hyperglycemia, perfusion restoration was significantly lower than in C57BL/6 mice. However, C57BL/6 mice are known for their excellent capacity to form collaterals and the measured difference between NOD and C57BL/6 mice could also be explained by the genetic dissimilarities between the two strains, rather than the presence or absence of DM type I.

In dogs undergoing repetitive coronary artery occlusion together with infusion of dextrose to increase blood glucose levels, collateral development was less than at normal glucose levels [106]. The authors accredited this effect to an increased MMP-9 activity and angiostatin expression in the myocardial interstitial fluid. MMP-9 is involved in tissue remodeling and forms angiostatin from plasminogen [107]. Angiostatin inhibits vascular growth by reducing endothelial cell proliferation [108, 109] and increasing apoptosis [110, 111] As shown in a hyperglycemic rabbit model an impaired shear induced vasodilatation of the blood vessels and an inhibited monocyte chemotaxis provides another explanation for the observed retarded collateralization upon chronic hyperglycemia [112].

However, whether these findings can be translated to DM type II patients with concomitant hyperinsulinemia and insulin resistance is not clear as the used model only applied hyperglycemia. Waltenberger provided a possible explanation how hyperglycemia could affect arteriogenesis [113]. Monocyte migration assays showed that monocytes of diabetic patients are less capable to migrate toward VEGF-A. Due to their central role in collateral remodeling, impaired monocyte migration could indeed result in a reduced arteriogenic response in DM patients. Supporting these findings, Zhou et al stated that elevated blood glucose levels attenuated the capacity of GM-CSF mobilized peripheral blood mononuclear cells to participate in blood vessel formation and remodeling [114].

Till now, modulation of arteriogenesis by hyperglycemia and/or insulin resistance seems plausible, but conclusions can only be drawn after more mechanistic and prospective patient studies with careful patient selection have been executed.

Obesity

Obesity is a growing pandemic [115] and the incidence of cardiovascular disease within obese patients is high [116]. While western societies fatten up, data on the link between obesity (Body Mass Index or BMI (weight in kg/ (length in meters)^2) > 30 kg/m^2) and arteriogenesis is scarce. Yilmaz et al. concluded that the coronary collateral circulation is poorer in obese patients with ischemic heart disease compared to patients with a normal BMI [117-119]. Possibly, the explanation for these findings can be found in the fact that the obese patients also had altered lipid profiles and probably insulin resistance too, reflecting an underlying metabolic syndrome. The metabolic syndrome is defined as having three or more of the following conditions: (1) abdominal obesity; (2) high triglyceride levels (>150mg/dl); (3) low HDL (High-density lipoprotein) -cholesterol levels (< 40mg/dl for men and < 50 mg/dl for women); (4) high blood pressure (systolic >130 mmHg or diastolic > 85 mmHg, or on antihypertensive drugs); and (5) a high fasting glucose concentration (> 110 mg/dl). From the aforementioned, an added effect of all of them can be expected on arteriogenesis. Consequently, the metabolic syndrome was found to be independently associated with a poorer collateral network [120, 121].

Thus, obesity itself might even be an innocent bystander with the accompanying factors and symptoms responsible for a decreased collateral growth.

Age

In case of an ischemic event (e.g. myocardial infarction, stroke), mortality in elderly patients is significantly higher than in their younger counterparts [122-124]. The cardiovascular changes are more often generalized and extensive and the accompanying co-morbidity makes this patient group less suitable for many treatment options. However, this patient group would particularly profit from a good collateral network. This makes it interesting and important to know how collateralization is influenced by aging.

In the senescent patient most physiological processes seem to change. Metabolism slows down, neurons are less excitable [125] and wounds take longer to heal [126], the latter already indicates reduced blood vessel growth capacities. Telomeres, DNA sequences capping the ends of all eukaryotic chromosomes, reduce in length during replicative aging of normal cells and after a critical reduction in telomere length cells stop dividing. Hence the attenuated responses of senescent cells [127]. Besides cell division, other cellular functions change over time. With increasing age, endothelial cells produce less NO after stimulation [128]. Aging is also associated

with an increased inflammatory activity in the blood [129] which could enhance angiogenesis and arteriogenesis. However, the response to an acute stimulus seems to be diminished. It has been postulated that an important part of human aging may be characterized by a declined ability to adapt to internal and external stressors [130]. The elderly are less likely to adequately respond to inflammatory triggers and this may also hold true for activation of inflammatory cells in case of a significant arterial occlusion, resulting in a reduced vascular remodeling capacity while having increased levels of circulating cytokines.

Indeed, preclinical and clinical studies showed that the ability to compensate for arterial occlusion is impaired with increasing age and collaterals seem to be present to a lesser extent [131-134]. A direct relation is difficult to prove and the exact mechanisms underlying the suppression of collateral growth by aging are poorly understood. Nowadays, being of high age almost always associates with the presence of other factors that could influence collateral growth and elucidating a clear link is difficult. Nevertheless, aging was recognized as an independent factor determining the amount of collaterals present at the time of vascular occlusion after multivariate analysis in a cohort of 1934 patients [135].

Having a reduced capacity to develop collaterals could be contributing to the worse outcome after ischemic events in the older patient, since the presence of collateral arteries is a known protective factor in case of a vascular occlusion [2, 136-138]. Despite the fact that aging itself cannot be influenced, older patients may still benefit from arteriogenic therapies as they seem to be still susceptible for such treatment 139. Extra awareness of treatment optimization is of special importance in this patient group, because of the clearly proven clinical importance in this fragile patient group [140-142].

Non traditional risk factors

Besides the traditional risk factors discussed above a number of other factors is associated with cardiovascular disease (non-traditional risk factors), such as chronic inflammation (e.g. auto-immune disorders) and anemia.

Several auto-immune diseases increase cardiovascular risk, mainly due to an altered and continuous activation of the immune system. For instance, rheumatoid arthritis and systemic lupus erythematosus patients have a highly increased risk of developing atherosclerosis [143]. Whether the increased inflammatory status in these patients also leads to more collateral development has not yet been documented. In contrast to the unspecific inhibition of inflammatory responses applied previously (e.g. corticosteroids) current treatments focus on specific inflammatory mediators, e.g. TNF-alpha. The latter is involved in many processes including chronic heart failure. Application of TNF-alpha antagonists (e.g. infliximab, etanercept) had deleterious effects in these patients [144]. Furthermore, TNF-alpha antagonists have also been shown to reduce collateral artery growth in a rabbit model [145]. Given this limited amount of data any conclusion on the effect of chronic inflammation on arteriogenesis would be more than speculative.

Data on the direct effects of anemia on blood vessel growth is also scarce. Severe anemia can lead to hypoxia, particularly in the extremities, possibly stimulating angiogenesis. No clinical data are available on the correlation between anemia and the presence of collateral vessel, though blood viscosity certainly can affect arterial remodeling. Scholz et al showed that structural collateral artery growth in mice overexpressing erythropoietin (EPO) was significantly increased due to an increased shear stress evoked by the high blood viscosity.

Table 1. Overview of potential influences of cardiovascular risk factors on collateral artery growth.

Risk Factor	Pathological change	Effect on arterio-genesis	Model	Reference
Hypercholes-terolemia	Reduced expression of VEGF	↓	hindlimb, mouse	(21)
	Reduced bioactivity of NO	↓	hindlimb, rat	(22)
	Reduced early monocyte macrophage influx, impaired response to growth factor therapy	↓	hindlimb, mouse	(23)
	Impaired monocyte chemotaxis	↓	*in vitro*	(24)
Hypertension	Increased fluid shear stress → increased NO production	↑	heart, dog ; mesentery, rat	(40, 41)
	Increased fluid shear stress → increased MMP activity	↑	carotid- jugular fistula, rabbit	(40)
	Increased angiotensin level → increased growth factor production	↑	*in vitro*	(50- 53)
	Unknown	↑	heart, human	(59)
	Increased angiotensin II → inhibition VEGF induced migration and decreased endothelial tube formation	↓	*in vitro*	(45, 46)
	Increased angiotensin II levels → increased ROS → endothelial dysfunction	↓	*in vitro*	(48)
	Unknown	↓	heart, human	(58)
Smoking	Endothelial dysfunction (reduced endothelial progenitor cell numbers and function)	↓	*in vitro*	(65)
	Declined bioavailability of VEGF in macrophages	↓	*in vitro*	(66)
	Impaired VEGF induced migration and tube formation, increased ROS	↓	*in vitro*	(67)
	Nicotine induced stimulation of EC proliferation migration and tube formation	↑	hindlimb, mouse	(69, 71)
	Unknown	↑	heart, human	(80)
	Unknown	=	heart, human	(76-79)
Hyperglyce-mia	Unknown	↑	heart, human	(85)
	Unknown	=	heart, human	(86)
	Unknown	↓	heart, human	(32, 78, 79, 82, 84, 87, 88)
	Unknown	↓	heart, human	(89)
	No changes significantly influencing arteriogen-esis	=	hindlimb, mouse	(94)
	Increased MMP-9 activity and angiostatin expres-sion	↓	heart, dog	(82)
	Reduced monocyte chemotaxis and impaired shear induced vasodilatation	↓	hindlimb, rabbit	(100)
	Impaired migration and participation of leuko-cytes	↓	*in vitro*	(87, 101)
Obesity	Unknown	↓	heart, human	(104- 106)
	Metabolic syndrome → insulin resistance and dyslipidemia	↓	heart, human	(89, 107)
Age	Reduced production eNOS	↓	hindlimb, rabbit mesentery, rat	(114, 118)
	Reduced VEGF expression	↓	hindlimb rabbit	(114)
	Unknown	↓	heart, human	(117, 119)
Non-tradi-tional	Anemia → over expression EPO → high blood viscosity	↑	hindlimb, mouse	(126)

Abbreviations used in the table: VEGF = Vascular endothelial growth factor; NO = Nitric oxide; ROS = Reactive oxygen species; MMP = Matrix metalloproteinase; EC = Endothelial cell; eNOS = Endothelial nitric oxide synthase

However, while vessel growth was enhanced, perfusion restoration was disturbed by the high blood viscosity as well [146]. This suggests an inhibiting effect of anemia on arteriogenesis though experimental and clinical proof of this hypothesis is lacking.

Lifestyle modifications

The majority of risk factors described above to some extent depend on lifestyle. Diet, exercise level, job and living habitat have a large impact on homeostasis. Exercise and its link with collateral vessel growth have been studied extensively. Among clinical therapies to induce collateral formation, exercise is still the most potent one, exceeding the effect of any drug treatment [147]. The effects of exercise on the cardiovascular system and vessel growth are manifold and are reviewed elsewhere [148-150].

CONCLUSION

Given the importance of the collateral circulation in patients suffering from cardiovascular disease, a better understanding of the factors influencing each patient's capability to develop a functional collateral network is needed. Cardiovascular risk factors present in this patient group are not necessarily negatively affecting collateral growth. In the best case, certain factors might even be promoting collateralization. Controversy still exists on the role of each individual risk factor and how it contributes to or hampers collateral growth. Nevertheless, at least part of the inter-patient variability can be explained by the presence of risk factors, as most of them alter vascular responses, each in its specific manner. Most evidence indicates that elderly and hypercholesterolemic patients seem to be most likely to have a poor collateral circulation. For diabetes, obesity and hypertension the present data are not yet conclusive. Smoking on the other hand does not seem to negatively influence arteriogenesis and thus is not a risk factor for collateral artery formation.

To reduce morbidity and mortality, whether or not to treat a patient's risk factors mostly poses no question. However, the choice between the available treatment options could account for its effect on collateral growth. In particular statins and ACE inhibitors are positively related with arteriogenesis and can improve collateral function and clinical outcome.

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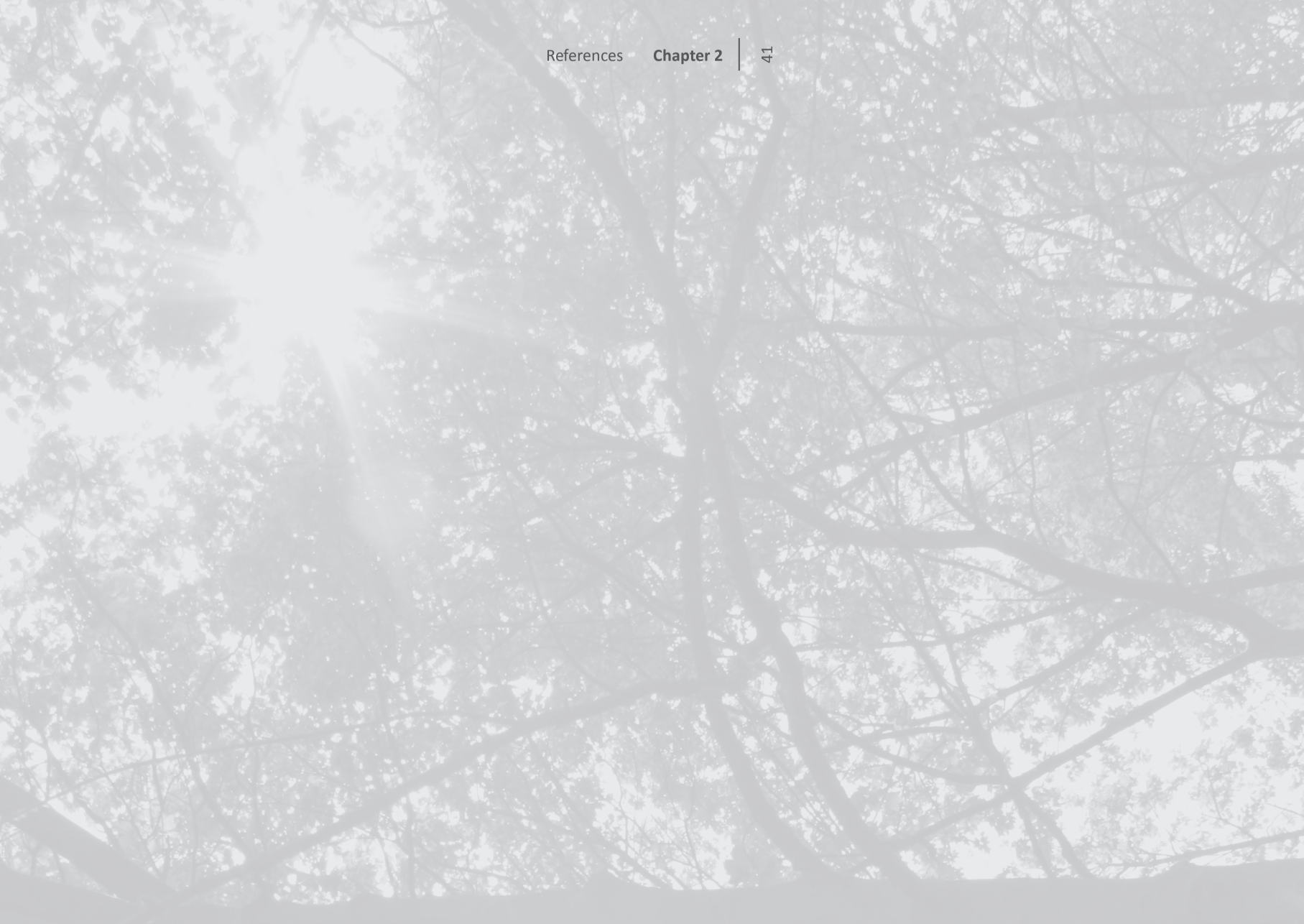

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3

Targeted deletion of the inhibitory NF-κB p50 subunit in bone marrow derived cells improves collateral growth after arterial occlusion

ABSTRACT

Aim Adaptive collateral artery growth (arteriogenesis) is an important mechanism to maintain tissue perfusion upon arterial obstruction. Leukocytes and inflammatory mediators play a crucial role in this process. Depletion of the Nuclear Factor kappa B (NF-κB) p50 subunit modulates inflammatory processes in cardiovascular disease. We hypothesized that NF-κB p50 is a regulator of the inflammatory response after arterial occlusion and subsequent collateral perfusion.

Methods & Results Unilateral femoral artery ligation was performed in NF-κB p50-/- and wild type (Wt, B6/129PF2) mice. Seven days after arterial occlusion, tissue perfusion restoration was significantly enhanced in NF-κB p50-/- mice compared to Wt mice (42.9±3.9 vs. 32.0±2.6 % perfusion recovery, p=0.04). Transplantation of NF-κB p50-/- bone marrow (bm) into Wt mice and vice versa showed that the effect of p50 subunit depletion can be predominantly attributed to the bone marrow derived circulating cells (NF-κB p50-/- bm in Wt mice 42.1±1.5%, Wt bm in NF-κB p50-/- mice 35.4± 1.5% perfusion recovery). Histological analyses revealed a more elaborate extravasation of monocytes in hindlimb tissue of NF-κB p50-/- mice. Chemotaxis assays confirmed the increased migration ability of NF-κB p50-/- monocytes, which may be due to an observed increased integrin expression. Upon stimulation of blood from NF-κB p50-/- and Wt mice more interleukin-6 was produced, confirming the pro-inflammatory phenotype in absence of the p50 subunit.

Conclusion Depletion of the NF-κB p50 subunit enhances collateral artery growth. Its absence in circulating cells improves tissue perfusion restoration after femoral artery ligation by increasing macrophage influx into the growing collateral vessels.

INTRODUCTION

Patients suffering from a reduced tissue perfusion due to atherosclerotic disease can greatly benefit from a well developed collateral network. These collaterals derive from pre-existing arterial/ arteriolar anastomoses after being triggered by changes in shear forces upon main arterial obstruction. Unfortunately, in fast progressing cardiovascular disease, the failure of sufficient collateral recruitment results in still high numbers of cardiovascular related morbidity and mortality. Nevertheless, naturally developed collateral networks are of incredible value in preventing tissue loss and enhancing collateral artery growth is a promising alternative, non-invasive therapy.

The underlying mechanisms of collateral artery formation (arteriogenesis) are not yet fully understood, but there is strong evidence that local inflammation plays a crucial stimulating role. Arteriogenesis is initiated by changes in blood flow and hence shear stress, sensed by the endothelial lining within the pre-existing collateral anastomoses [1, 2]. These changes activate the endothelial cells (ECs) and induce an intra-cellular signaling cascade involving the transcription factor nuclear factor- kappa B (NF-κB) [3, 4]. This leads to chemokine production and leukocyte attraction5 to facilitate the subsequent remodeling process [6-8]. Consequently, leukocyte concentration, adhesion and extravasation are important determinants of arteriogenesis [9-15]. Toll Like Receptor (TLR) mediated monocyte activation with the TLR4 ligand lipopolysaccharide (LPS) has previously been reported to enhance collateral artery growth by attracting more macrophages to the developing collateral [16, 17]. Both shear stress and TLR4 stimulation result in NF-κB activation and subsequent inflammatory gene transcription, indicating a functional role of NF-κB in collateral artery formation.

The transcription factor NF-κB regulates the expression of several genes involved in immune reactions and inflammatory processes [18]. It is composed of 2 members of the Rel superfamily, which consists of in total 5 members: p65 (Rel A), Rel B, and c-Rel, p50 (NF-κB-1) and p52 (NF-κB-2). All members contain a highly conserved N-terminal Rel homology domain (RHD) that facilitates their dimerization and binding to the DNA [19]. The p50 and p52 subunits are distinctively different from the other subunits, as they lack a transactivation domain, rendering them incapable to activate DNA transcription on their own. p50 and p52 homodimers are therefore considered to act as a brake by binding and occupying the target sites [20-22]. In addition, p50 depletion has been shown to enhance inflammation-driven processes such as outward arterial remodeling and ventricular remodeling after myocardial infarction [23, 24].

Since arteriogenesis highly depends on shear stress induced gene transcription and inflammation, we hypothesized that targeted deletion of the NF-κB p50 subunit enhances the arteriogenic response upon arterial occlusion in a mouse hindlimb model.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and prior approval was given by the Animal Ethical Committee of the faculty of Medicine, Utrecht University, the Netherlands. To investigate the role of the p50

subunit after femoral artery ligation. 60 B6;129P2-NF-κB^{tm1Bal}/J (NF-κB p50-/-) and 75 B6/129PF2 wild-type (Wt) mice (10-12 weeks old) were studied. Male as well as female mice were used and they were equally distributed between both study groups to exclude any gender effect. In the NF-κB p50-/- mice a PFG-neomyacin resistance cassette was inserted into exon 6, the exon encoding for the precursor of p50 (p105). Therefore, no functional NF-κB p50 could be generated and we refer to these mice in the rest of our article as knock-outs.

Surgical protocol

To study collateral artery growth, 10 NF-κB p50-/- and 10 Wt mice underwent ligation of the right femoral artery as previously described [25]. The left femoral artery was sham-operated and served as an intraindividual control. Analgesia and anesthesia were induced by subcutaneous injection of 0.1 mg/kg buprenorphine and isoflurane inhalation. Body temperature was maintained during the operation by placing the animals on a heating pad.

Perfusion measurements

Seven days after femoral artery ligation, microsphere-based perfusion measurements were performed to assess collateral dependent perfusion restoration. In short, the animals were anesthetized and heparinized. The abdominal aorta was cannulated and fluorescent microspheres (Fluospheres, 15µm, Molecular Probes Inc., Eugene Oregon) were infused at several different perfusion pressures under adenosine induced maximal vasodilatation [26]. Afterwards, hindlimb muscle samples were collected and weighed. The muscle tissue was digested and homogenized and the number of microspheres per sample was quantified by FACS[27]. Tissue perfusion restoration in the occluded hindlimb was defined as a percentage of the perfusion in the sham-operated hindlimb.

Bone marrow transplantation

Thirty 6 week old mice (15 Wt and 15 NF-κB p50-/-) were irradiated at a dose of 700cGray (1030 monitor units), for autologous bone marrow eradication. Ten donor mice (5 per strain) were sacrificed to isolate bone marrow for subsequent transplantation. The bone marrow was aseptically collected by flushing the femoral, tibial and humeral bones with RPMI 1640 (Invitrogen). After filtering (70 micron Falcon cell strainer, BD Bioscience, San Jose, USA) and washing, the bone marrow was injected ($5x10^6$ cells) into the tail vein of the recipient mouse. p50 -/- mice received Wt bone marrow and vice versa to study the influence of NF-κB p50 depletion in bone marrow derived cells during arteriogenesis. Wt → Wt transplantation was performed to exclude direct effects of the irradiation procedure on collateral artery formation.

After bone marrow transplantation, the animals were allowed to recover for 6 weeks before undergoing right femoral artery ligation and subsequent perfusion measurements as described above.

Chimerization efficiency

Using a puncture of the vascular plexus of the cheek, 100µl blood was collected in EDTA tubes 6 weeks after transplantation to test the efficacy of bone marrow transplantation. DNA was isolated using the Gene Mole system (Mole genetics, Norway) according to the manufacturer's protocol. The presence or absence of the p50 gene in these samples was measured by RT-PCR.

The following primers were used: a common forward primer 5'-GCA AAC CTG GGA ATA CTT CAT GTG ACT AAG-3', a reverse primer recognizing the Wt (normal) NF-κB p50 gene 5'-ATA GGC AAG GTC AGA ATG CAC CAG AAG TCC-3' and a reverse primer directed to the defective NF-κB p50 gene 5'-AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG-3'. Conditions followed: step 1 = 1.5 minutes at 94˚C, step 2 = 30 seconds at 94˚C, step 3 = 1 minute at 68˚C, step 4 = 1 minute at 72˚C (repeat step 2-4 for 35 cycles), step 5 = 2 minutes at 72˚C, step 6, hold at 100C. Both Wt and NF-κB p50 PCRs were done on 1ng/ul DNA of all samples. Chimerisation efficiency was calculated by using the following method. Full Wt and full NF-κB p50-/- genomic DNA was mixed at known ratios, from a 100% Wt, via 90% Wt and 10% NF-κB p50-/-, …, to 100% NF-κB p50-/- DNA, generating a 'reference standard' to which the samples could be compared. Each chimeric sample was then matched to the corresponding reference standard to show the relative amount of Wt and NF-κB p50 gene present.

Immunohistochemistry

At day 3 and 7 after femoral artery occlusion, hindlimb muscles were collected and snap frozen in liquid nitrogen (10 mice per strain/ time point). After embedding in Tissue Tek (Sakura, Zoeterwoude, the Netherlands) 7μm sections were cut and fixed in acetone. Blocking was performed with Cytomation Biotin blocking system (DAKO) followed by 1 hour incubation in 5% milk/PBS. The following antibodies were used: rat monoclonal MOMA2 (1mg/ml, Millipore) 1:50 in 1% milk/PBS, goat anti rat-biotin (1mg/ml, Southern Biotech) 1:250 in 1% milk/PBS, streptavidin-alexa fluor 555 (Invitrogen) 1:1000 in 1% milk/PBS and monoclonal to αSMA-FITC (Sigma) 1:300 in 1%BSA/PBS. All sections were counterstained with Hoechst (trihydrochloride trihydrate 33342, working solution 1:10 000, Invitrogen, Oregon, USA). Isotype rat IgGb (ABserotec) was used as negative control. The sections were inspected under a microscope (Olympus BX60) and pictures were taken (Olympus DP71) at a 400x magnification. Monocyte/macrophages accumulating in the perivascular tissue were counted by two independent blinded observers in at least 6 tissue sections/ animal and expressed as the number of MOMA2 positive cells/ αSMA positive artery.

Monocyte migration experiments

Whole blood of 19 NF-κB p50 -/- and 19 Wt mice was aseptically collected via cardiac puncture using a heperanized syringe. Blood composition and cell counts were analyzed using an automated hematologic analyzer (Cell Dyn 1800, Abbott Laboratories, Illinois, USA) on small samples of the blood. NF-κB p50 -/- and Wt peripheral blood mononuclear cells (PBMCs) were isolated from the remaining blood samples by using Ficoll- Paque ™ plus (Greiner BioOne), according to the manufacturer's protocol and stored in liquid nitrogen in RPMI supplemented with 10% Fetal Bovine Serum (FBS) and 10% Dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA).

Migration of NF-κB p50-/- and Wt PBMCs was assessed in a 5μm pore size polycarbonated Transwell system (Corning inc., New York, USA). All samples were measured in duplicates. Cells were thawed in RPMI (10%FBS) and left to recover for 1 hour at 37 degrees in an incubator (5% CO_2.). Cell numbers and viability was checked using a Countes™ automated cell counter (Invitrogen, Oregon, USA) and trypan blue stain 0.4% (Invitrogen, Oregon, USA). Only samples with less than 10% dead cells were used to avoid any effect of dead cells on the assay. The concentration viable cells was set at $1*10^6$ cells/ml. 150 μl of the PBMC in RPMI (10%FBS) was

then added to the upper inserts. The lower chambers were filled with increasing concentrations of mouse MCP-1 (Peprotech, London) (0ng/ml, 10ng/ml and 40ng/ml) in RPMI (10%FBS). The inserts were placed onto the lower chambers and were left at 37˚C and 5% CO_2 for 5 hours.

Subsequently, the inserts were carefully removed and the content of the lower wells was collected after adding 1ml of PBS-EDTA (2mM) and placing the plate on a shaker for 15 minutes. Migrated cells were stained with F4/80- Alexa647 (rat- anti mouse; AbDserotec, Germany) and CD11b-FITC (rat- anti mouse; Bioconnect, the Netherlands) and quantified by flow cytometry. Cells positive for both F4/80 and CD11b were identified as migrated monocytes.

Cell number and adhesion marker quantification

Whole blood samples of 10 NF-κB p50 -/- and 10 Wt mice were examined for the expression of integrins on the cell surface. The following antibodies were used: CD11a (PECy7, rat- anti mouse; Abcam, United Kingdom), CD11b (FITC, rat- anti mouse; Bioconnect, the Netherlands), CD49d (alexa 444, rat- anti mouse: AbSerotec, Germany) and F4/80 (Alexa 647, rat- anti mouse; AbSerotec, Germany). All samples were incubated with the antibodies for 45 minutes, washed and the erythrocytes were lysed. The remaining leukocytes were analyzed by flow cytometry. Monocytes were selected on F4/80 and scatter properties.

Cytokine production assay

Heparinized whole blood from 6 NF-κB p50-/- and 6 Wt mice was stimulated with lipopolysaccharide (LPS, E. Coli 055-B5, Sigma-Aldrich, St. Louis, USA) 1ng/ml, 10ng/ml in PBS or only PBS (negative control) at 37˚C and 5% CO_2. Samples were spun down and plasma and cells were separated. To measure the extent of NF-κB dependent gene transcription, an ELISA for mouse IL-6 (Quantikine immunoassay, R&D systems, Minneapolis, USA) was performed on the plasma according to the manufacturer's protocol.

Statistics

All statistics were performed using SPSS 15.0 (SPSS Inc.). Mann Whitney U test was used to compare differences between two groups. A one way ANOVA with post-hoc Bonferoni test was used to compare differences between multiple groups. The values are presented as mean ± SEM and p-values < 0.05 were regarded as significant.

RESULTS

The NF-κB p50 subunit is involved in arteriogenesis

Seven days after unilateral femoral artery occlusion tissue perfusion restoration was assessed in the NF-κB p50-/- and Wt mice during maximal vasodilatation. A significant increase in hindlimb perfusion was measured in the NF-kB p50-/- animals compared to the Wt (perfusion occluded/non-occluded hind limb: NF-κB p50-/-: 42.9 ± 3.9%; Wt: 32.0 ± 2.6%; p=0.04) (figure 1A). None of the animals suffered from gangrene or limb loss after the operation. There were no evident differences regarding hind limb function between NF-κB p50-/- and Wt mice.

The absence of the p50 subunit of NF-κB in circulating cells is responsible for the enhanced perfusion restoration

Since both, vascular cells and circulating cells express NF-κB, we wanted to investigate which of these cells were mainly responsible for the observed increase in perfusion recovery. Therefore, we performed bone marrow transplantations in a cross-over design, enabling us to distinguish between the contribution of circulating cells and resident cells or their combination to hind limb collateral growth. NF-kBp50-/- who received Wt bone marrow (organ knockout) showed similar perfusion recovery as complete knockouts (35.4 ± 1.5%) In contrast, transplantation of NF-κB p50-/- bone marrow into Wt animals (blood knock-outs) had a similar

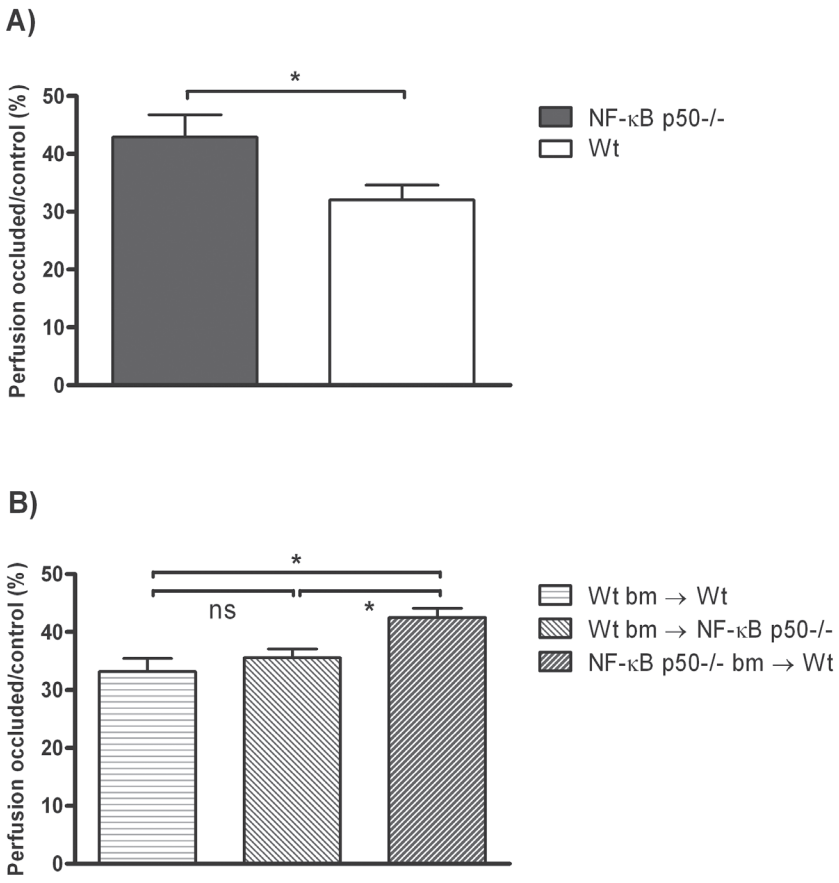

Figure 1. Microsphere-based perfusion measurements 7 days after femoral artery ligation
A) The NF-κB p50-/- mice demonstrated a better perfusion restoration compared to wild type (Wt) mice (n =10/strain). In each animal, the left (sham-operated) hind limb served as an intraindividual control, representing normal perfusion (100%). (p<0.05). B) NF- κB p50 subunit deficient bone marrow transplanted in Wt mice (n=13) completely mimicked the effect of a total knock-out, indicating that the absence of NF-κB p50 in circulating cells is the key factor responsible for the improved arteriogenic response. p50 -/- mice receiving Wt bone marrow (n=15) did not show any significant improvement of collateral growth. Wt → Wt transplantation confirmed that bone marrow transplantation itself did not affect collateral growth, as the perfusion recovery resembles the recovery of a normal Wt animal (n=11). (* p<0.05)*

effect on perfusion restoration as observed in the full NF-κB p50-/- (42.1 ± 1.5 %) and showed a significantly increased perfusion recovery compared to the NF-κB p50 organ knockouts (figure 1B). To exclude any potential effect of irradiation and bone marrow transplantation on arteriogenesis, we transplanted Wt bone marrow into Wt mice. There were no significant differences in perfusion restoration compared to non- irradiated Wt mice (33.1 ± 2.3%; p= ns), indicating no direct effect of the chimerization protocol on collateral vessel growth. The efficacy of the bone marrow transplantation was confirmed by measuring the presence of the NF-κB p50 gene in the bloodcells. After transplantation of NF-κB p50-/- bone marrow into Wt mice, NF-κB p50 gene expression in circulating cells was reduced by 92% (± 1.4 %).

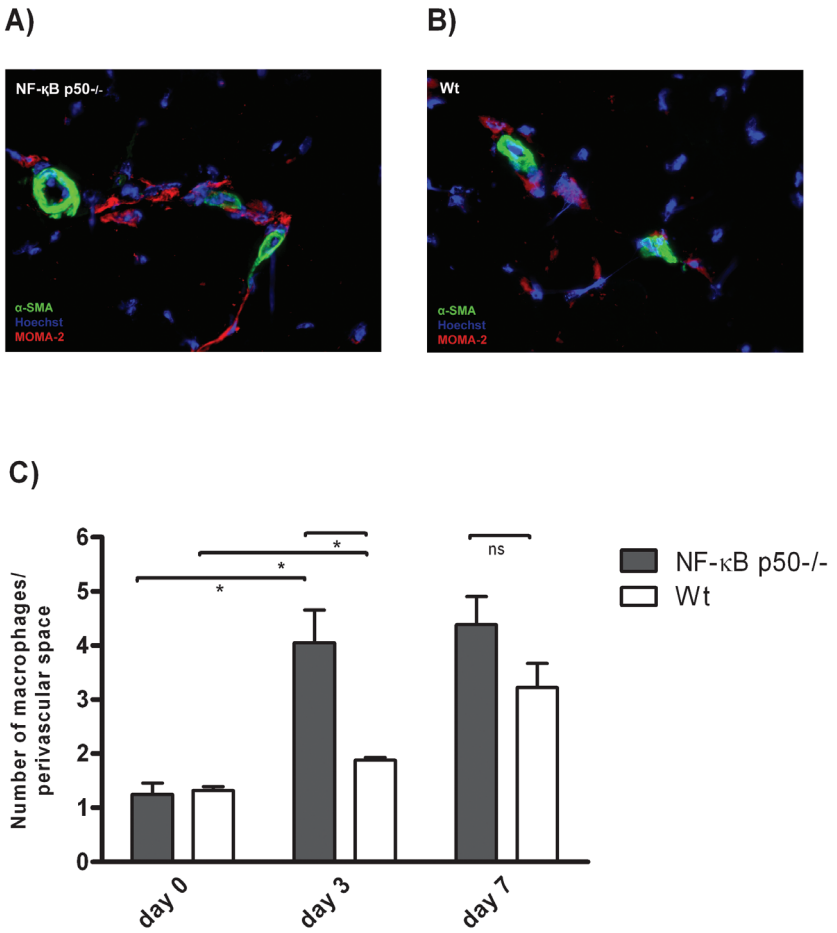

Figure 2. Staining for macrophages (red), smooth muscle cells (green) and cell nuclei (blue) on adductor hindlimb muscle tissue

The macrophages are clearly visible in the perivascular spaces of the NF-κB p50-/- (A) and wild type (B) animals. C) Quantification of macrophages in the perivascular space of the arterial anastomoses. At baseline equal numbers of macrophages were present in both strains. After arterial occlusion, the influx of macrophages was more elaborate in the NF-κB p50 -/- animals compared to their wild types, especially early after occlusion (day 3). (p< 0.05; **p<0.01).*

Vice versa, Wt bone marrow transplantation into NF-κB p50 -/- mice increased p50 gene expression to at least 83% (± 1.6 %) of normal expression levels in Wt mice.

Macrophages are more prevalent around developing collaterals of the p50 knock-out animals

After establishing their key role during arteriogenesis in this model, the circulating cells were further examined. Monocyte/macrophage stainings were performed at different time points after femoral occlusion in both NF-κB p50-/- and Wt. Under baseline conditions, i.e. prior to femoral ligation, the pre-existing collateral anastomoses were accompanied by a minor number of macrophages, comparable in both strains (number of macrophages/vessel: NF-κB p50 -/-: 1.4 ± 0.3; Wt: 1.7 ± 0.2, p= ns). After femoral artery ligation, all animals showed an increase of

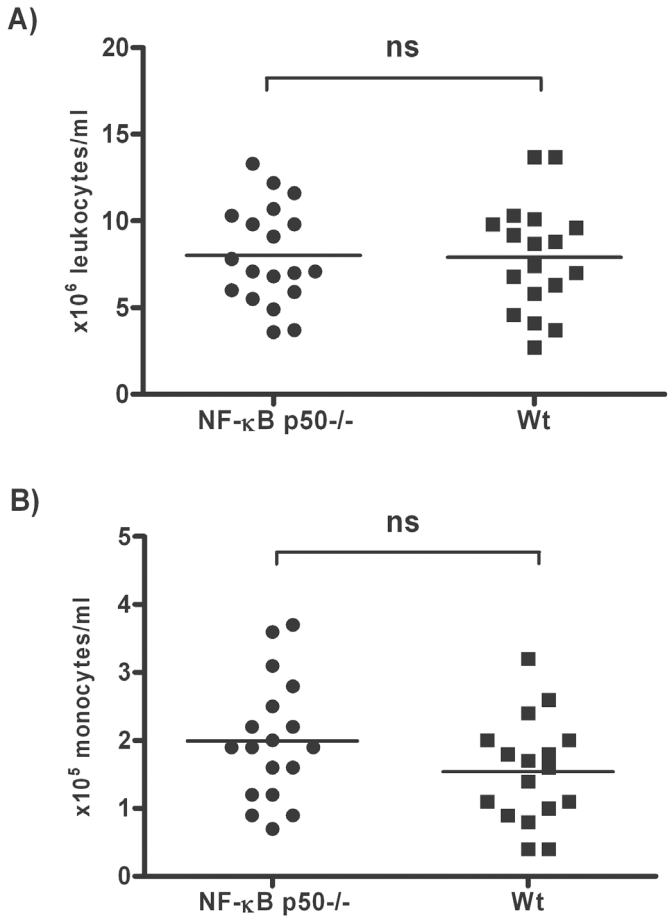

Figure 3. Quantification of cell number in whole blood of the two mice strains (n=19/ strain)
Both total white blood cell count A) and its monocyte content B) were not significantly different between the mice strains.

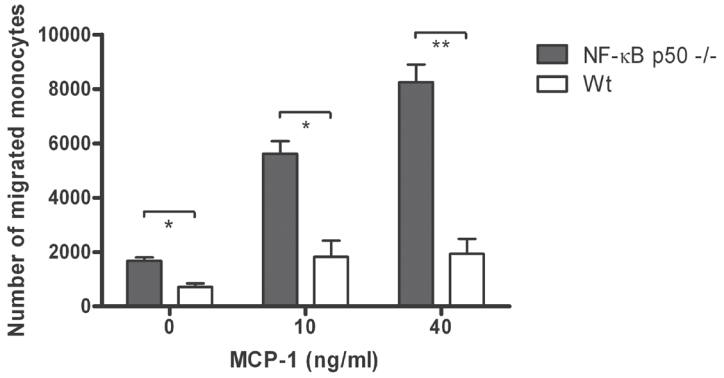

Figure 4. *In vitro* migration of monocytes to monocyte chemoattractant protein -1 (MCP-1)
NF-κB p50-/- and Wt monocytes were allowed to migrate to increasing concentrations of MCP-1 in a 5μm pore size Transwell system. The tests were done twice with all samples in duplicates. The numbers of migrated monocytes were quantified by FACS analysis (F4/80 and CD11b positive cells). The NF-κB p50-/- cells already showed a difference in migratory capacity at baseline, which increased even to extreme high concentrations of MCP-1 (40ng/ml), whereas the Wt cells reach a plateau phase at a lower concentration. (p<0.05)*

monocytes/ macrophages in the perivascular space (NF-κB p50-/- day 3: 4.05 ± 0.61, p=0.007; day 7: 4.38 ± 0.52, p=0.005) (Wt day 3: 2.16 ± 0.35, p=0.011; day 7: 3.23 ± 0.44, p=0.043). However, the NF-kB p50 animals showed a more elaborate and faster response since there was a marked difference between the amount of cells already present at day 3 (NF-κB p50-/- versus Wt day 3 p=0.008) (figure 2)

Monocyte migration accelerates in absence of the p50 subunit of NF-κB

The higher number of monocytes/ macrophages accumulating around the vessels of NF-κB p50-/- mice suggests a difference in either monocyte numbers or monocyte function compared to Wt. Neither total white blood cell count, nor the monocyte count differed significantly between the two strains of mice (figure 3). To test whether they showed an improved influx and migratory capability, transmembrane monocyte chemotaxis and migration was assessed in a transwell system. Even without addition of a chemoattractant factor to the lower well, NF-κB p50-/- cells showed more transmembrane movement compared to Wt (p= 0.04). In the presence of MCP-1 the migratory response of NF-κB p50-/- was significantly increased compared with Wt cells, resulting in an almost 3 fold increase in migrated cells. Furthermore, NF-κB p50-/- cells were able to respond to increasing MCP-1 concentrations, while Wt cells quickly reached a plateau level (figure 4).

A possible explanation for these differences in the migratory response could be a different expression of monocyte adhesion molecules. Depletion of the NF-κB p50 subunit enhanced the expression of the integrins CD11a ([MFI] p50 -/-: 26.6 ± 1.5; Wt: 20.6 ± 1.4; p=0.037) (figure 5A) CD11b ([MFI] p50 -/-: 51.5 ± 1.6; Wt: 44.8 ± 3.0; p=0.044) (figure 5B) and CD49d ([MFI] p50 -/-: 30.4 ± 0.7; Wt: 26.8 ± 0.9; p=0.009) (figure 5C). This increased expression of several integrins on the surface of NF-κB p50-/- mononcytes probably enables cells to better adhere

to the membrane (*in vitro*) and to endothelial cells (*in vivo*), facilitating their transmigration and subsequent extravasation. FACS quantification of monocyte numbers, selected on F4/80 and scatter properties, confirmed no significant differences in cell counts between the strains.

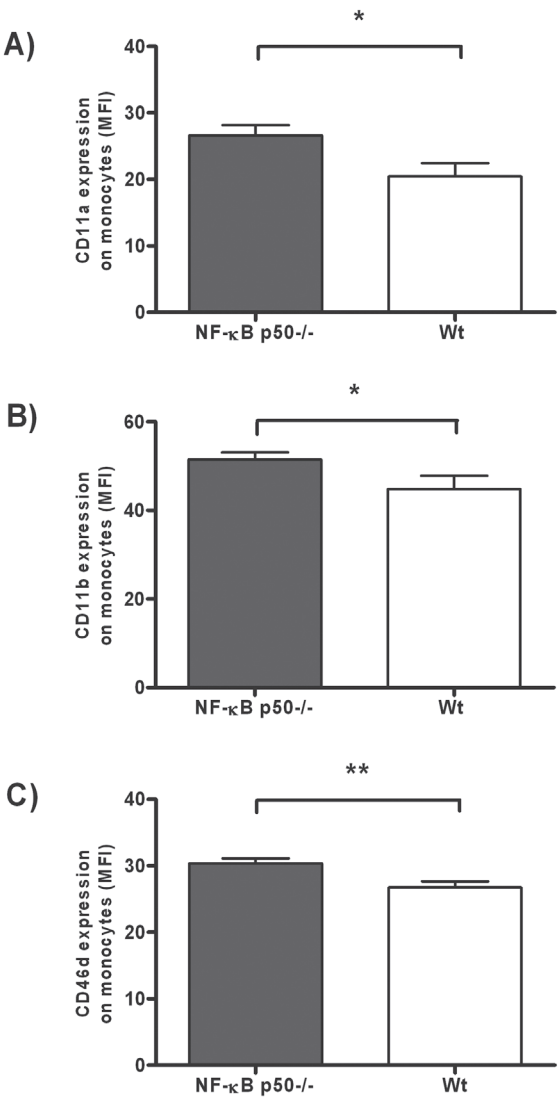

Figure 5. Expression of integrins on the monocyte surface
*Integrin expression on monocytes of both mouse strains was measured with FACS analysis and is expressed as mean fluorescent intensity (MFI).The integrins CD11a (A)) CD11b (B)) and CD46d (C)) were significantly more present on the surface of NF-κB p50 -/- monocytes compared to the wild type cells (n=10/strain) (*p<0.05, ** p< 0.01)*

Depletion of NF-kB p50 enhances the inflammatory response upon stimulation

The pro- inflammatory properties of NF-κB p50-/- cells were once more confirmed as the p65 content in the nucleus increased more after stimulation in the NF-κB p50-/- cells then in the Wt cells (6.9± 2.0 versus 1.9±0.6 increase compared to baseline, p=0.01). Also the IL-6 production after LPS stimulation was significantly higher compared to Wt cells (figure 6). The baseline IL-6 production did not significantly differ, illustrating the need for NF-kB activation before any differences will become apparent.

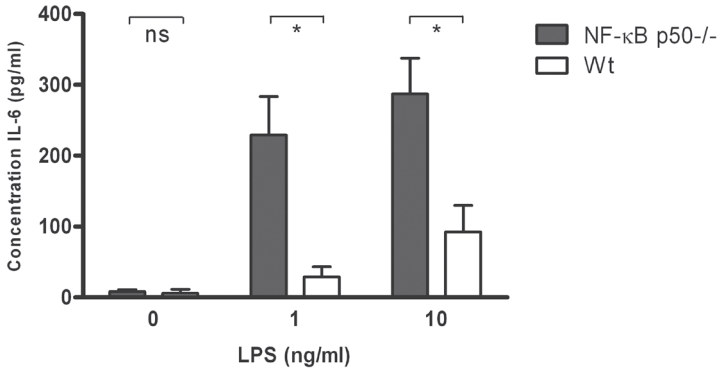

Figure 6. Interleukin-6 (IL-6) production after LPS stimulation of NF-κB p50 -/- and Wt blood
Whole blood of both strains (n= 6/strain) was stimulated with only PBS, or LPS in PBS (1ng/ml or 10ng/ml). PBS incubation evoked no additional cytokine production, while adding LPS dose dependently increased IL-6 production. In absence of the p50 subunit significantly more cytokine was produced. (p<0.05)*

DISCUSSION

This study demonstrates that the absence of the p50 subunit of NF-κB accelerates hindlimb tissue perfusion restoration after occlusion of the femoral artery in mice. The observed effect seems to be attributable to the absence of NF-κB p50 in circulating cells, as normal mice with NF-κB p50 depleted blood cells show similar perfusion restoration as the full NF-κB p50 knock-outs.

Arteriogenesis is considered to be an inflammatory process. Shear stress change and subsequent endothelial activation initiate the remodeling of the anastomosis [28, 29], after which inflammatory cells are recruited to the area. Stimulation of any of these steps will lead to enhancement of collateral growth [30, 31]. As the p50 subunit of NF-κB functions like a brake on NF-κB mediated gene transcription, NF-κB p50-/- mice show pro-inflammatory characteristics [32-34] and hence have an increased arteriogenic response.

Although the shear stress alterations in response to the arterial occlusion activate NF-κB in endothelial cells [35], depletion of NF-κB p50 in tissue alone did not lead to any functional improvement in perfusion recovery. In contrast, bone marrow derived circulating cells seem directly involved in arteriogenesis as transplantation of NF-κB p50-/- bone marrow is enough to improve the reperfusion capacity of a Wt mouse to that of a full NF-κB p50-/-. This is in agreement with previous finding of our group, showing a comparable pivotal role of circulating cells during inflammatory signaling after myocardial ischemia/ reperfusion [36].

During histological examination of the hindlimb tissue, more monocytes or macrophages were found in the vicinity of growing collaterals of the NF-κB p50-/- animals. The quantification of MOMA-2 positive cells in the perivascular space revealed an accelerated and enhanced monocyte extravasation in the NF-κB p50-/- animals, most prominent in the early phase (day 3). This is in concurrence with the time period of maximum monocyte accumulation after acute femoral artery occlusion [37]. After their extravasation, the migrated leukocytes create a local inflammatory environment by producing cytokine in an NF-κB dependent manner. Hence, the observed increased monocyte/macrophage accumulation may be secondary to leukocyte derived chemotactic factors. Therefore, we tested the migratory capacity of NF-κBp50 -/- and Wt cells in an *in vitro* transmembrane migration assay. NF-κB p50-/- monocytes showed an increased migration, which is in accordance with the higher number of macrophages in the tissue. This difference was already evident without addition of chemoattractants. When creating a concentration gradient to MCP-1, more NF-κB p50-/- cells migrated towards this chemoattractant. MCP-1 is a monocyte chemoattractant which signals via CCR2 and previous research has emphasized its involvement in arteriogenesis [38, 39]. Interestingly, NF-κB p50-/- cells even responded to very high concentrations of MCP-1.

The p50 subunit has been described to be responsible for the down regulation of immune receptors after repeated stimulation, such TLR down regulation upon continued LPS stimulation [40]. The CCR2 receptor is regulated by TLR activation [41]. We therefore speculate that the lack of the p50 subunit prevents down regulation of the CCR2 receptor and thus continued chemoattraction can take place.

The increased migratory capability of the NF-κB p50-/- cells could be due to the enhanced integrin expression on the monocyte surface. Integrins are adhesion molecules which are required for the attachment of leukocytes to the vascular wall. This is an important step in the

extravasation of the leukocyte from the blood stream into the tissue. The adhesion process is normally initiated with rolling of the leukocytes over the endothelium slowing them down. For the subsequent firm adhesion to the vessel wall integrins are critically important [42]. Only after this firm adhesion, the cells are arrested and can migrate to the perivascular tissue [43, 44]. The integrin receptors CD11a, CD11b and CD49d were more abundantly present on the surface of the NF-κB p50-/- cells, confirming their enhanced binding capacities.

In conclusion depletion of the NF-κB p50 subunit, particularly in circulating cells, enhances inflammatory cytokine production, monocyte/macrophage migration and accumulation and thereby increases collateral artery growth in response to femoral artery occlusion.

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CONFLICT OF INTEREST

None Declared

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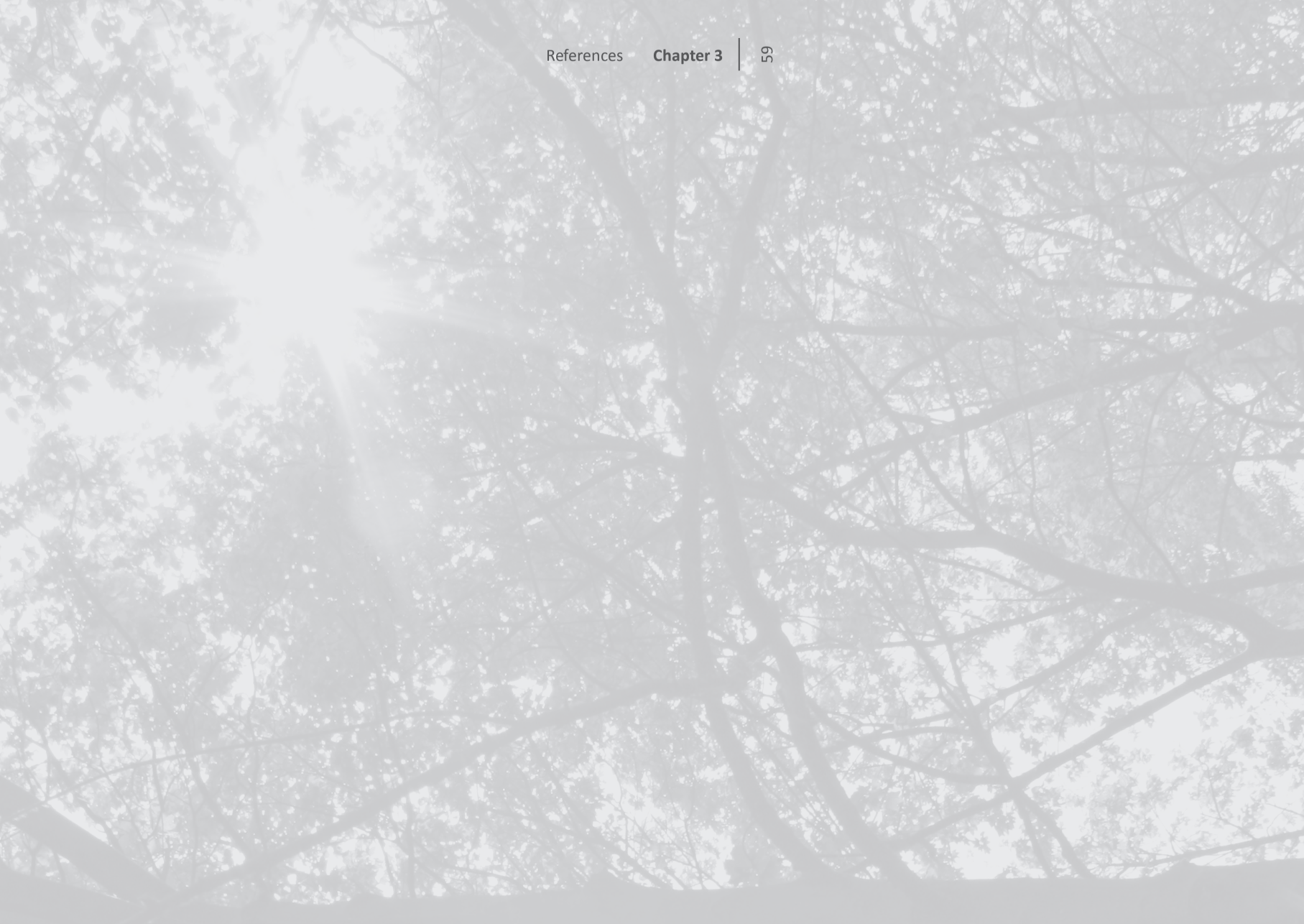

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Cardiovascular risk factors and collateral artery formation Arteriogenesis Requires Toll-Like Receptor 2 and 4 Expression in Bone-Marrow Derived Cells

ABSTRACT

Adaptive collateral growth (arteriogenesis) is an important protective mechanism against ischemic injury in patients with cardiovascular disease. Arteriogenesis involves enlargement of pre-existent arterial anastomoses and shares many mechanistic similarities with inflammatory processes.

Although infusion of the Toll-like receptor (TLR)4 ligand Lipopolysaccharide (LPS) has shown to result in a significant stimulation of arteriogenesis and both Toll-like receptor 2 and 4 are involved in structural arterial adaptations, the requirement for TLRs in arteriogenesis has not yet been established. We therefore subjected TLR2 null and TLR4 defective mice to unilateral femoral artery occlusion. At 7 days, both TLR2 null and TLR4 defective mice showed a significant reduction (~35%) of collateral perfusion. Histological staining showed that TLR2 and TLR4 expression during arteriogenesis is mostly restricted to infiltrating leukocytes. To distinguish between the functional importance of vascular and leukocytic TLRs in arteriogenesis, cross-over bone marrow transplantation was performed 6 weeks before femoral artery occlusion. Perfusion measurements showed that transplantation of wild-type bone marrow into TLR2 null and TLR4 defective mice rescued the impaired arteriogenesis, while injection of TLR2 null and TLR4 defective bone marrow into wild-type mice significantly reduced collateral vessel growth to levels of TLR null/defective mice. RT-PCR analysis demonstrated a significant upregulation of two endogenous TLR ligands EDA and Hsp60 (91.7 fold and 1.9 fold respectively) in regions of collateral vessel formation. This study illustrates the involvement of TLR2 and TLR4 in adaptive collateral artery growth and shows the importance of TLR2 and 4 expression by bone-marrow derived cells for this process.

INTRODUCTION

Adaptive collateral artery growth (arteriogenesis) is an inflammatory process involving the expression and release of pro-inflammatory cytokines, migration of circulating cells into the perivascular tissue and production of growth factors that trigger the proliferation and the maturation of small anastomoses toward functional collateral arteries [1]. During early arteriogenesis, i.e. shortly after an arterial obstruction has become hemodynamically relevant, different leukocyte populations (monocytes, lymphocytes) accumulate in the perivascular space. Previous studies have shown that, besides the changes in hemodynamic forces (altered shear stress patterns), these inflammatory cells are particularly important for an adequate arteriogenic response. Their absence [2, 3] or the blocking of their adhesion and migration [4] significantly hampers arteriogenesis. Vice versa, collateral artery growth can be significantly enhanced by attracting circulating leukocytes to the growing collateral vessels and by promoting their activation, which increases their adhesiveness to the activated endothelium [5-7]. Recent studies support the pivotal role of bone-marrow derived leukocytes as major source of growth factors in a mouse hind limb ischemia model, as bone marrow transplantation of e.g. PlGF null or Bmx null -strains into wild-type mice leads to comparable reductions in vessel growth as observed in the respective null strain [8, 9].

The innate immune system responds non-specifically to the exposure of pathogen associated molecular patterns (PAMPs) and is an important determinant in arterial restructuring. Within the innate immune system, Toll Like Receptors (TLR) play a major role. TLRs do not only recognize PAMPs but also endogenous ligands produced in response to stress or injury such as Heat shock protein 60 (Hsp60) [10-12] and Extra Domain A of alternatively spliced fibronectin (EDA) 13. This indicates that the tasks of the innate immune system are much more extensive than protection from the outside world only [14].

As part of the innate immune system, monocytes/macrophages can be activated by TLR ligand binding. Particularly the membrane bound TLR 2 and TLR 4 have been shown to be involved in leukocyte activation, triggering a cascade ultimately resulting in NFKB activation and inflammatory cytokine release. Among these cytokines, a number of factors have previously been identified as potent angiogenic and arteriogenic mediators (e.g. TNF-alpha, IL-8 or MCP-1). Exogenous activation of TLR 4 by infusion of lipopolysaccharide (LPS) into the collateral network of New Zealand White rabbits after femoral artery occlusion has previously been shown to significantly increase collateral conductance as a measure of collateral artery growth [15], indicating the involvement of exogenous TLR ligands during arteriogenesis.

TLR 2 and TLR 4 have also been shown to be involved in structural arterial adaptations such as neointima formation and arterial remodeling [16-18]. These processes are accompanied by an increased expression of the endogenous TLR ligands Hsp60 and EDA. Fibronectin expression is increased in growing collateral arteries [19, 20], indicating its potential role as an endogenous TLR ligand in this process.

The first event in arteriogenesis is a change in flow within the collateral anastomoses. It has previously been shown that TLR 4 is involved in flow induced outward arterial remodeling [21]. Considering the involvement of TLR 2 and TLR 4 in adaptive arterial remodeling and the eminent role of TLR expressing leukocytes in collateral artery growth, we furthermore hypothesized that arteriogenesis is impaired in the absence of leukocytic TLR 2 or TLR 4.

The data presented in this study provide genetic evidence for the involvement of TLR 2 and TLR 4 in arteriogenesis. Moreover, they show a pivotal role of bone-marrow derived TLR expression and point to a role for endogenous TLR ligands during collateral artery growth.

MATERIALS AND METHODS

This study was performed after securing appropriate institutional approvals. It conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Mouse hind limb model and hemodynamic perfusion measurements

All mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The right femoral artery was ligated under sterile conditions in 6 TLR 2 null (Tlr 2^{tm1Kir}, N9 backcross onto C57BL/6J), 8 TLR 4 defective mice (C.C3-Tlr 4^{LPS-d}, N20 backcross onto BALB/c) [22, 23] and their respective background (n=10 C57BL/6J, n=6 BALB/c) distal to the inguinal ligament as previously described [21]. The contra lateral hind limb was sham-operated.

Seven days after femoral artery occlusion animals were again anesthetized and heparinized for hemodynamic perfusion measurements to assess collateral artery growth as previously described [24]. In short, the abdominal aorta was cannulated with the tip of the catheter located proximal to the aortic bifurcation. For assessment of collateral artery function, the aortic canula was connected to a pressure controlled perfusion system and both hind limbs were perfused with fluorescent microspheres at different perfusion pressures. Maximal vasodilatation of the collateral network was achieved by continuous adenosine infusion (1 mg kg^{-1} min^{-1}). Differently labeled fluorescent microspheres were injected at different pressure levels. Perfusion restoration is expressed as percentage of the perfusion in the occluded hind limb relative to the non-occluded hind limb.

Immunofluorescence staining

Frozen tissue sections (7 μm) from thigh muscles obtained 3 days after femoral artery occlusion were fixed in ice-cold acetone and stained with a polyclonal rabbit anti-mouse antibody against TLR 2 (Imgenex, San Diego, U.S.A) or a polyclonal rabbit anti-mouse antibody against TLR 4 (Abgent, San Diego, U.S.A.) respectively. An Alexa555-labeled goat anti-rabbit antibody (Invitrogen) was used as a secondary reagent. Collateral arteries were visualized with a FITC-conjugated mouse anti-alpha smooth muscle actin antibody (Sigma Chemical Company, St. Louis, MO) and nuclei were stained with Hoechst 33342 dye. Pictures were taken at a 400-fold magnification on an Olympus BX60 microscope and fluorescent images were adjusted (filter overlay, background subtraction) using analySIS software 3.0 (Soft-Imaging, Muenster, Germany).

Monocyte/macrophage accumulation in the perivascular tissue was detected as previously described [4], using a mouse monocyte/macrophage specific antibody against MOMA-2 (Abcam, Cambridge, UK). Collateral vessels were visualized as described above. Photomicrographs were taken at 400-fold magnification. The number of monocytes/macrophages around muscular collateral arteries was counted by a blinded observer and expressed as number of MOMA-2 positive cells/collateral vessel.

Bone marrow transplantation

Bone marrow transplantations were performed in a cross-over design. At the age of 6 weeks, mice were lethally irradiated at a dose of 700 cGray (1030 monitor units) before bone marrow transplantation. Bone marrow was isolated from 4 donor mice by flushing the femoral and the humeral bone with RPMI 1640 (Invitrogen) medium. Each mouse received 4.5 million cells via the tail vein. The animals were allowed to recover for 6 weeks before femoral occlusion. To check whether the bone marrow transplantation and the associated irradiation itself influenced the arteriogenic response, wild-type strains, i.e. BALB/c (n=10) and C57BL/6J (n=7), were treated in the same manner, but received wild-type bone marrow instead of the TLR deficient bone marrow. Perfusion measurements were performed 7 weeks after bone marrow transplantation as described above.

Flow cytometric analysis bone marrow transplantation efficacy

The efficacy of the bone marrow transplantation was tested by flow cytometric whole blood analysis (FC500, Beckman Coulter, Miami, FL, USA) at the time point of the perfusion measurements. Whole blood was collected in heparinized tubes and stained for TLR 2 (anti-mouse TLR 2, FITC conjugate, eBioscience, San Diego, CA, USA) or TLR 4 (anti-mouse TLR 4, PE conjugate, eBioscience) respectively. Monocytes were identified by F4/80 staining (PE-Cy5 conjugate, Invitrogen, Breda, Netherlands) and their specific scatter properties. Transplantation efficacy was determined by the percentage of TLR 2 or TLR 4 expressing monocytes respectively.

Flow cytrometric analysis of TLR expression on leukocytes

TLR 2 and TLR 4 expression was measured on leukocytes at day 3 after operation. Heparinized whole blood samples were stained for 45 min with the following antibodies: TLR 2 (anti-mouse TLR 2, FITC conjugate, eBioscience, San Diego, CA, USA), TLR 4 (anti-mouse TLR 4, PE conjugate, eBioscience), F4/80 (Alexa 647, rat- anti mouse; AbSerotec, Germany) and CD3 (alexa700, eBioscience, San Diego, CA, USA). After erythrocyte lysis and washing with phosphate buffered saline the remaining leukocytes were analyzed by flow cytometry. Monocytes were selected on being F4/80 positive and scatter properties. T-lymphocytes were recognized as CD3 positive. Granulocytes were gated on their destinctive scatter properties.

Quantitative RT-PCR

Tissue samples from the adductor muscle of the occluded and the sham-operated hind limb were homogenized under liquid nitrogen using pestle and mortar. From this residue total RNA was isolated using Tripure® reagent (Roche) according to the manufacturer's instruction. After isolation, total RNA was treated with DNAse (GE Healthcare, Freiburg, Germany) to remove possible genomic DNA. The presence of genomic DNA was tested by PCR without reverse transcriptase. cDNA was then created using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) on 500 ng of total RNA. cDNA was subjected to quantitative reverse transcriptase polymerase chain reaction (RT-PCR), as described before [25], using a dilution series of the cloned amplicon for the generation of standard curve. The following oligonucleotide primers were used: EDA (forward: 5'-acgtggttagtgtttatgctc-3'; reverse: 5'-tggaatcgacatccacatcag-3') and Hsp60 (forward: 5'-accgtctattgccaaggag-3'; reverse: 5'- cagcaattacagcatcaacag-3'). All mRNA expression levels were normalized for 18s rRNA (forward: 5'-tcaacacgggaaacctcac -3'; reverse:

5'-accagacaaatcgctccac-3') and expressed as pg plasmid. The identity of the amplified PCR product was confirmed by automated sequence analysis (Hubrecht Laboratory, Utrecht, Netherlands)

Statistical analysis

Results are presented as mean ± SD. All statistical intergroup comparisons except RT-PCR analyses were performed with SPSS 15 software (SPSS Inc., Chicago) using Student's t-test for 2 groups and one way analysis of variance (ANOVA) and post-hoc Bonferroni correction for comparisons of more than two groups. Wilcoxon signed ranks test was used for paired comparisons of mRNA expression (paired analysis of the expression levels of the occluded and the sham-operated hind limb per animal).

RESULTS

Perfusion restoration in TLR 2 null and TLR 4 defective mice

Seven days after femoral artery ligation, microsphere-based perfusion measurements under maximal vasodilatation showed a significant reduction of perfusion restoration in mice lacking TLR 2 compared to wild type (relative perfusion: C57BL/6J: 54.9 ± 5.29%; TLR 2 null: 36.7 ± 1.77%; $p<0.001$) and mice without functional TLR 4 compared to wild type (BALB/c: 25.6 ± 5.07%; TLR 4 def.: 15.8 ± 5.67%; $p<0.001$) (figure 1).

As previously described, C57BL/6J mice showed a faster restoration of collateral perfusion compared to BALB/c mice [26-28].

TLR expression in arteriogenesis

Since not only leukocytes (monocytes, granulocytes) but also e.g. endothelial cells have been shown to express TLRs, we performed immunofluorescence staining to determine the localization of TLR 2 and TLR 4 in collateral arteries. Sections from murine thigh muscles were stained for TLR 2 and TLR 4. Staining of both TLR 2 and TLR 4 was observed in infiltrating cells only present in the perivascular tissue of hind limbs subjected to prior femoral artery ligation (figure 2a).

In the circulation, TLR 2 and 4 were highly expressed on monocytes (95 ± 2%) and to lesser extent on the granulocytes (77± 16%). Lymphocytes hardly showed any TLR 2 or 4 staining (6± 13%). Single receptor expression as well as co-expression of both TLR 2 and TLR 4 was observed. Monocytes had both TLR 2 and TLR 4 in 30± 7% of the cells, 64± 7% only expressed TLR 2 and just 1± 1% of the population expressed only TLR 4.

Monocyte/macrophage accumulation around collateral arteries

Monocyte/macrophage migration and accumulation around the growing collateral arteries plays an important role during arteriogenesis. Previous studies have indicated the functional involvement of TLRs, in particular TLR 2 and TLR 4 in monocyte/macrophage migration and accumulation [29-31]. Therefore, we quantified monocytes/macrophage accumulation in the surrounding tissue of growing collateral vessels on day 3 after femoral artery occlusion in the different mouse strains. Both, TLR 2 null and TLR 4 defective mice showed significantly reduced monocyte/macrophage numbers compared to the corresponding wild type mice (monocytes/

macrophages per collateral artery: C57BL/6J: 2.85 ± 0.15; TLR 2 null: 1.81 ± 0.31; p<0.05; BALB/c: 2.33 ± 0.20 TLR 4 def.: 1.63 ± 0.12; p<0.05) (figure 3).

To check whether baseline blood levels of monocytes were influencing the observed differences we tested the amount of circulating monocytes in all mouse strains. There were no differences found between the C57B6 and TLR2 null (1.32±0.35 x 10^5 versus 1.16±0.13 x 10^5 monocytes

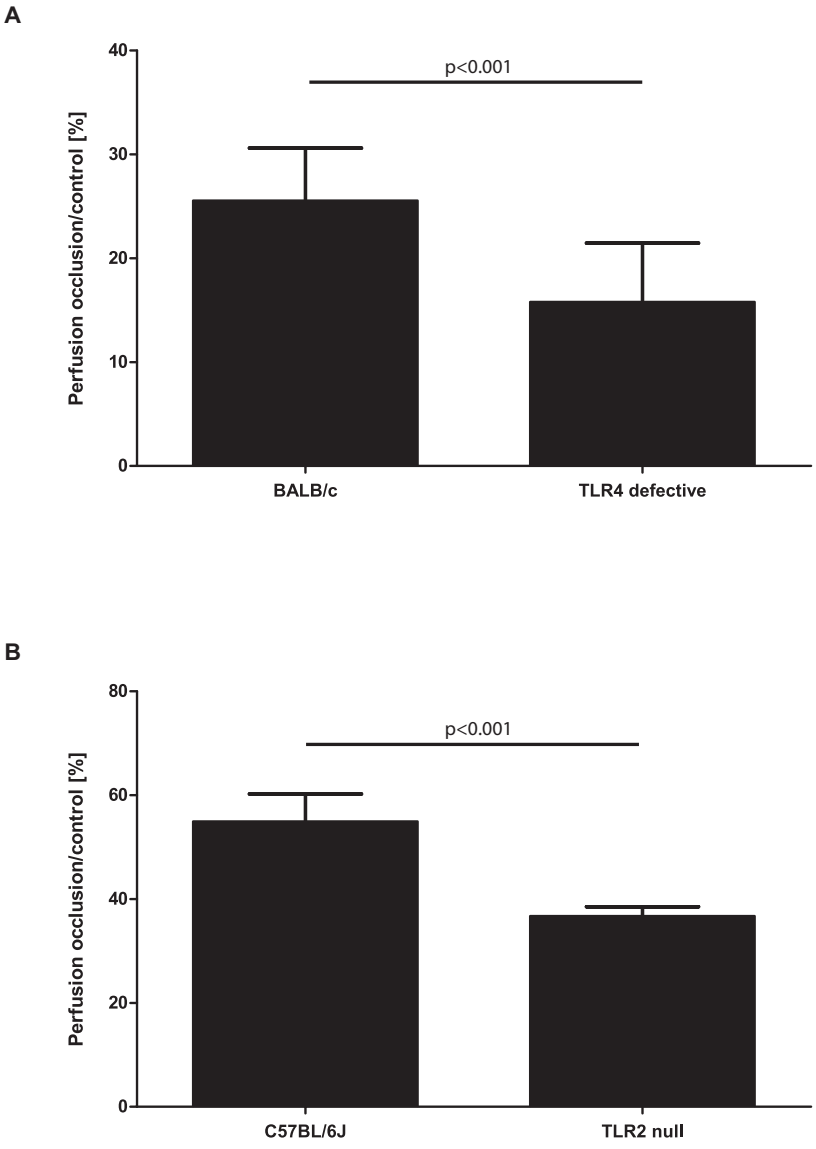


Figure 1.

Microsphere-based perfusion restoration one week after femoral artery occlusion in wild-type (C57BL/6J n= 10, BALB/c n= 6) and TLR 2 -/- (n= 6) and TLR 4 defective (n= 8) mice respectively. Perfusion recovery is expressed as a percentage of the perfusion of the non-occluded, sham-operated hind limb. The values are expressed as mean +/- SEM and statistical analysis was performed with a student's t-test. p < 0.05 was regarded as significant.

per ml blood, p= ns) or the BALB/c and TLR 4 defective mice (0.80±0.21 x 10^5 versus 0.82±0.08x 10^5 monocytes per ml blood, p=ns) (supplemental figure 1). There were however more monocytes in the animals of a C57B6 background (p=0.03), which could correspond with the higher macrophage levels in the tissue.

Perfusion restoration after bone marrow transplantation

Having determined that TLR 2 and TLR 4 are involved in arteriogenesis and that infiltrating cells show the strongest TLR 2 and TLR 4 staining in the surrounding of growing collateral arteries, we examined whether the observed impaired arteriogenic response in TLR-deficient mice can be attributed to TLR expression on leukocytes rather than to expression by other vascular cell types (e.g. endothelial cells, smooth muscle cells). Therefore, bone marrow transplantation was performed in a cross-over design.

To determine if bone marrow transplantation was successful, flow cytometry analysis of TLR expression on peripheral monocytes was performed at day 7 after femoral artery occlusion. After wild-type bone marrow transplantation into TLR 2 null mice, 71.8 ± 14.1 % of circulating monocytes exhibited TLR 2 expression (supplemental figure 2).

Animals with less than 70% TLR 2 positive cells were excluded from the perfusion measurements. Transplantation of TLR 2 null bone marrow in C57BL/6J mice led to an almost

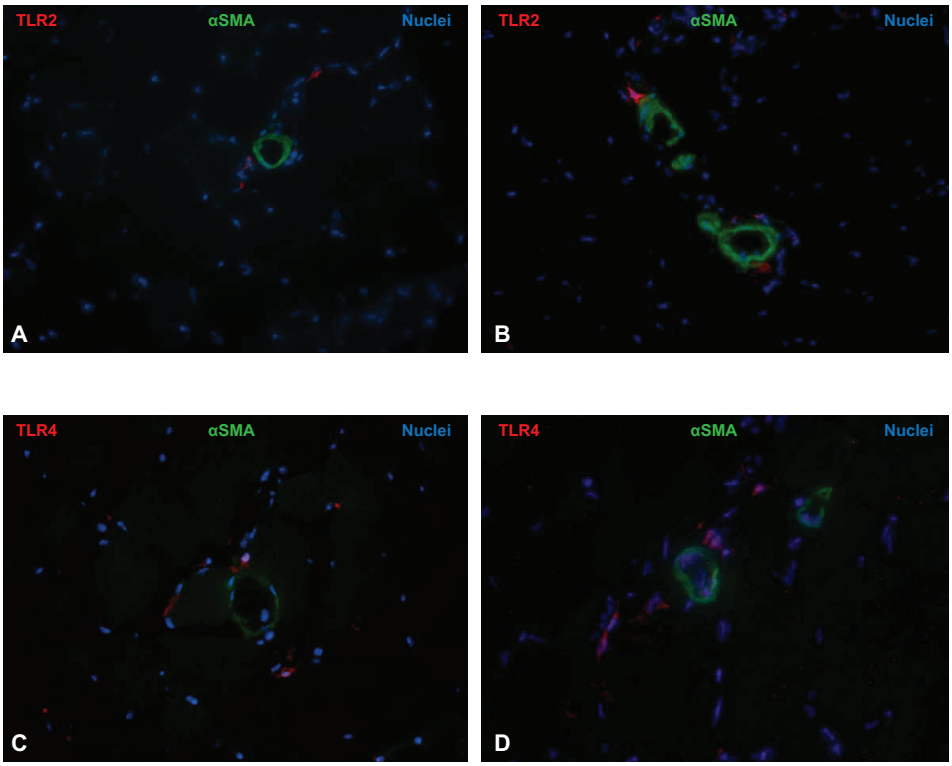


Figure 2.
Immunofluorescence staining for TLR 2 (A, B) and TLR 4 (C, D) on hindlimb adductor muscle. The pictures were taken of representative sections at a 400x magnification. Arteries were identified by alpha smooth muscle actin staining (green). TLR expression (red) was mainly limited to infiltrating cells. Nuclei were stained with Hoechst 33342 (blue).

complete deficiency for TLR 2, as only 3.29 ± 2.40% of monocytes expressed TLR 2 (supplemental figure 2). Flow cytometry analysis of TLR 4 expression in the respective mice is not conclusive as, despite the functional defect of the TLR 4 receptor, antibodies still detect the receptor (data not shown). Therefore, all animals were included in the study population.

Quantification of perfusion restoration showed that, after injection of TLR 2 null or TLR 4 defective bone marrow into wild-type mice, collateral perfusion significantly decreased to levels that did not differ from non-irradiated TLR null mice (TLR 2 null bm into C57BL/6J, n=9: 38.8 ± 3.23%; TLR 4 defective bm into BALB/c, n=9: 16.7 ± 1.57%). Injection of wild-type bone marrow into TLR deficient animals almost completely restored the arteriogenic deficit to levels comparable to those of wild-type animals (C57BL/6J bm into TLR 2 null, n=8: 46.5 ± 5.55%; BALB/C bm into TLR 4 defective, n=9: 22.6 ± 3.34%) (figure 4).

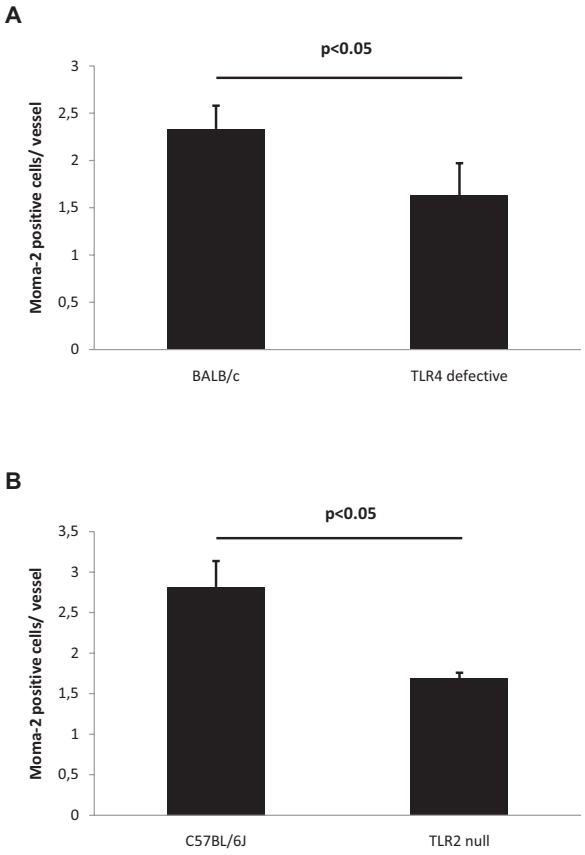


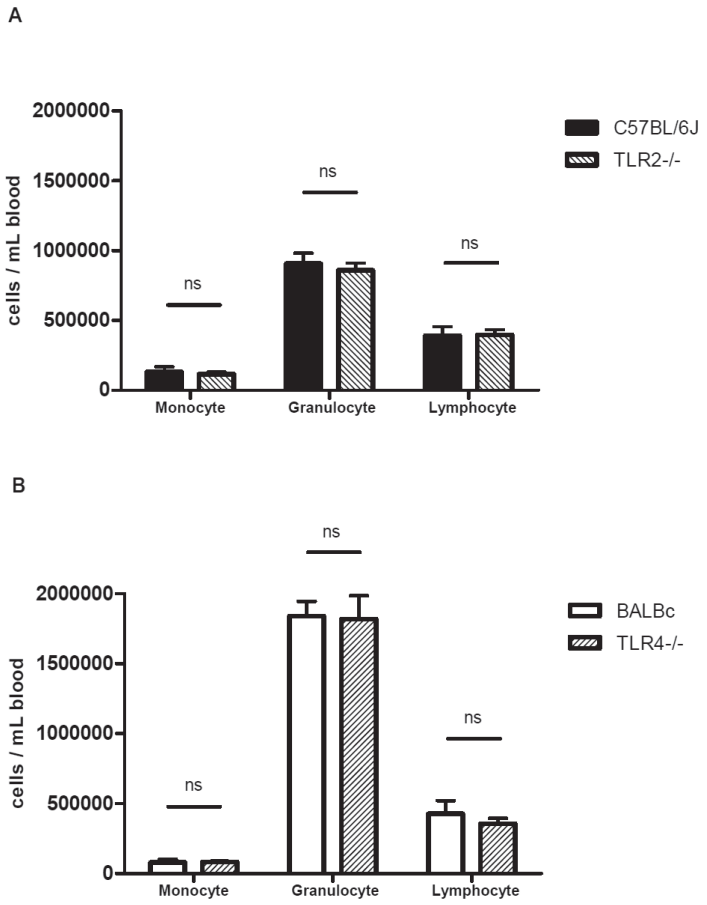
Figure 3.

Quantitative analysis of monocyte/macrophage accumulation in the perivascular tissue of growing collateral arteries. Collateral arteries were visualized by an αSMA staining and a combination of MOMA-2 and Hoechst were used to identify the monocytes/ macrophages. Six random sections were counted per mouse and the average per mouse was used for the analysis. In TLR 4 defective mice (figure A) and TLR 2 -/- (figure B), monocyte/macrophage accumulation was significantly reduced compared to their respective wild type mice. The number of mice which were included in the analyses: 8 TLR4 defective, 11 BALB/c, 6 TLR2 -/- and 10 C57BL/6J mice. The values are expressed as mean +/- SEM and statistical analysis was performed with a student's t-test. p < 0.05 was regarded as significant.

Transplantation of wild-type bone marrow in wild-type mice showed no difference with non-irradiated wild-type mice (relative perfusion: C57BL/6J bm, into C57BL/6J, n=7: 49.7 ± 2.59%; BALB/c bm into BALB/c, n=10: 23.1 ± 1.01%), showing that the transplantation protocol did not significantly influence collateral artery growth.

Expression of potential endogenous TLR ligands during arteriogenesis

The observed reduction in collateral dependent perfusion in mice lacking either functional TLR 2 or TLR 4 strongly suggests the presence of an endogenous TLR ligand expressed during arteriogenesis.



Supplemental figure 1.
Blood leukocyte composition was analyzed by flow cytometry. Heparinized blood samples of 8 mice per group were analyzed for the presence of monocytes, (T-) lymphocytes and granulocytes. There were no differences observed between the TLR 2 null (A) or the TLR 4 deficient (B) mice and their corresponding wild types (respectively C57BL/6J and BALB/c) in any of the cell lineages. In this experiment monocytes were gated as F4/80 positive and on their specific scatter properties. (T-) lymphocytes were CD3+ and granulocytes were defined as Ly6G positive in combination with a high sideward scatter. Quantification of the cells was obtained by adding a known concentration of counting- beads to the sample prior to the measuring and calculation of cells concentration with help of the bead reference afterwards. A Student's T test was performed for each comparison and p< 0.05 was regarded as significant.

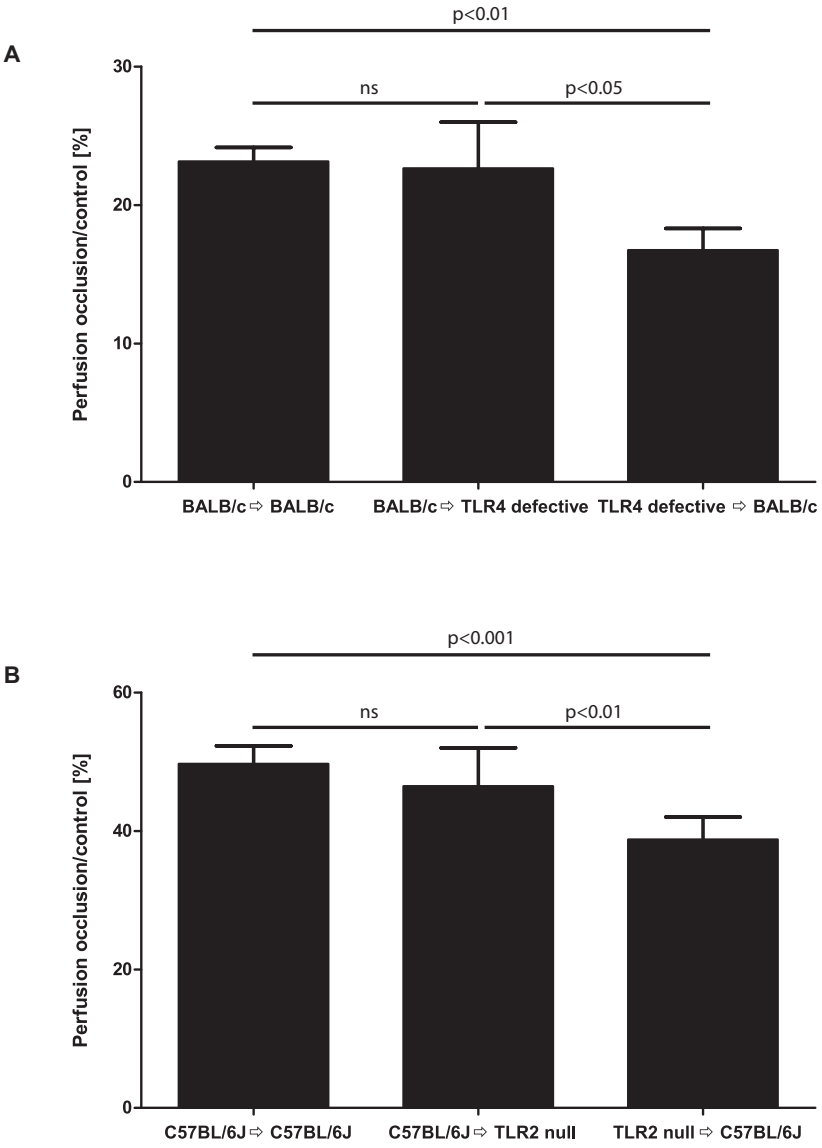


Figure 4.

Perfusion restoration one week after femoral artery occlusion in mice 6 weeks after bone marrow transplantation, measured with fluorescent microspheres. While there was no significant difference between untreated WT mice (n= 6 for BALB/c and n= 10 for C57BL/6J) and WT mice that were irradiated and subsequently received WT bone marrow (n=10 for BALB/c and n= 7 for C57BL/6J), TLR deficient bone marrow transplantation into WT mice significantly reduced collateral perfusion (n= 9 for TLR4 deficient into BALB/c and n=9 for TLR2 null into C57BL/6J). Vice versa, WT bone marrow transplantation into TLR deficient mice restored the arteriogenic response (n= 9 for BALB/c into TLR4-/- and n= 8 for C57BL/6J into TLR2-/-).
The data is presented as a percentage of sham hindlimb perfusion, which was set at a 100%. All data presents the mean value ±SEM and a one way analysis of variance (ANOVA) with Bonferroni correction was done to test for differences. P< 0.05 was regarded as significant.

Therefore, we performed RT-PCR analysis of mRNA expression of the extra-domain A (EDA) of fibronectin as a known TLR 4 ligand and of Hsp60 as a known TLR 2 ligand on day 3 after femoral artery occlusion. RT-PCR analysis showed that after femoral artery occlusion both TLR ligands are significantly upregulated as compared to sham-operated hind limbs (EDA expression: occlusion: 0.88 ± 0.77; sham: 0.0096 ± 0.012 pg plasmid) (Hsp60 expression: occlusion: 0.0050 ± 0.0049; sham: 0.0026 ± 0.0021 pg plasmid) (figure 5).

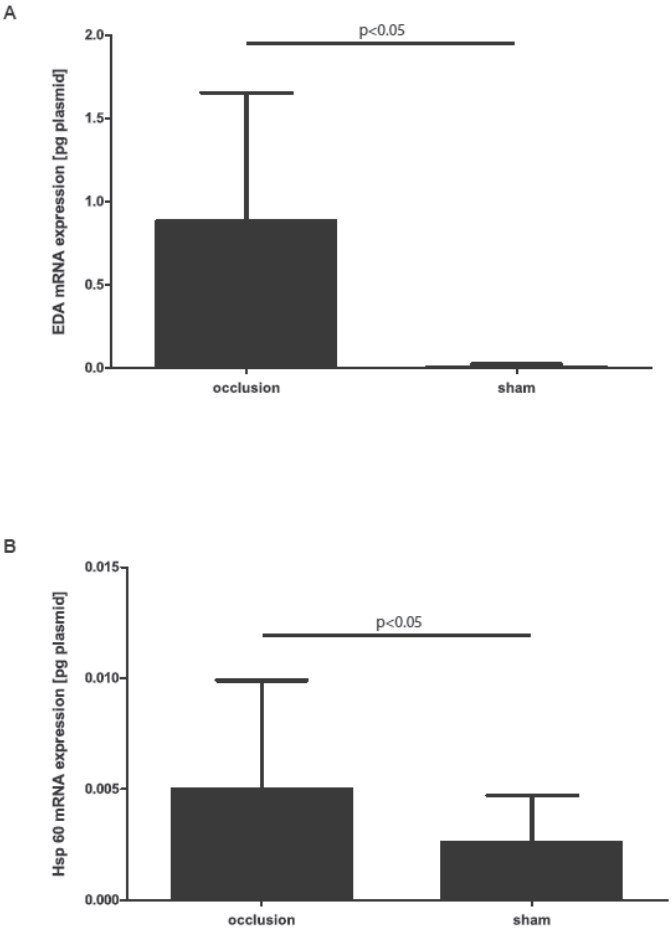
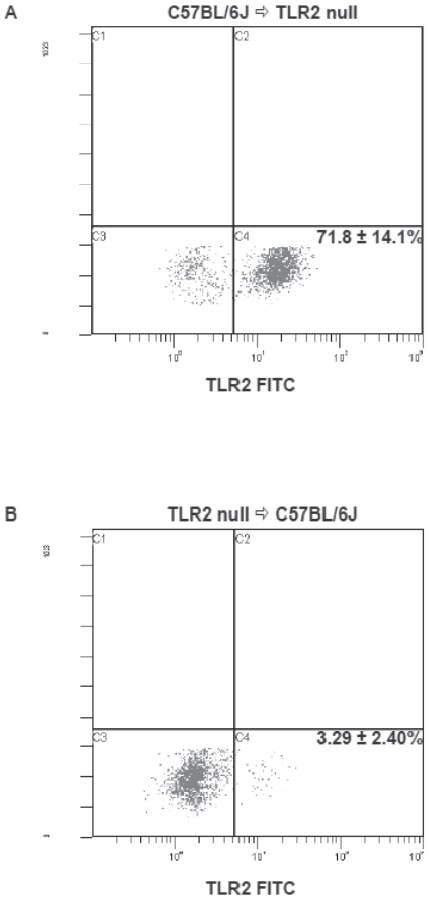


Figure 5.

Quantitative RT-PCR of the endogenous TLR ligands Hsp60 (n=9) and extra domain A of fibronectin (n=7) in WT mice. After femoral artery occlusion both ligands are significantly upregulated in the adductor muscle. The contralateral hind limbs were sham-operated to rule out any effect of the surgery on ligand expression and served as control. The presented values are mean ± SEM and differences were tested by a Wilcoxon signed ranks test (paired analysis of the expression levels of the occluded and sham-operated hind limb per animal). P< 0.05 was regarded as significant.



Supplemental figure 2.

FACS analysis of TLR 2 expression on peripheral blood monocytes 6 weeks after bone marrow transplantation. Only a minor percentage of cells were still derived from the host organism, indicating the efficacy of the transplantation protocol. C57BL/6J transplantation into TLR2 null mice resulted in 71.8 ± 14.1 % of circulating monocytes exhibiting TLR 2 expression and vise versa transplantation of TLR 2 null bone marrow in C57BL/6J mice led to only 3.29 ± 2.40% of monocytes expressing TLR 2. The images show representative pictures of the all samples analyzed (n=9 for TLR2 null into C57BL/6J mice and n= 8 for C57B6 into TLR2 null mice)

DISCUSSION

Collateral growth can be induced by infusion of an exogenous TLR 4 ligand. However, the role of TLRs themselves in this process yet remains unknown. Our results show the involvement of TLR 2 and 4 in collateral artery growth. Bone marrow transplantation showed that TLR 2 and 4 present on bone marrow derived cells are pivotal for collateral formation and endogenous ligands of the Toll-like receptors 2 and 4 are expressed at the site of collateral artery growth.

Involvement of TLRs in arteriogenesis

Lipopolysaccharide (LPS), a membrane component of gram negative bacteria is the classical TLR 4 ligand. It induces a strong inflammatory response by binding to a receptor complex of TLR 4, CD14 and MD-2 [32], increasing the expression and the release of pro-inflammatory cytokines such as TNF-alpha [33] or the C-C chemokines RANTES and MCP-1 [34]. These chemokines in turn lead to the attraction of leukocytes to the site of the highest concentration to limit the extent of the damage. Ligand binding to TLR 2 induces a comparable response.

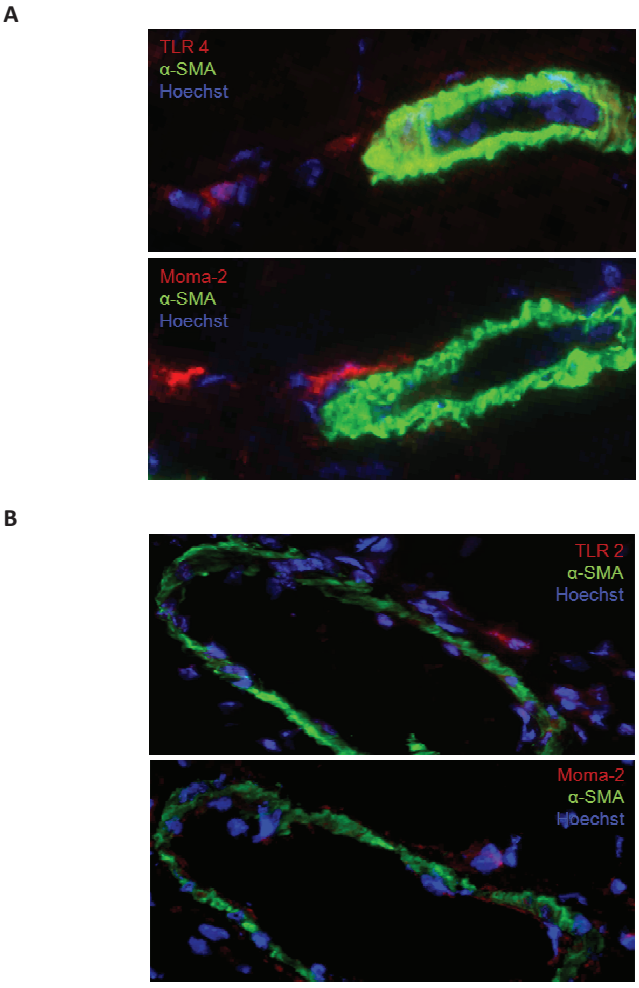
Leukocyte migration and accumulation is a typical feature of a variety of processes that can be observed in cardiovascular pathophysiology, e.g. atherosclerosis but also post-natal vessel growth, i.e. angiogenesis and arteriogenesis. The common downstream signaling molecule of many TLRs (-1, -2, -4, -5, -6, -7, -9 and -11), MyD88 proves to be important in wound healing, by influencing blood vessel growth [35]. Activation of TLR 2 or TLR 4 increases the expression of angiogenic factors like VEGF [36-38]. Furthermore, several studies have shown that the TLR system is involved in atherosclerotic plaque development and plaque progression. TLR 4 is also involved in flow-induced outward arterial remodeling [21]. In this model, arterial enlargement of the carotid artery is induced by occlusion of the contralateral one. The compensatory increase in shear stress in the non-occluded artery induces growth and lumen expansion. Arteriogenesis after arterial occlusion shares many common mechanisms with the latter situation, in particular the dependence on increased shear stress in pre-existing arteries [39]. Our studies investigating the arteriogenic response in TLR 2 null and TLR 4 defective mice further support these findings and are in concordance with a previous study in a rabbit hind limb model showing an improved arteriogenesis upon LPS injection [15]. The second important component of arteriogenesis - besides flow changes - is the invasion of circulating leukocytes into the perivascular tissue of growing collateral arteries. TLR activation results in expression of cytokines such as MCP-1 or RANTES, guiding and attracting leukocytes to the site of inflammation. These cells provide the milieu and growth factors needed for the maturation of the collateral vessels. In our model TLR 2 and 4 expression on circulating cells seemed to appear in two populations: either expressing only TLR 2, or expressing TLR 2 and TLR 4 combined. Further research is needed to investigate the actual role of those two subsets of monocytes in arteriogenesis. Both, TLR 2 null and TLR 4 defective mice showed significantly reduced monocyte/macrophage accumulation in the perivascular tissue of growing collateral arteries compared to their wild types. This decreased extravasation is likely to be responsible for the attenuated perfusion restoration. This effect might either be due to the absence of leukocytic TLR or secondary to TLR mediated chemokine expression in the collateral vessels.

The observed difference in tissue macrophages between the two wild type mice strains (e.g. C57BL/6J and BALB/c) has not been reported before, but could be explained by the genetic backgrounds. [26, 27, 40] Blood monocyte counts between BALB/c and C57BL/6J differ at baseline

(supplemental figure 1) and in combination with the previously reported discrepancies in endothelial reaction to stimuli this could be an explanation for the observed difference in tissue macrophages [41, 42]. As cell counts in wild type and knock-out blood were similar, good comparisons could be made between those two.

Role of leukocytic TLR expression

Considering the central role of leukocytes in arteriogenesis, it seems consistent that leukocytic TLR expression is responsible for the observed effect. This is supported by previous studies [8, 9]. Besides leukocytes a variety of other cells involved in vessel growth are also able to express TLRs, e.g. endothelial cells [43]. In atherosclerotic lesions, TLR expression is largely confined to



Supplemental figure 3.
Staining for Moma-2, TLR 2 and TLR 4 on consecutive mouse hindlimb sections. The TLR 2 and TLR 4 are expressed on Moma-2 positive cells. Figure A shows consecutive sections stained for TLR 4 and Moma-2 and figure B for TLR 2 and Moma-2. The arteries are depicted in green, nuclei in blue and TLR 4/ TLR 2 or Moma-2 in red.

endothelial cells and macrophages [44]. In the growing collateral arteries after femoral artery occlusion, TLR expression was mainly restricted to the invading leukocytes (figure 2 and supplemental figure 3). This is in line with a recent study showing that TLRs in non-atherosclerotic arteries are predominantly expressed in the adventitial layers [45]. Nevertheless, we cannot exclude lower TLR expression levels of the endothelium. Therefore, we created chimeric mice by bone marrow transplantation of TLR deficient mice into wild-type mice and vice versa.

A comparable approach recently revealed that both endothelial as well as leukocytic TLR 2 expression are involved in ischemia-reperfusion injury in mice [46]. However, during arteriogenesis TLR expression of bone-marrow derived cells seems to be of major importance since transplantation of TLR deficient bone marrow into WT mice significantly reduces the arteriogenic response to the level of TLR null or defective mice, whereas transplantation of WT bone marrow almost completely restores collateral perfusion in TLR null/defective mice. The differences between WT bone-marrow in TLR 2 null and TLR 4 defective mice and complete WT mice as well as the differences of TLR 2 null and TLR 4 defective bone-marrow in WT mice compared to TLR 2 null and TLR 4 defective mice respectively are not significant. We can, however, not completely rule out contribution of TLR expression on other cells such as endothelial cells. Our findings are supported by the pivotal role of leukocytes in arteriogenesis and previous findings that indicate the importance of TLRs for leukocyte activation and the adhesion and migration of these cells into surrounding tissue [30, 47, 48].

Endogenous ligands

Arteriogenesis, the formation of collateral arteries, is considered to be a sterile process. Hence, the significant decrease in perfusion restoration in TLR 2 null and TLR 4 defective mice in our study cannot simply be ascribed to potential TLR activation by infectious agents. Furthermore, arteriogenesis takes places in spatial distance from the site of arterial obstruction making it even more unlikely that the TLRs are triggered by invading microbes. Considering the observed effects, this implies TLR activation during collateral vessels growth by endogenous ligands.

Although there are many endogenous TLR ligands suggested nowadays we just tested two potential candidates to present as an example: the extra domain A of fibronectin (EDA) and Heat Shock protein 60 (HSP60). While the latter is still controversially discussed, there is general consensus that EDA activates TLR 4 [49-52]. Also, EDA seems to be involved in TLR2 signaling. As we previously could show, absence of functional TLR 2 or TLR 4 results in the abolishment of cell activation by EDA [53]. This finding suggests co-signaling of TLR 2 and TLR 4 upon EDA ligation.

HSP60 and EDA transcription are significantly increased upon femoral artery occlusion as opposed to sham operation. However, whether these ligands are the only endogenous TLR ligand and whether they are indeed actively involved in collateral artery growth needs to be further investigated.

Study limitations

The two TLR deficient strains used in this study are derived from a different genetic background strain, making direct comparisons between them difficult; in particular since BALB/c and C57BL/6J mice differ significantly in their ability to form collateral arteries [23, 24]. However, the aim of this study was not to compare the quantitative contribution of the single TLRs, but to

provide insight into the role of the innate immune system per se during arteriogenesis and the cells involved, which is independent from the genetic background.

Our results only give insight in the involvement of leukocyte TLR2 and 4 receptor in arteriogenesis. Whether the process is impaired or just delayed can not be concluded for this study.

The search for endogenous ligands of the process is very interesting, especially in respect to therapeutic options. For now, we limited the study to show the expression of 2 potential ligands after hindlimb ligation. From this, however, no conclusion can be drawn that these ligands are actually involved in the process.

CONCLUSION

Our study demonstrates an important role for TLR 2 and TLR 4 in collateral artery growth. Furthermore, we show that TLR expression by bone-marrow derived cells play a decisive role in arteriogenesis. This adds to the increasing evidence that bone-marrow derived TLR expression is not only necessary for the defense against exogenous pathogens but also is of eminent importance for physiological adaptive processes such as arteriogenesis. A further elucidation of the beneficial effects of TLR ligands may in the long term result in the development of new tools for the treatment of cardiovascular disease.

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Conflict of interest

None declared

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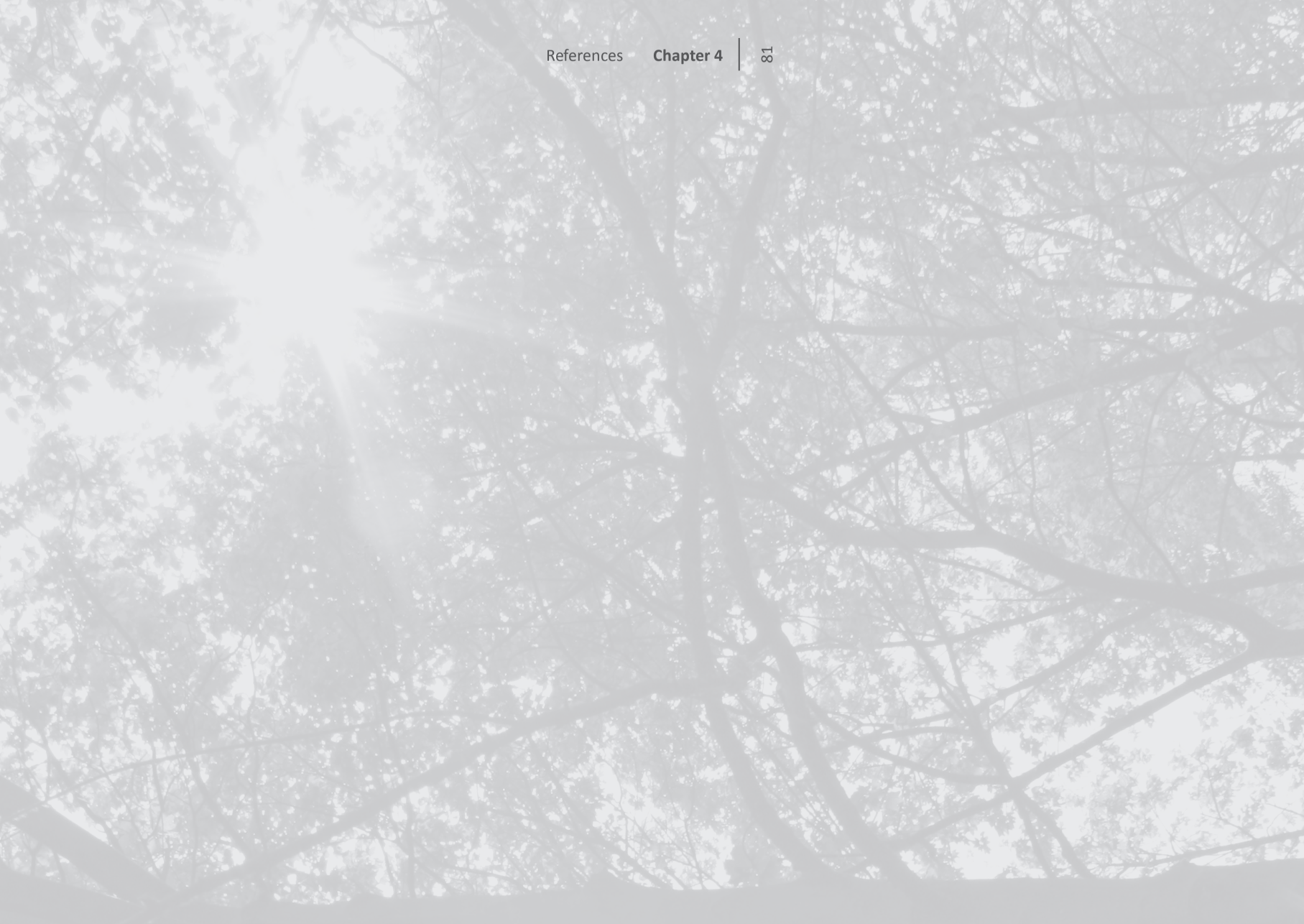

Manuscript in preparation

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5

Absence of Extra Domain A of Fibronectin diminishes perfusion restoration after arterial occlusion

ABSTRACT

Introduction Toll like receptors (TLR) 2 and 4 are involved in perfusion restoration after arterial occlusion. During tissue ischemia and subsequent perfusion restoration the expression of extra domain A of fibronectin (EDA), an endogenous ligand of TLR 2 and 4, is increased. In the present study we explore the role of EDA in perfusion restoration after arterial occlusion .

Methods 44 EDA-/- mice and 44 wild type (WT, Balb/c) mice were subjected to right femoral artery ligation to evoke an arteriogenic response. The left hindlimb was sham-operated and served as the control. At day 3 and 7 tissue was collected for RNA extraction and macrophage staining. Perfusion restoration was measured at day 7, using fluorescent microspheres. Blood was collected at baseline and day 1, 2, 3 and 7 for flow cytometric analysis.

Results EDA mRNA was increased at the site of collateral growth compared to sham (occlusion versus sham: 0,131 versus 0,034 EDA mRNA/ pg plasmid, $p<0.001$). The EDA-/- mice showed an impaired perfusion restoration compared to the WT (22.0 % versus 30.8 %, $p= 0.011$) together with lower macrophage numbers surrounding the collaterals. Monocyte levels in the blood of EDA-/- mice were also lower at baseline and during the perfusion restoration.

Conclusion Absence of EDA results in diminished perfusion restoration showing the involvement of EDA in this process. This is probably due to lower monocyte levels in the blood and a subsequent diminished influx of macrophages into the perivascular tissue, which is essential for proper perfusion recovery.

INTRODUCTION

Toll like receptors (TLR) 2 and 4 have been identified as essential receptors for proper perfusion restoration, due to collateral artery growth (arteriogenesis) after arterial occlusion [1]. This shear stress induced remodeling of pre-existing arterial connections, is an extremely important process for cardiovascular disease patients, suffering from atherosclerosis. The collaterals protect tissue from ischemia and necrosis due to their function as natural bypasses. They are predictive for short and long term outcome, in patients having coronary artery disease [2].

Arteriogenesis is an inflammatory process. Especially monocyte recruitment to the premature collaterals has proven to be important to facilitate the arterial remodeling. Absent TLR2 or 4 results in a reduced monocyte extravasation at the site of collateral growth, thereby leading to a diminished perfusion restoration.

TLRs are receptors of the innate immune system. They are present on all leukocytes, but also on several other cell types, like endothelial cells. The innate immune system is the first line of defense against exogenous pathogens. TLRs classically recognize pathogen associated molecular patterns (PAMPS). Recent studies, however, have shown that the innate immune system also plays an important role in sensing and responding to hazards from the inside via endogenous activators of toll like receptors. The extra domain A of fibronectin (EDA) has been identified as an endogenous ligand for TLR 2 and 4 [3-5].

Fibronectin is an important structural protein present in all vertebrates. It exists in two forms: a soluble form floating in abundance in the plasma and a cellular variant, which is a major component of the extra cellular matrix and highly expressed at the site of collateral growth. Fibronectin is produced from a single gene, but alterations in the splicing of the cellular variant can give rise to several isoforms [6]. Extra domain A of fibronectin (EDA) is such a splice variant. After birth, EDA is only produced during biological processes involving substantial migration and proliferation of cells. Examples of these conditions are vascular stress, tissue damage and ischemia. The presence of the EDA fibronectin enhances the affinity of integrins to bind to the fibronectin [7]. Integrins and transmembrane receptors present on all leukocytes, are principle mediators of interaction between cells and the extra-cellular matrix. The integrins involved in the binding to fibronectin (mainly alpha-5 beta-1), mediate leukocyte adhesion but also provides proliferative signals to vascular cells [8].

Fibronectin-EDA mRNA levels has been shown to increase after collateral growth and outward arterial remodeling [9]. Furthermore, fibronectin-EDA can lead to TLR activation, an important processes in perfusion restoration. Based on this, we hypothesized that EDA is involved in perfusion restoration. Until now, it is unknown if EDA plays a causal role in this process.

In the current study we investigated the involvement of EDA in arteriogenesis, by studying the process in mice which genetically lack the ability to form the EDA splice variant (EDA-/- mice) and their corresponding wild type (Balb/c) mice. The identification of a causal role for EDA-fibronectin in perfusion restoration makes it a potential target for future arteriogenic therapies, in patients suffering from arterial obstructive disease.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and prior approval was given by the Animal Ethical Committee of the faculty of Medicine, Utrecht University, the Netherlands. To investigate the role of EDA in arteriogenesis 44 EDA-/- (UC Davies School of Medicine, California) and 44 Balb/c wild-type (Wt) mice (Harlan, the Netherlands) (10-14 weeks old) were studied. Male as well as female mice were used and they were equally distributed between both study groups to exclude any gender effect.

Mouse hind limb model and hemodynamic perfusion measurements

To study collateral growth, 11 EDA-/- and 11 Balb/c mice underwent ligation of the right femoral artery distal to the inguinal ligament, as previously described [10]. The contralateral artery was sham-operated and served as an intra- individual control. Analgesia and anesthesia were induced by subcutaneous injection of 0.1 mg/kg buprenorphine and isoflurane inhalation. Body temperature was maintained optimal during the operation by placing the animals on a heating pad.

Seven days after the arterial occlusion animals were again anesthetized and heparinized for hemodynamic perfusion measurements to assess collateral artery growth [11]. In short, the abdominal aorta was cannulated with the tip of the catheter located proximal to the aortic bifurcation. For assessment of collateral artery function, the aortic canula was connected to a pressure controlled perfusion system and both hind limbs were perfused with fluorescent microspheres (Fluospheres, 15µm, Molecular Probes Inc., Eugene Oregon) at different perfusion pressures. Maximal vasodilatation of the collateral network was achieved by continuous adenosine infusion (1 mg kg^{-1} min^{-1}). Differently labeled fluorescent microspheres were injected at different pressure levels. After infusion of the microspheres, both right and left calf muscles were harvested, after which they were weight and digested. The number of microspheres in each sample was determined by flow cytometry (Beckman Coulter, Miami, Fl, USA). Perfusion restoration was expressed as percentage of the perfusion in the occluded hind limb relative to the non-occluded hind limb.

Quantitative RT-PCR

Tissue samples from the adductor muscle of the occluded and the sham-operated hind limb of 11 Balb/c mice were snap frozen in liquid nitrogen and homogenized using pestle and mortar. From this, residue total RNA was isolated using Tripure® reagent (Roche) according to the manufacturer's instruction. After isolation, total RNA was treated with DNAse (GE Healthcare, Freiburg, Germany) to remove possible genomic DNA. The presence of genomic DNA was tested by PCR without reverse transcriptase. cDNA was then created using the iScript cDNA synthesis kit (Bio-Rad, Herculus, CA) on 500 ng of total RNA. cDNA was subjected to quantitative reverse transcriptase polymerase chain reaction (RT-PCR), as described before [12], using a dilution series of the cloned amplicon for the generation of standard curve. The following oligonucleotide primers were used: EDA forward: 5'-acgtggttagtgtttatgctc-3' and reverse: 5'-tggaatcgacatccacatcag-3'. All mRNA expression levels were normalized for 18s rRNA (forward:

5'-tcaacacgggaaacctcac -3'; reverse: 5'-accagacaaatcgctccac-3') and expressed as pg plasmid. The identity of the amplified PCR product was confirmed by automated sequence analysis (Hubrecht Laboratory, Utrecht, Netherlands)

Immunofluorescense staining

Frozen tissue sections (7 μm) from thigh muscles, obtained 3 days after femoral artery occlusion (11 mice per stain), were fixed in ice-cold acetone. Blocking was performed with Cytomation Biotin blocking system (DAKO, Heverlee, Belgium) followed by 1 hour incubation in 5% milk/ PBS. To identify monocyte/macrophage accumulation in perivascular tissue we used a mouse specific antibody against these cells: rat anti mouse monoclonal MOMA-2 (1mg/ml, Millipore) 1:50 in 1% milk/PBS. This was followed by goat anti rat-biotin (1mg/ml, Southern Biotech, Alabama, USA) 1:250 in 1% milk/PBS and streptavidin-alexa fluor 555 (Invitrogen, Breda, the Netherlands) 1:1000 in 1% milk/PBS. Collateral arteries were visualized with a FITC-conjugated mouse anti-alpha smooth muscle actin antibody (Sigma Chemical Company, St. Louis, MO) and nuclei were stained with Hoechst 33342 dye. Isotype rat IgGb (ABserotec) was used as negative control. The sections were inspected under a microscope (Olympus BX60) and pictures were taken (Olympus DP71) at a 400x magnification. The fluorescent images were adjusted (filter overlay, background subtraction) using analySIS software 3.0 (Soft-Imaging, Muenster, Germany). Monocyte/macrophages accumulating in the perivascular tissue were counted by two independent blinded observers in at least 6 tissue sections/ animal and expressed as the number of MOMA2 positive cells/ αSMA positive artery.

Blood monocyte counts and receptor expression

Monocyte numbers and activation were determined in the WT and EDA-/- mice. Whole blood samples were collected before and after arterial ligation by puncturing the vascular plexus of the cheek. A total of 22 EDA-/- and 22 Balb/c mice donated blood, either at day 0, 2 and 3, or day 1 and 7 after ligation and samples were collected in heparinized tubes to prevent coagulation. The following antibodies were added to the samples: F4/80- alexa647 (rat- anti mouse; AbSerotec, Germany), CD11b-FITC (rat- anti mouse; Bioconnect, the Netherlands). All samples were incubated for 45 minutes in the dark. Counting beads were added as a reference before washing and lyses of the erythrocytes. The remaining leukocytes were analyzed by flow cytometry (FC500, Beckman Coulter, Miami, Florida, USA). Monocytes were selected on being F4/80 and CD11b positive, combined with their scatter properties. The amount of receptors on the monocyte surface were expressed by their mean fluorescent intensity (MFI).

Statistical analysis

Results are presented as mean ± SD. All statistical intergroup comparisons except RT-PCR analyses were performed with SPSS 17 software (SPSS Inc., Chicago) using Mann-Whitney U test for 2 groups and Kruskal Wallis test of more than two groups.

RESULTS

Fibronectin-EDA mRNA levels are increased after arterial occlusion

Three days after right femoral artery ligation, adductor muscle tissue was collected to determine EDA mRNA levels during perfusion restoration. This was compared to a sham operation of the left hindlimb, to exclude any effect of the operation and manipulation of the femoral artery. The ligated site showed higher EDA mRNA levels in comparison to the sham-operated side (occluded vs. sham ligated side: 0,131 vs. 0,034 EDA mRNA/ pg plasmid, p<0.001) (figure 1).

Perfusion restoration after arterial occlusion is decreased in EDA knock-out mice

To test whether the presence of EDA after arterial occlusion plays a causal role in arteriogenesis, we examined the perfusion restoration in mice lacking fibronectin-EDA. At 7 days after ligation, EDA-/- mice showed only 22% perfusion restoration in their right ligated hindlimb (compared to the left leg, which was set at 100% perfusion). This was significantly lower than the 30.8% in the wild type (WT) mice (p= 0.011) (figure 2).

Number of perivascular macrophages around collaterals are lower in the EDA -/- than in wild type mice

Monocytes/macrophage accumulation in the surrounding tissue of growing collateral vessels were determined before ligation and at day 3 and 7 after femoral artery ligation in EDA -/- and WT mice. Prior to femoral artery ligation, the pre-existing anastomoses were already accompanied by a low number of macrophages (number of macrophages /artery: EDA-/- 1.26 ± 0.15; WT 1.65 ± 0.18, p=0.045). After arterial occlusion both strains showed an increase in the number of macrophages in their perivascular space. However, in the EDA-/- animals, the number of cells per collateral artery stayed significantly lower, when compared to the Balb/c (EDA-/- vs. Balb/c day 3, 2.02 ± 0.27 vs. 3.04 ±0.38, p= 0.038) (figure 3).

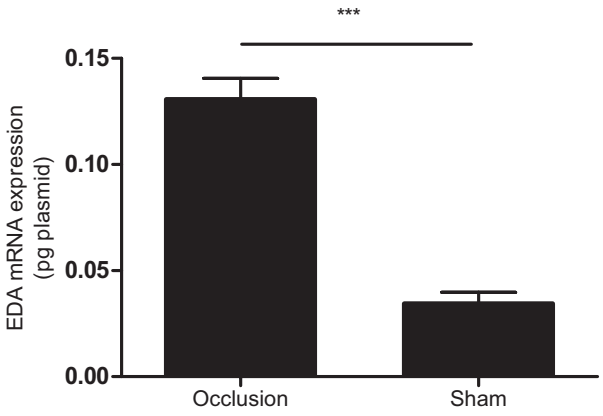

Figure 1. EDA mRNA in the hindlimb muscle

*After arterial ligation there is significantly more EDA mRNA present in the adductor muscle tissue on the occluded side (*** p<0.001), when compared to muscle from the sham-operated side (n= 11 Balb/c mice) The data is normalized for 18s and represents the mean +/- SEM.*

Blood monocyte count and CD11b surface levels in EDA-/- and wild type mice

EDA, as a TLR ligand, can initiate inflammatory responses. Having established that perfusion restoration and macrophage influx was diminished in EDA -/- mice, we determined whether the absence of EDA affected blood monocyte counts and monocyte CD11b activation.

In EDA -/- mice, the number of monocytes in the blood was lower, when compared to WT (EDA-/- vs. WT: 68 000 ± 8 000 vs. 123 000 ± 18 000, p= 0.004). In response to the arterial ligation, an initial increase in monocyte numbers was seen in both EDA-/- and WT mice. In the EDA-/- mice, this was only between day 0 and 1, after which monocyte numbers gradually returned back to baseline levels at day 7. In the WT animals, monocyte count increased between day 0 and 2, resulting in a higher number at day 2 (EDA-/- vs. WT, p= 0.001). This difference remained significantly different at day 3 (p=0.017) and 7 (p= 0.0011) (figure 4a).

CD11b, a classical activation marker, was measured at baseline (day 0) and at day 1, 2, 3 and 7 days after femoral ligation. Baseline CD11b levels were significantly lower in the EDA-/- animals, when compared to WT (EDA-/- vs. WT (MFI): 40.1 ±1.8 vs. 49.8 ± 1.8, p=0.005). After femoral ligation, CD11b monocyte surface expression increased more in the EDA-/- mice and was even higher than in the WT mice (EDA-/- vs. WT (MFI): day 1: 90.7± 2.3 vs. 62.4± 1.8 <0.001; day 2: 88,5± 2.9 vs. 65.7± 2.3; p<0.001; day 3: 61.6± 3.3 vs. 51.2± 1.8, p=0.03). At day 7, the EDA-/- animals again expressed less CD11b on their monocyte surface (EDA-/- versus WT(MFI): day 7: 35.0± 2.5 vs. 57.7± 4.1, p=0.002) (figure 4b).

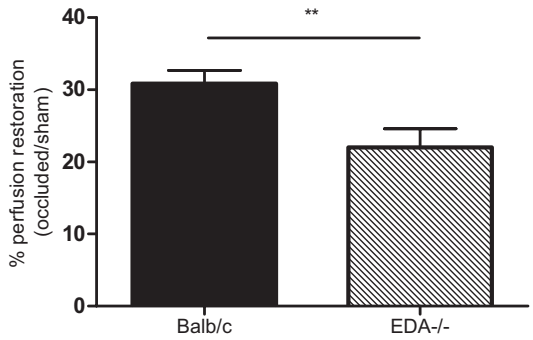

Figure 2. Perfusion restoration after femoral artery occlusion

*Seven days after femoral artery occlusion microsphere-based perfusion restoration was measured in 11 Balb/c mice and 11 EDA-/- mice. The graph shows the perfusion in the right ligated hindlimb as a percentage of the normal left (sham-operated) hindlimb perfusion. EDA-/- mice restore to only 22%, which is significantly less than the 31% of the Balb/c mice (** p<0.01)*

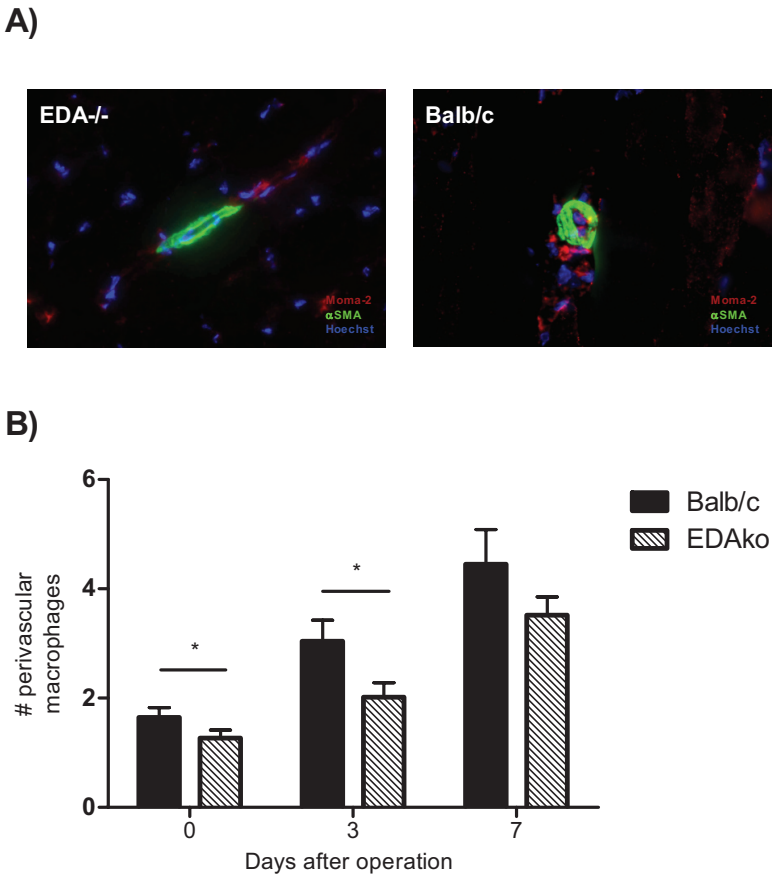

Figure 3. Perivascular macrophage accumulation during collateral artery growth in EDA-/- and wild type mice

A) Monocytes/ macrophages accumulate around developing collateral arteries during arteriogenesis. Monocytes/ macrophages are stained by Moma-2 staining (red). Collateral arteries were identified as αSMA positive (green) and nuclei were stained blue. The pictures are taken at a 400x magnification and the images represented here are representative pictures of both strains. B) Quantification of macrophage accumulation in the perivascular space (per developing artery). The data represent the average of 11 animals/ strain of which at least 5 random sections were counted by 2 independent observers. The results are depicted as mean +/- SEM (p < 0.05)*

DISCUSSION

Perfusion restoration after occlusion is an extremely important process, as it prevents tissue damage. For up to 20% of the coronary artery disease patients does not qualify for the classical treatment modalities, such as percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) [13]. The current known stimulators of arteriogenesis (e.g. FGF-2, GM-CSF) did not yield the positive results hoped for, when tested in clinical studies [14, 15]. Also, serious safety concerns were raised, because most arteriogenic agents also stimulate atherosclerosis and cause plaque destabilization [16].

Although perfusion restoration occurs naturally in response to significant arterial narrowing, the process often cannot keep pace with progression of the underlying disease. Therapeutic stimulation of collateral artery growth could therefore be beneficial for many patients. Large efforts are made to further unravel the mechanisms behind collateral artery growth and search for potential new candidates for therapeutic applications.

This study focuses on the endogenous TLR ligand fibronectin EDA. The hampered perfusion restoration after femoral artery ligation in EDA-/- mice shows that EDA plays a causal role in perfusion restoration. The absence of EDA in the EDA-/- mice resulted in a lower number of macrophages around collaterals compared to WT. In 1976, the monocytes were first identified as major players in arteriogenesis [17] and since then experimental research has further stressed their importance in collateral artery growth [18, 19]. Monocyte and macrophages are important cells of the innate immune system and express TLRs. The number of monocytes in the blood directly influence perfusion restoration, with a high number corresponding to a good response and low numbers with poor outcome [20]. In response to arterial ligation both the EDA-/- mice and the Balb/c mice showed an increase in monocyte numbers in the blood, but the extent and duration of this increase was different. The EDA-/- animals had lower circulating numbers and a quicker return to baseline. Although we do not know the exact reason for the lower monocyte levels in the EDA-/- animals, the absent EDA mediated TLR activation could be responsible. The subsequent synthesis of cytokines, stimulates the release of leukocytes into the bloodstream. The lower blood monocyte levels can correspond with lower macrophage counts in the perivascular tissue. This is supported by several papers, that found a direct link between the number of monocytes present in the blood and the amount of macrophages in the tissue around collateral arteries [21-23].

The presence of EDA in fibronectin enhances the affinity for alpha-5-beta-1 integrin. The EDA itself serves as a binding domain. The alpha-5-beta-1 integrin axis is activated in collateral growth [24]. Alpha-5-beta-1 mediates cell adhesion, but also provides proliferative signals to vascular smooth muscle cells [25]. Although the reduced monocyte blood count could be solely responsible for the decreased monocyte numbers in the tissue, a reduced integrin binding and thereby a reduced extravasation is also plausible. Wheter this also playes a role in our model, remains to be elucidated

Although lacking an endogenous ligand of TLRs, EDA-/- monocytes surface CD11b expression was higher after femoral ligation. This was somewhat surprising, since CD11b is regulated by TLR signaling. TLR2 and 4 are, however, not the only activation pathway involved in the regulation of CD11b. It responds to several different chemokines [26] and also interacts with other integrins by a mechanism called 'integrin cross-talk' [27, 28]. Since EDA mice exhibited less perfusion recovery, they would also have more ischemia. Differences in chemotactic gradients could therefore be present. Interestingly, the increased expression of CD11b in the EDA-/- mice could also have contributed to the decreased monocyte migration into the tissue. CD11b retards migration of leukocytes through the subendothelium and extracellular matrix. There is an inverse relationship between the density of CD11b on the cell surface and cell migration to fibronectin, i.e. increasing density of CD11b results in a progressive decline in migration [29]. Also, in CD11b-/- mice, it was demonstrated that neutrophils showed enhanced migratory properties and up to 3-folt greater number of neutrophiles accumulated at the inflammatory sites, when compared to the wild type mice [30-32].

The causal role for EDA in perfusion restoration makes this protein a potential candidate for

therapeutic stimulation of perfusion restoration. For this, involvement of EDA in atherosclerosis is important. EDA is expressed in atherosclerotic lesions and higher levels in the plaque are associated with a more stable plaque phenotype [33]. However, both over expression and deletion are associated with a reduction in plaque progression [34, 35] pointing to a double role for EDA. Therefore, EDA as an arteriogenic stimulator should be extensively investigated in the appropriate models.

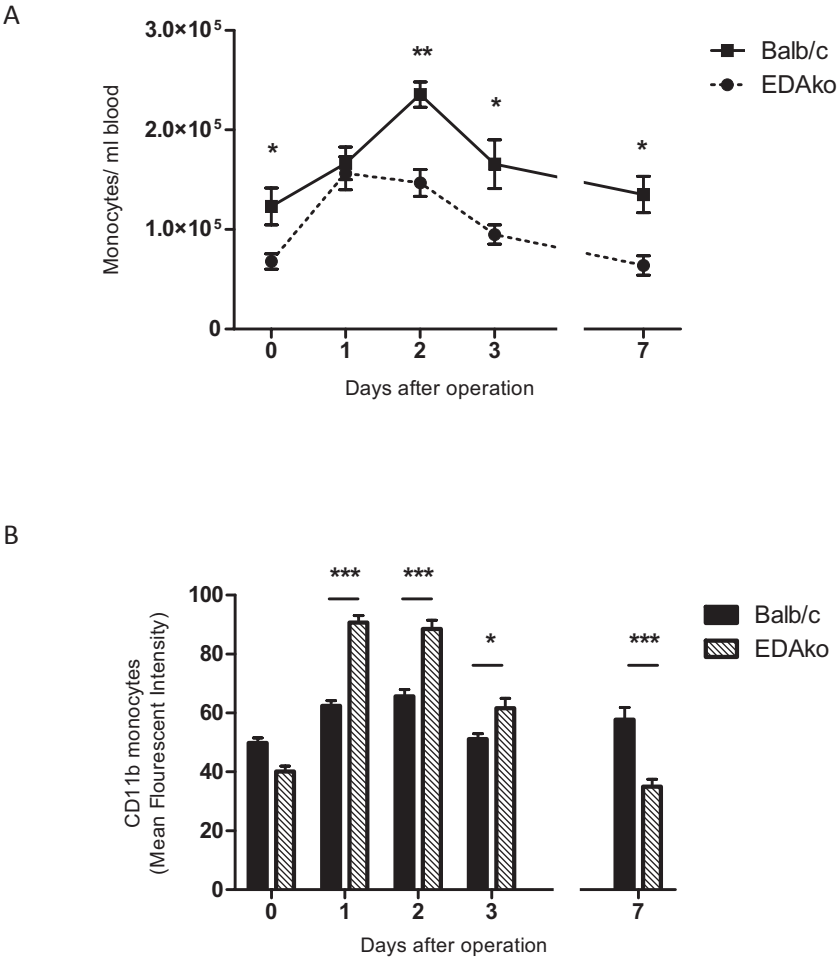

Figure 4. Blood monocyte count and activation before in response to arterial ligation

A) The total blood monocyte count was measured by flow cytometry at baseline and at day 1, 2, 3 and 7 after right femoral artery ligation (n=11/ strain/ time point). EDA-/- mice have less monocytes at baseline. Upon arterial occlusion both strains show an increase in the monocyte count, however EDA-/- mice peak already at day 1, while Balb/c mice continue to rise until day 2. At day 7 the levels are normalized again in both strains. (p< 0.05, ** p< 0.01) B) CD11b expression on monocytes was quantified by flow cytometry at baseline and at day 1, 2, 3 and 7 after right femoral artery ligation. The mean fluorescent intensity represents the mean intensity of the CD11b signal per cell. At baseline, EDA-/- animals have lower CD11b expression compared to balb/c animals). In response to the arterial occlusion the knock-out animals upregulate the receptor more than balb/c animals resulting in higher levels at day 1, 2 and 3 . This difference is again reversed in favor of the balb/c animals at day 7. (* p< 0.05, ** p< 0.01, *** p< 0.001)*

In summary, absence of EDA reduces the perfusion restoration after arterial occlusion. Lower levels of circulating monocytes in EDA-/- mice and a decrease in the number of macrophage around developing collateral arteries are potentially responsible for the decrease in perfusion recovery. This demonstrates for the first time an causal role for EDA in arteriogenesis and makes EDA a potential target for therapeutic stimulation of collateral growth.

Acknowledgements

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Submitted

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6

Age dependent reduction in perfusion restoration is partly depending on circulating leucocytes

ABSTRACT

Introduction The inflammatory response of the immune system towards infection is less prominent with increasing age. Collateral artery growth (arteriogenesis) depends on inflammatory responses and could therefore be impaired in the elderly. We investigated the impact of a high age on perfusion restoration after acute arterial occlusion and the role of circulating leucocytes in this process.

Methods Young (8-12 weeks) and old (>18 months) C57BL6/J mice underwent unilateral femoral artery ligation. Perfusion restoration in the affected hindlimb was measured by fluorescent microspheres at day 7. Bone marrow transplantations in a cross- over design (old↔ young) were performed to investigate the involvement of aging of bone marrow derived cells, e.g. circulating leucocytes, in the process of arteriogenesis. Alterations in monocyte function were examined *ex vivo* and *in vivo*, by looking at cytokine production, surface receptor expression and tissue infiltration.

Results Old mice showed a reduced perfusion restoration compared to young (42.0± 3.2% vs. 57.5± 2.0%, p=0.027). Transplantation of old bone marrow cells in young mice reduced perfusion restoration. Transplantation of young bone marrow cells in old mice, however, could not improve perfusion restoration. *In vivo*, old mice have more circulating monocytes, increased cytokine levels and higher CD11b expression, but showed a decrease in monocyte extravasation around developing collateral arteries (monocytes/ artery: 3.1± 0.3 versus 4.3± 0.3, p=0.022). *Ex vivo* stimulation by LPS showed that, monocytic responses where similar in old and young, except for ICAM-1 expression, which was lower in the aged mice (MFI: 57.9± 7.5 versus 82.2± 4.0, p=0.01).

Conclusion Aging reduces perfusion restoration. This is the result of a decreased influx of monocytes in the tissue, which was probably partly due to age related changes in the circulating leucocytes. However, the decline in collateral growth at high age could not be rescued by transplantation of young bone marrow, indicating that future treatments should focus on both resident and circulating cells.

INTRODUCTION

Aging is one of the most important risk factors for the development of atherosclerosis [1]. The incidence and prevalence of atherothrombotic disease increases with advancing age. Progression of atherosclerosis leads to cardiovascular events, like stroke, myocardial infarction and occlusion of the larger peripheral arteries. Morbidity and mortality of these cardiovascular events are caused by a sudden loss of tissue perfusion. The body tries to respond to a perfusion deficit by recruiting collateral arteries that serve as natural bypasses. The collaterals develop from an already existing non-functional network of vascular connections (vascular anastomoses) and play an important protective role by saving tissue from ischemia. For this, the amount of collaterals present after arterial occlusion determine outcome [2-4].

Since cardiovascular events occur predominantly in elderly patients, this group would require the benefits from an optimal functioning collateral network. Unfortunately, there is accumulating evidence that aging not only increases the risk for severe cardiovascular events, but also reduces the recruitment of collateral arteries. Several observational studies have shown a negative relation between advancing age and the amount of collaterals present [5, 6]. It remains, however, unclear whether the aging itself, or co-existing factors like medication use and risk factors are responsible for the observed reduction in collateral growth. Sprouting of capillaries, as occurs during angiogenesis, has been shown to decrease during aging [7] and is probably due to a reduced T-cell migration into the tissue and less vascular endothelial growth factor (VEGF). If this also applies to arteriogenesis is still unknown.

Like angiogenesis, arteriogenesis depends on the function of inflammatory cells [8, 9]. Especially the bone marrow derived monocytes have proven to play a central role, since changes in monocyte number or function results in direct changes in perfusion recovery [8, 10-15]. Aging leads to changes in the immune system. At high age, high levels of pro-inflammatory cytokines continuously circulate in the blood [16]. A decline in immune function is considered a hallmark of aging, as demonstrated by the increased susceptibility of elderly individuals to various infections [17, 18]. Although the inflammatory potential of the blood plays a very important role in arteriogenesis, so far it is unclear if aging does affect collateral artery growth via circulating leucocytes.

We hypothesize that high age leads to lower perfusion recovery due to the aging of circulating leucocytes. To investigate this, we used young and old C57Bl/6J mice and performed a femoral artery ligation to evoke collateral artery growth. To get more insight in the role of the circulating leucocytes in collateral formation, we transplanted bone marrow in a cross-over design between the young and old mice. This showed that high age results in less perfusion recovery, which was partly due to circulating leucocytes.

METHODS

Animal model and surgical protocol

All animal experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and prior approval was given by the Animal Ethical Committee of the faculty of Medicine, Utrecht University, the Netherlands. To investigate the influence of high

age on arteriogenesis we used young (8-12 weeks) and old (>18 months) C57Bl/6J mice. 34 young and 35 old mice underwent ligation of the right femoral artery, as previously described [19]. In these animals the right femoral artery was surgically occluded, just below the inguinal ligament, by double ligation. During the operation, analgesia and anesthesia were induced by subcutaneous injection of 0.1 mg/kg buprenorphine and isoflurane inhalation. Body temperature was maintained by placing the animals on a heating pad.

Perfusion measurements

Seven days after femoral artery ligation, microsphere-based perfusion measurements were performed on six young and eight old mice, for assessment of collateral dependent perfusion restoration [19]. During this protocol, the animals are anesthetized and heparinized. The abdominal aorta is exposed and cannulated to infuse fluorescent microspheres (Fluospheres, 15µm, Molecular Probes Inc., Eugene Oregon) at several different perfusion pressures. During the experiment, maximal vasodilatation was obtained by continuous adenosine infusion. After the introduction of the microspheres, the right and left hindlimb muscles were collected and weighed. The tissue was digested and homogenized. The number of microspheres per sample are quantified by flow cytometry (FC500, Beckman Coulter, Miami, FL, USA) [20]. Perfusion restoration in the occluded hindlimb was defined as a percentage of the normal, left hindlimb perfusion.

Immunohistochemistry

Day 3 after femoral artery occlusion, hindlimb muscles were collected and snap frozen in liquid nitrogen (9 old and 10 young mice). After embedding in Tissue Tek (Sakura, Zoeterwoude, the Netherlands) 7µm sections were cut and fixed in acetone. Blocking was performed with Cytomation Biotin blocking system (DAKO, Heverlee, Belgium) followed by 1 hour incubation in 5% milk/PBS. The following antibodies were used: rat monoclonal MOMA2 (1mg/ml, Millipore) 1:50 in 1% milk/PBS, goat anti rat-biotin (1mg/ml, Southern Biotech) 1:250 in 1% milk/PBS, streptavidin-alexa fluor 555 (Invitrogen) 1:1000 in 1% milk/PBS and monoclonal to αSMA-FITC (Sigma) 1:300 in 1%BSA/PBS. All sections were counterstained with Hoechst (trihydrochloride trihydrate 33342, working solution 1:10 000, Invitrogen, Oregon, USA). Isotype rat IgGb (AbD Serotec, Dusseldorf, Germany) was used as negative control. The sections were inspected under a microscope (Olympus BX60) and pictures were taken (Olympus DP71) at a 400x magnification. Monocyte/macrophages accumulating in the perivascular tissue were counted by a blinded observer in at least 5 tissue sections/ animal and expressed as the number of MOMA2 positive cells/ αSMA positive artery.

Bone marrow transplantation

Nine young and eight old mice were irradiated at a dose of 700cGray (1030 monitor units), for eradication of the autologous bone marrow. Four donor mice (2 old and 2 young) were sacrificed to isolate bone marrow for subsequent transplantation. The bone marrow was aseptically collected by flushing the femoral, tibial and humeral bones with RPMI 1640 (Invitrogen), after which it was filtered (70 micron Falcon cell strainer, BD Bioscience, San Jose, USA) and washed. 5x106 bone marrow derived cells were injected into the tail vein of the recipient mouse and the mice were left to recover for at least 6 weeks. After this 6 week period, the animals underwent the femoral artery ligation protocol as described above. Young mice

received old bone marrow and vice versa to study the influence of aging of the circulating or resident cells on arteriogenesis. Because no effect of the irradiation and transplantation was ever seen by our group [11, 21], we did not repeat the control transplantations, to reduce animal use and suffering.

Monocyte number and response

To investigate *in vitro* monocyte responsiveness in old and young mice, whole blood samples were collected (cardiac puncture) from both groups at baseline (n= 6/group). Heparin was used to prevent coagulation. The blood was placed in an incubator at 370C and 5% CO2 either in the presence of lipopolysaccharide 10ng/ml (LPS, E. Coli 055-B5, Sigma-Aldrich, St. Louis, USA) or PBS. After two hours, 100ul of each sample was collected and stained with the following antibodies: F4/80 -alexa 647 (AbSerotec, Germany), CD11b -FITC (Bioconnect, the Netherlands), TLR2 –FITC (eBioscience, Frankfurt, Germany), TLR4 -PE (eBioscience, Frankfurt, Germany), CD54 (ICAM-1)-PE (BD Biosciences, Breda, the Netherlands). Counting beads were added to all samples to facilitate the quantification of monocytes. The samples were analyzed by flow cytometry (FC500, Beckman Coulter, Miami, FL, USA). Monocytes were defined as being F4/80 and CD11b positive in combination with their scatter properties.

After 24 hours of stimulation the remaining blood samples were taken from the stove and centrifuged at 300G for 5 minutes. The plasma was collected and used to measure cytokine levels (Mouse IL-6 Quantikine immunoassay, R&D systems, Minneapolis, USA and Mouse TNF-alpha ELISA, Bender MedSystem, Vienna, Austria).

Three days after ligation of the femoral artery, again, blood was collected (n= 9/group). To investigate the *in vivo* response of the monocytes to the ligation, the same protocol was followed as described above, but now without adding any additional *in vitro* stimulus (no LPS).

Statistics

All statistics were performed using SPSS 17.0 (SPSS Inc.). Mann Whitney U test was used to compare differences between two groups. All values are presented as mean ± SEM and p-values < 0.05 were regarded as significant.

RESULTS

Aged mice have a reduced perfusion recovery and macrophage influx after femoral artery ligation

Perfusion recovery was measured with fluorescent microspheres one week after occlusion of the femoral artery. In aged mice, perfusion recovery was significantly reduced compared to the young mice (42.0± 3.2% vs. 57.5± 2.0%, p=0.027) (figure 1). No hindlimb necrosis was seen in either group, however, delayed would healing was observed in the aged mice. One old animal died in the first day after operation, probably due to old age in combination with the anesthesia. The adductor muscle is the most prominent location for collateral artery growth in the hindlimb model. Tissue sections of these muscles were stained for monocyte and macrophages. In the perivascular tissue surrounding developing collateral arteries, less macrophages were detectable in old animals (number of monocytes/ artery, old vs. young mice: 3.1± 0.3 vs 4.3± 0.3, p=0.022) (figure 2).

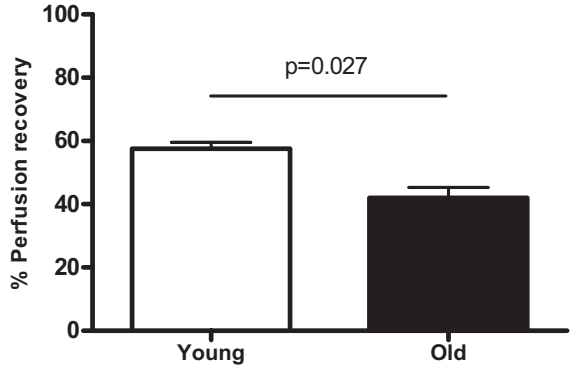

Figure 1. Aging reduces perfusion recovery after arterial occlusion

Perfusion recovery was measured with fluorescent microspheres, 7 days after right femoral artery ligation in 6 young (white bar), 8 old (black bar) C57Bl/6J mice. Perfusion restoration (right leg) was expressed as a percentage of normal hindlimb perfusion (left leg), which was set at 100%.

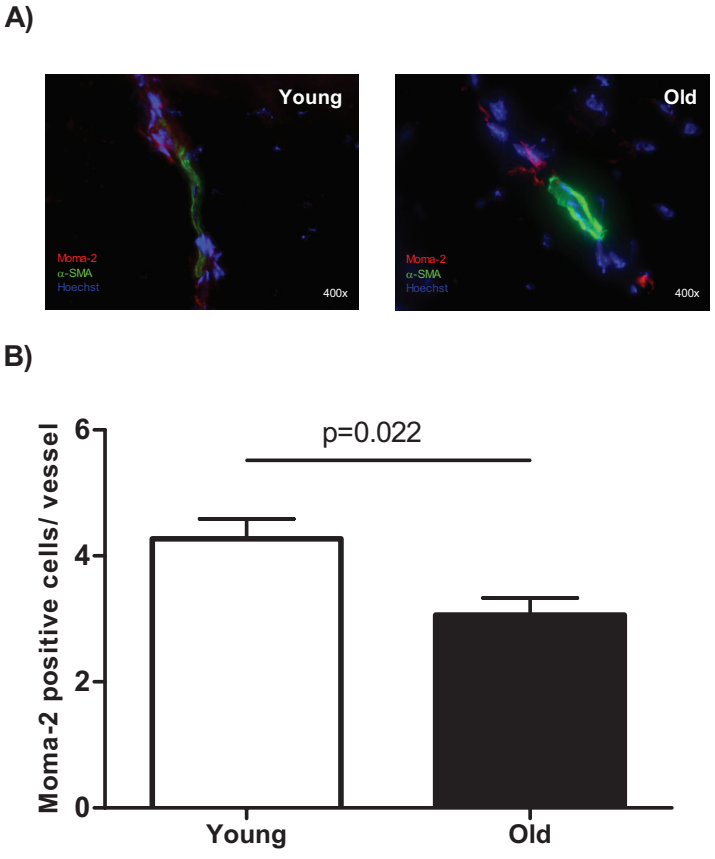

Figure 2. Aged mice show less macrophages around developing collaterals

Tissue sections of the right adductor muscle were stained for monocytes/macrophages (red), α smooth muscle cells (green) and nuclei (blue). At least 5 sections of 9 old and 10 young mice were analyzed for the presence of macrophages in the perivascular space. Representative images of a young (left) and old (right) mouse are show at a 400x magnification (A). The average number of moma-2 positive cells per artery is presented (B).

To determine the role of circulating leucocytes in perfusion recovery, bone marrow transplantations were performed. The transplantation of old bone marrow into young mice resulted in a reduction in perfusion recovery, when compared to normal young animals (47.6± 1.1% vs. 57.5± 2.0%, p=0.016). Vice versa, no significant improvement was found in old mice that received young bone marrow, when compared to normal old mice (45.4± 4.0% vs. 42.0± 3.2%, p= ns) (figure 3). In previously published work we show that the transplantation protocol has no effect on arteriogenesis, since the transplantation of young bone marrow in a young animals resulted in a normal perfusion recovery [11, 22].

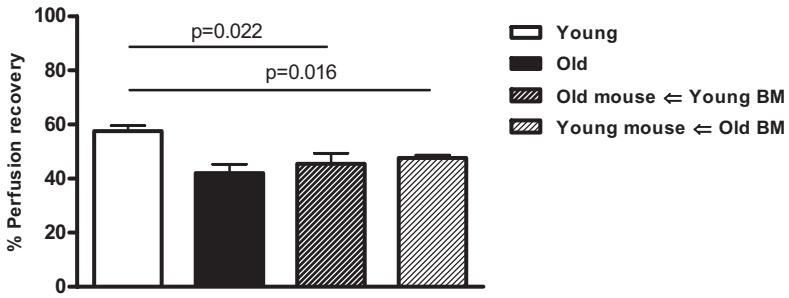

Figure 3. The age related perfusion recovery and bone-marrow derived cells
Perfusion recovery was assessed in mice who first underwent a bone marrow (BM) transplantation in a cross-over design. Eight old mice with young circulating cells (old mouse <= young BM) and 9 young mice with old circulating cells (young mouse <= old BM) were measured with fluorescent microspheres, 7 days after right femoral artery ligation and compared to 6 young (white bar) and 8 old (black bar) C57Bl/6J mice without BM transfer. Perfusion restoration (right leg) was expressed as a percentage of normal hindlimb perfusion (left leg), which was set at 100%.

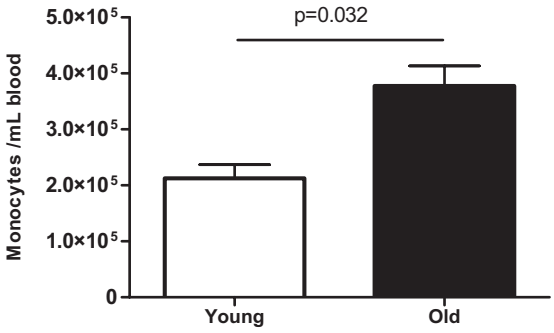

Figure 4. Old mice have increased blood monocyte numbers
Blood monocyte numbers of 6 old and 6 young mice were counted by flow cytometry. After blood collection at basline, fluorescent counting beads were added to a fixed volume of blood. All samples were stained with F4/80 and CD11b. Monocytes were identified as being positive for both markers, in combination with specific scatter properties. All counts were converted to numbers per mL blood.

Having demonstrated a role of circulating leucocytes in perfusion recovery, we assessed monocyte number and function in young and aged mice. In old mice, almost twice as many monocytes were present in the blood (old vs. young mice, per mL blood: 380 000± 36 000 vs. 210 000± 25 000, p=0.032) (figure 4). At baseline and 3 days after femoral artery ligation, we measured monocyte responses *in vivo* and *ex vivo*.

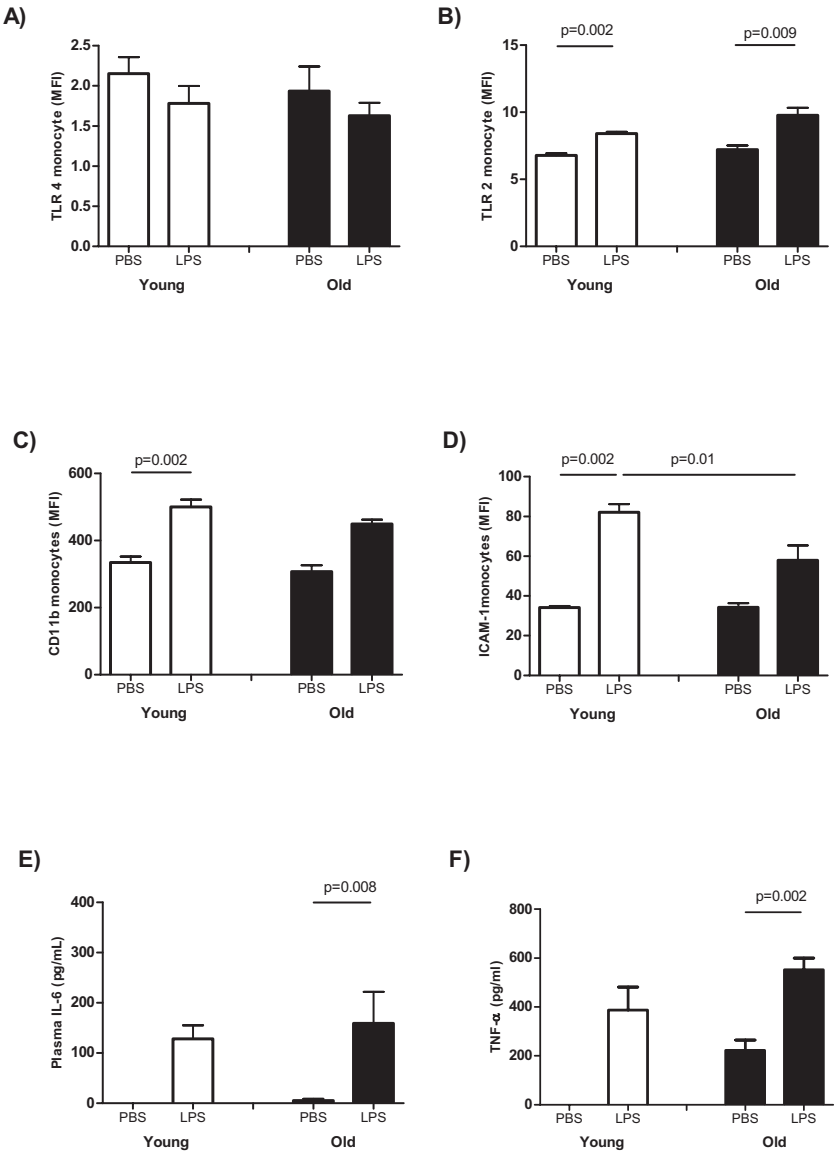

Figure 5. Baseline monocyte function and *ex vivo* stimulation

Heparinized whole blood samples of 6 old and 6 young mice were stimulated with either PBS or LPS (10ng/ml). After 2 hours, samples were collected and stained for TLR4, TLR2, CD11b and ICAM-1 and mean fluorescent intensity (MFI) of the receptors on the monocyte surface was measured by flow cytometry (A-D). After 24 hours, samples were centrifuged and plasma was collected with subsequent measurement of TNF-α and IL-6 levels. TNF-α and IL-6 are presented after correction for leukocyte number (E-F).

Whole blood samples, collected at baseline, were measured with and without (*ex vivo*) stimulation with LPS. No baseline differences were found in the expression of TLR2, TLR4 or CD11b on the monocyte surface between old and young mice, with equal up (CD11b and TLR2) or down (TLR4) regulation of the receptors in response to the LPS (figure 5A-C). Intracellular adhesion molecule-1 (ICAM-1) baseline expression at the surface of monocytes was equal between old and young mice, but upon activation the old mice were less capable of presenting ICAM-1 on their cellular surface (MFI: 57.9± 7.5 vs. 82.2± 4.0, p=0.01) (figure 5D).
In the baseline samples, the pro-inflammatory cytokines TNF-α and IL-6 were already detectable in the old mice (TNF-α (pg/mL) 222.5± 42.3 ; IL-6 (pg/mL) 5.1± 3.3), while in young mice, these cytokines were below the detection limit. In the samples stimulated with LPS, no significant differences in cytokine production were seen between the old and young mice, when corrected for cell count (old vs. young; TNF-α (pg/mL) 553± 47 vs. 338± 94, p= ns; IL-6 (pg/mL) 159± 63 vs. 128± 27, p= ns) (figure 5E-F).

After arterial ligation, old mice showed much higher plasma cytokine levels, with 7 fold higher levels of IL-6, compared to young mice that underwent the same procedure (old vs. young (pg/mL): 100.0± 8.1 vs.14.5± 6.5, p=0.002). This resulted in an increased cellular activation in the old mice (CD11b (MFI) old vs. young: 544.5± 26.9 vs. 388.3± 16.3, p= 0.004). Although in the old mice, the ICAM-1 transcription should be increased by pro-inflammatory cytokines, ICAM-1 in old animals only reached the same levels as the young mice, which had far less pro-inflammatory cytokine production (figure 6).

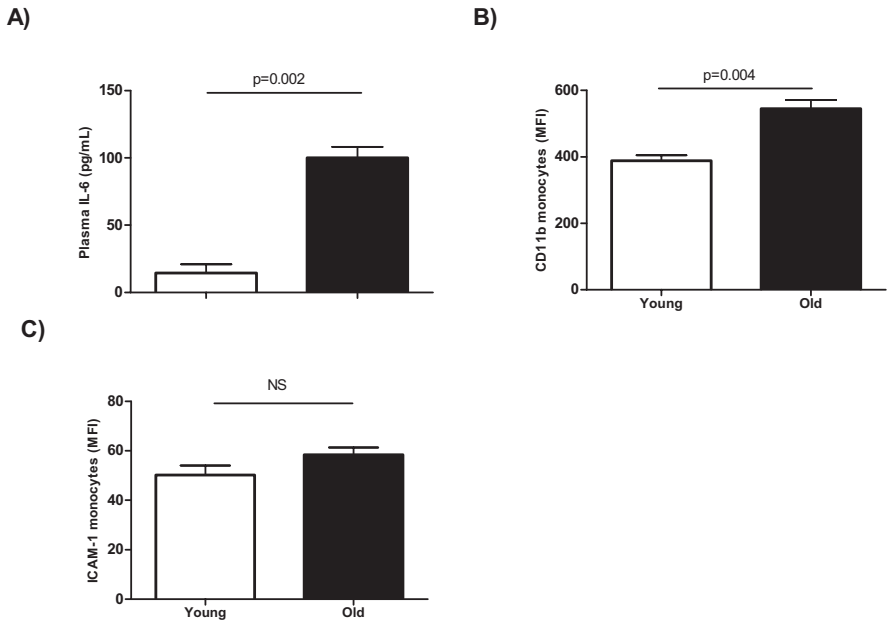

Figure 6. Monocyte CD11b and ICAM-1 expression and plasma cytokine levels after arterial ligation
Heparinized whole blood samples of 9 old and 9 young mice were collected, 3 days after femoral artery ligation. Plasma was collected to measure IL-6 levels in vivo (A). Furthermore, cells were stained for the activation marker CD11b and ICAM-1. The mean fluorescent intensity (MFI) of the receptors on the monocyte surface was measured by flow cytometry (B-C).

DISCUSSION

Regaining adequate tissue perfusion is essential for all patients suffering from atherosclerotic disease. The natural growth of collateral arteries represents an important process for maintaining and restoring the perfusion, hereby preventing ischemic damage. For the elderly patient, this process is even more important because they are less likely to qualify for the (standard) invasive treatment modalities, due to the frequent presence of co-morbidities at high age.

In this study we show that high age leads to a reduction in the perfusion restoration after an arterial occlusion. This is most likely due to a reduction in collateral growth, because collateral arteries are the most important contributors to perfusion restoration following Hagen-Poiseuilles law. This in accordance with the work of Westvik and colleagues [23], who found in old mice, less collaterals in response to iliac ligation. Furthermore, they found that high age increased angiogenesis, which was in contrast to the original work of Rivard in 1999. Although angiogenesis will definitely be present after femoral artery ligation, the majority of the measured restoration will be due to collateral dependent flow. The decreased monocyte extravasation, around the developing collateral arteries of the old mice, is likely to be responsible for the reduction in collateral growth. Macrophages play a crucial role in creating the ideal environment for the remodeling of pre-existing anastomoses into collateral arteries [8].

The transplantation of old bone marrow in young mice resulted in a perfusion restoration similar to old mice, showing that old bone marrow derived cells negatively influence collateral growth and contribute to the age dependent decrease in perfusion recovery. Transplantation of young healthy bone marrow in old mice, however, did not restore the deficit in perfusion recovery in the old mice. This shows that also non-bone marrow derived cells contribute to the age dependent decrease in perfusion recovery. Possible explanations are endothelial dysfunction [24, 25] and reactive oxygen species (ROS) formation in the tissue [26]. As a consequence, future attempts to target the age derived reduction in perfusion should not only focus on targeting circulating cells, or resident cells, since both contribute to the process.

Alterations in the immune system at high age are frequently described [27-30]. There is a general consensus that the adaptive immune functions are diminished [30]. There is, however, no consensus if and how the innate immune system changes during aging [27-29]. The monocytes and toll like receptors (TLRs), which have shown to be important for arteriogenesis are part of the innate immune system

Having demonstrated that circulating cells are partly involved in the age-dependent decrease in of perfusion recovery, we compared monocyte number, responsiveness, surface expression and plasma TNF-α and IL-6 levels between young and old mice. Old mice have twice as many monocytes circulating their blood. Their baseline expression of toll like receptor 2 and 4, CD11b and ICAM-1 on the monocyte surface was equal to that of the monocytes in young mice. Baseline levels of the pro-inflammatory cytokines TNF-α and IL-6 were already measurable in old mice, while in young mice these cytokines were below detection limit. This is in concordance which previous reports, stating that in the senescent patient a continuous state of low grade inflammation is present, corresponding with higher levels of pro-inflammatory cytokines [16]. The chronic activation of the circulating cells, has its reflections on the levels of peripheral blood leukocytes [31]. The high number of circulating monocytes in our old mice could be an example of this.

In response to arterial ligation, pro-inflammatory Il-6 levels were higher in old animals as well as monocyte surface CD11b levels. CD11b is an integrin that plays an important role in

the firm cellular adhesion to the blood vessel wall. High CD11b surface expression, has been described to be responsible with decreased migration of cells [32] This identify CD11b as one of the possible contributing factors for the decreased migration of monocytes in the old mice. Within the current accepted models of cell migration, optimal cell migration occurs with intermediate integrin expression, intermediate substrate concentration and intermediate integrin activation [33].

The monocyte surface ICAM-1 levels did not differ between old and young animals at baseline. Although pro-inflammatory cytokines are known to stimulate ICAM-1 production, no difference in expression was found between old and young, indicating a difference response to cytokines in old mice. To investigate this, blood was stimulated *ex vivo* with LPS. This lead to equal responses between old and young for CD11b, TLR2, TLR4 expression and the production of the cytokines TNF-α and IL-6. ICAM-1 expression, however, was significantly lower in old monocytes compared to the young cells. This defect in ICAM-1 upregulation at high age has been previously described by Hobden et al in corneal infections [34]. In their model, the inability of aged mice to upregulate ICAM-1 resulted in a delayed migration of inflammatory cells into the cornea. Collateral artery growth proceeds via an ICAM-1 dependent mechanism. In absence of ICAM-1 perfusion restoration is abolished, due to inadequate monocyte adhesion to the endothelium and subsequent migration [35]. A reduced presence of ICAM-1 on aged monocytes could result in a reduction in monocyte extravasation, thereby influencing collateral growth negatively. However, more research is needed for this, as not only ICAM on the circulating monocyte, but also the interaction with the non-bone marrow derived endothelial cell is important [35] as is shown in the bone-marrow transplant from young to old mice. Also, the interaction with platelets might be involved in the reduced influx of monocytes [36], but has not been studied.

Another limitation is the health of the mice. Although both young and old mice were treated the same and all animals appeared healthy, age related processes could potentially influence arteriogenesis. In humans, almost all elderly cardiovascular disease patients have extensive risk factors present and take medication for their atherosclerosis. This can influence arteriogenesis [37], thereby acting as potential confounders. In our mice, we did not observe any difference in blood glucose or lipid metabolism (data not shown), two important risk factors for atherosclerosis, which frequently alter during aging. However, we cannot exclude that in our mice unknown confounders were present potentially influencing the process in an undesired manner.

In summary, high age leads to a reduction in perfusion restoration after arterial occlusion and less monocyte infiltration in the perivascular tissue. Although rejuvenation of the bone marrow derived cells could not rescue this defect, old circulating cells do contribute to the negative effect on collateral artery growth. Initial experiments point to a role of high CD11b levels and low ICAM-1 levels on old circulating monocytes but more research is needed for this.

The identification of age as a negative factor influencing the restoration of tissue perfusion has important implications for elderly cardiovascular disease patients. Following our observations, more emphasis should lay on active revascularisation strategies in the elderly patient, since the natural growth of a good collateral network is less likely to happen. Therapeutically enhancing collateral growth could be beneficial for old patients, however, the targeting should focus on both resident and circulating cells, since both seem to play a role in the reduction of perfusion recovery at high age.

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Cardiovascular risk factors and collateral artery formation Assessment of Collateral Artery Function and Growth in a Pig Model of Step Wise Coronary Occlusion

ABSTRACT

Background Therapeutic stimulation of collateral artery growth is a promising approach for treatment of cardiovascular diseases. Unfortunately, translation into clinical practice yet remains cumbersome. Cardiovascular physiology and anatomy are major determinants of vascular growth processes. Hence, large animal models are needed to improve clinical translatability of pre-clinical research. Furthermore, acute complete occlusions are mostly applied in experimental research, while stepwise occlusions are more often observed in human disease.

Methods We developed a model of coronary collateral artery growth in which: a) the artery is occluded in a step wise approach and b) effects of local treatment can be measured individually for each supplying coronary vessel. A hemodynamically relevant stenosis was created by implantation of a tapered stent at day 0 (d0) in the left circumflex artery (LCX), followed by complete arterial occlusion at day 14 (d14). Fluorescent microspheres were injected for demarcation of perfusion territories at each time point. Three and 4 weeks after induction of stenosis, collateral conductance measurements were performed for each coronary artery separately using differently labeled fluorescent microspheres.

Results Post-mortem angiography after acute LCX occlusion confirmed the presence of pre-existent coronary anastomoses in the pig. The tapered stent created a hemodynamically significant stenosis immediately post- placement (fractional flow reserve 0.70±0.03). Between day 21 and 28, collateral conductance significantly increased in both the left anterior descending (LAD) and the right coronary artery (RCA) supplied collateral dependent territories ([$ml*min^{-1}*g^{-1}*100mmHg^{-1}$]: LAD d21: 0.77±0.14, LAD d28: 1.35±0.12; RCA d21: 0.88±0.29, RCA d28: 1.70±0.16), indicating collateral artery growth.

Conclusion We here describe a new translational minimally invasive model of coronary collateral artery growth in pigs according to a defined protocol of LCX-stenosis and subsequent occlusion, allowing pre-clinical evaluation of arteriogenic therapies.

INTRODUCTION

Therapeutic angiogenesis and arteriogenesis, i.e. stimulation of capillary and collateral growth, has been envisioned as an alternative treatment modality for cardiovascular diseases for several years, but has not yet found its way into daily clinical practice [1]. Many chemical and biological entities have been proposed as putative agents in experimental studies, while clinical effects yet remain small. Several reasons for this divergence have been identified, such as anatomy, physiology, occlusion rates, delivery issues and co-morbidities, which frequently differ between patients and pre-clinical models.

The majority of experimental studies are nowadays performed in small animals (mice, rats) due to their relatively low costs, easy housing and the availability of transgenic strains. However, organism size limits the technical possibilities and thereby feasibility of more specialized models. Especially coronary artery disease models prove to be difficult, as they are invasive, associated with a significant mortality rate and do not have easy read-out parameter. Therefore peripheral artery disease models are favored in most experimental research. The acute occlusion of the femoral or iliac artery serves a good basis for mechanistic research into peripheral collateral growth, however clinical studies often focus on coronary disease patients. In these patients the arterial lumen narrowing occurs in a very different pattern than in peripheral disease, following a more step wise progression. Hence, a huge gap yawns between pre-clinical studies and clinical reality, supporting the need for appropriate animal models to reflect the patients' situation better.

Upon acute coronary artery occlusions, humans develop large myocardial infarction, as their pre-existent collateral network, although present, is not well developed. When given enough time though, the pre-existent anastomoses can grow and mature into functional collateral arteries (arteriogenesis), significantly reducing infarct size in case of an acute event, thereby improving myocardial function and survival. One of the requirements for large animal collateral growth models therefore is a comparable pattern of salvaging otherwise infarction prone tissue by growth of coronary collateral vessels.

Since the hallmark experiments of Maxwell et al. in 1987, pigs have been known for their poorly developed pre-existent network compared to dogs or guinea pigs [2, 3]. In combination with their similarity regarding heart size, weight and cardiovascular anatomy, pigs are considered as the ideal model to mimic acute myocardial infarction in humans. Furthermore, Maxwell's studies have been used as argument against the use of pigs for coronary collateral growth studies despite their sole focus on the pre-existing network rather than the ability to form functional collaterals upon coronary stenosis. More recent studies indicate the pig's potential to develop appropriate coronary collateral vessels, which are not inferior to dog coronary collaterals with respect to perfusion of the ischemic myocardial territory [4-11].

The coronary tree in humans and most large mammals can be divided into 3 large coronary arteries: right coronary artery (RCA), left anterior descending artery (LAD) and left circumflex artery (LCX). Occlusion of one of these vessels leads to enlargement of collateral vessels, mainly originating from the other 2 coronary arteries. However, as the coronary anatomy may vary significantly, the relative amount of collateral blood flow can also differ considerably. Therefore, a coronary collateral model should offer the possibility to differentiate collateral function for different source vessels, also enabling the measurement of selective local treatment effects.

In the current study we therefore aimed to create a large animal model for the investigation of

coronary collateral artery growth and testing of arteriogenic and angiogenic factors fulfilling the above described requirements, without inducing massive myocardial infarction.

MATERIAL AND METHODS

Stent Design

In order to create a fixed stenosis with a defined diameter, the proximal 2/3 of bare metal stents (3.5 x 18mm) were partly wrapped with an ePTFE membrane (thickness 0.1 mm, W. L. Gore and Associates, Flagstaff, AZ). The distal 1/3 was left unchanged to provide sufficient anchoring in the vessel after stent expansion. In the middle of the stent, a metal ring (technical department, UMC Utrecht, the Netherlands) was placed and fixed with 6-0 prolene (Ethicon, Somerville, NJ). The ring diameter (1.8 to 2.0 mm) was chosen after baseline angiography of the target vessel segment. After balloon inflation, the ring prevented stent expansion beyond ring diameter minus ePTFE membrane diameter. The ePTFE membrane in the proximal part of the stent ensured that blood flow was limited to the inner lumen of the stent (figure 1A, B).

Animal model, Fractional Flow Reserve

The present study was performed after having obtained appropriate institutional approvals. It conforms to the *Guide for the Care and Use of Laboratory Animals published by The US National Institute of Health* (NIH Publication No. 85-23, revised 1996). Twenty Dalland landrace pigs (body weight 65.3 ± 2.19 kg) were subjected to step wise occlusion of the left circumflex coronary artery (LCX). To minimize the risk of fatal arrhythmias during the experiment, pigs received amiodarone 300mg/day starting 10 days before surgery and 300mg daily afterwards. Clopidogrel 75mg/d was given from preoperative day -3 until 3 days before occlusion. Prior to the operation, the animals received intramuscular premedication (10 mg/kg ketamin, 0.4 mg/kg midazolam, 0.5 mg atropine), followed by intravenous induction of anesthesia with 4 mg/kg thiopental and antibiotic prophylaxis (amoxicillin/clavulanic acid) and intubation. Throughout the surgical procedures, animals were mechanically ventilated and anesthesia was maintained intravenously with 0.5 mg/kg/h midazolam, 2.5 µg/kg/h sufentanil and 0.1 mg/kg/h pancuronium. After median incision of the neck, the right carotid artery was carefully dissected and a sheath catheter (8F) was inserted. Acetylsalicylic acid (500mg) and heparin (100 IU/kg) were given intravenously before coronary angiography.

Baseline coronary angiography was performed using a 6F JL 3.5 guiding catheter. Subsequently, baseline perfusion territories were marked by selective injection of differently labeled fluorescent microspheres (Fluospheres, 15µm, Molecular Probes Inc., Eugene Oregon) into the coronary arteries (RCA, LAD, LCX, $1*10^6$ microspheres each) after pre-mixing the microsphere with arterial blood. Afterwards, the guiding catheter was replaced with an 8F JL 5.0 guiding catheter. An intracoronary pressure wire (Certus, St. Jude Medical, St. Paul, MN) was used as intracoronary guide wire and the stent was placed in the target segment. Proximal and distal arterial pressures were continuously monitored and recorded throughout the placement procedure (figure 1C-E), also allowing the calculation of pressure-derived collateral flow indices (CFI=(pdistal-CVP)/psystemic-CVP)) during balloon inflation. After balloon inflation and retraction, the fractional flow reserve (FFR) was measured under maximal vascodilatation (continuous intravenous adenosine infusion,10mg/min). The fractional flow reserve or FFR is

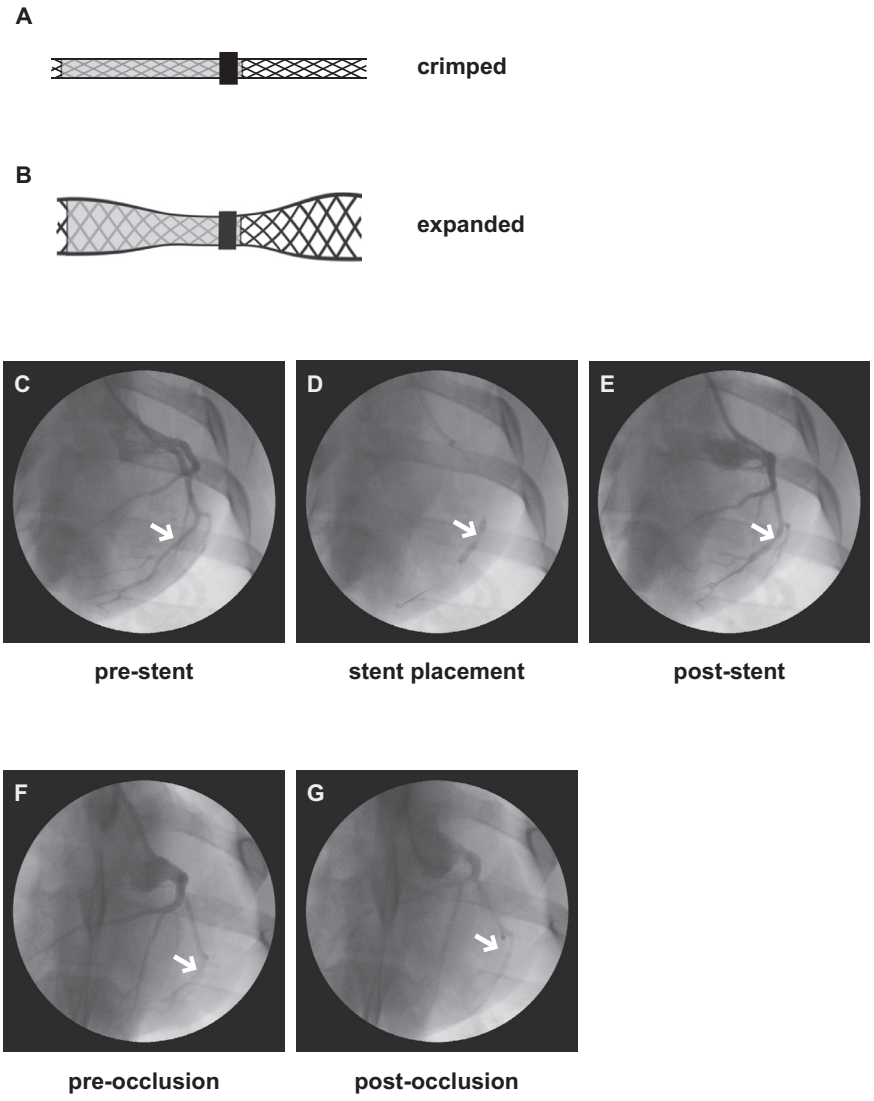


Figure 1. Design and placement of the stenosis stent

Crimped bare metal stents were covered with a ePTFE membrane and a metal ring was fixed to the stent to prevent complete deployment of the stent upon balloon inflation (A). When deployed, the stent acquired an hourglass shape (B), preventing blood flow through the stent struts. The smallest lumen diameter was defined by the ring diameter minus ePTFE coating and stent thickness. Stent placement (C-E) and occlusion 14 days later (F-G) of the LCX. (C) shows the LCX anatomy before intervention. Using the FFR wire as guiding, the stent was placed and the balloon was inflated, hindered from full inflation (arrow) by the stent design (D). Directly after stent placement, a significant stenosis was created at the site of stent implantation (E).Fourteen days after implantation, the stenosis was still evident (F). After occluding the stent, only minimal residual flow equivalent to a TIMI Grade of I was visible (G).

the ratio of maximum flow in the presence of a stenosis to normal maximum flow. This is a lesion-specific index of stenosis severity that can be calculated by simultaneous measurement of mean arterial and distal coronary pressure during pharmacological vasodilation (FFR= p_{distal})/$p_{systemic}$). After removal of all catheters, the carotid artery was carefully sutured, the skin incision was closed and the animals were allowed to recover.

Occlusion, 2 weeks perfusion

Two weeks after stenosis induction, the animals were anesthetized again and prepared for coronary angiography as described above. The LCX was selective cannulated with an 8F JL 5.0 catheter and a stainless steel ball (diameter 2.0 mm) was flushed into the stent, thereby occluding the vessel (figure 1F, G). To measure collateral function at this time-point, fluorescent microspheres were selective injected into the collateral feeding arteries (LAD: orange, RCA: yellow-green) as follows. Vasodilatation was achieved by intravenous adenosine infusion (10mg/min). An intracoronary flow wire (FloWire, Volcano, San Diego, CA) was introduced after selective cannulation of either RCA or LAD with a 6F JL 3.5 guiding catheter. Intracoronary flow was measured and recorded simultaneous to the pump-driven injection of microspheres into the coronary arteries after pre-mixing the microspheres (1*106) with blood.
Afterwards, wounds were surgically closed and the animals received 0.5mg/kg protamine sulphate to antagonize excess heparin before allowing the animals to recover.

Hemodynamic measurements

Three (n=6) and 4 weeks (n=9) after coronary stenosis creation, animals were again anesthetized for *in vivo* hemodynamic measurements of collateral artery function. An additional group of 3 animals received an acute LCX occlusion before the hemodynamic measurements to determine baseline collateral perfusion. The left carotid artery was dissected and a 6F sheath catheter was inserted and connected to a pressure transducer. A ventricular pacing electrode was introduced via the right jugular vein to increase heart rate (80 bpm) if warranted. The thorax was opened and the proximal LAD and RCA as well as the LCX distal to the site of stent implantation were carefully dissected. Distal (LCX) perfusion pressure was measured by cannulating the vessel with a polyethene catheter (inner diameter: 0.86mm, outer diameter 1.27mm) connected to a pressure transducer. Before cannulation of the proximal RCA and LAD, the abdominal aorta was dissected and cannulated, serving as feeding vessel for the subsequent coronary perfusion measurements. Directly after insertion of the cannulas into RCA and LAD respectively, coronary flow was maintained and controlled using two independent roller pumps, one for each coronary artery. Proximal perfusion pressures and coronary flows were continuously monitored and recorded for subsequent analysis and calculation of collateral conductance. Blood flow reference samples were withdrawn at the cannulation site using a syringe pump (sample collection for 3 minutes, withdrawal speed 30ml/hr)
During the microsphere-based perfusion measurements, maximal vasodilatation was achieved by continuous adenosine infusion (1mg/kg heart weight/min) via the roller-pump-driven shunt between abdominal aorta and coronary arteries. At 3 different perfusion pressure levels, differently labeled fluorescent microspheres were injected into the pump-driven shunt (LAD: yellow, blue-green, green; RCA: red-orange, scarlet, crimson $1*10^6$ each). Afterwards, the heart was removed and cut into 6 slices. To visualize the myocardial viability the slices were incubated in 1% triphenyltetrazolium chloride in 0.9% potassium chloride (NaCl) at 37˚C.

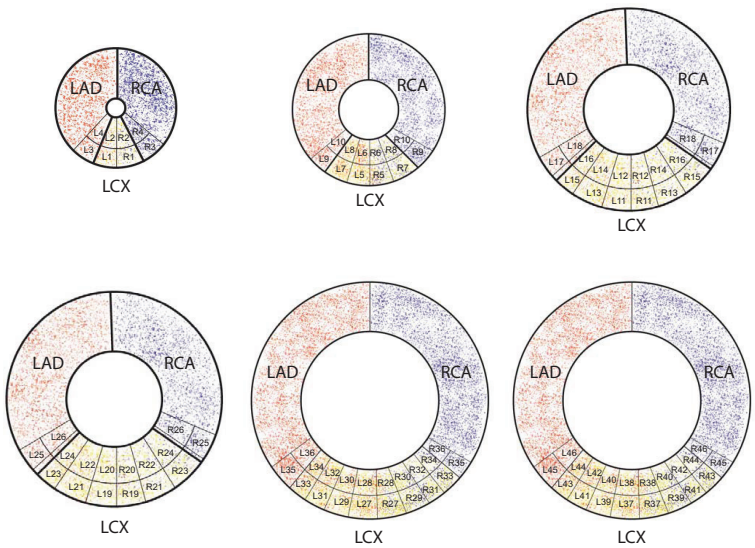
All slices were photographed on both sides and the tissue that stained red was regarded as viable. The area at risk was visualized by UV-light, marked and divided into supposedly RCA and LAD supplied areas. Per area, a total of 46 tissue samples (0.3 - 0.6g/sample) were collected for further analysis, starting from the center of the area at risk (supplementary figure 1). The remaining part of the LAD and the RCA supplied area was weighed and another 12 tissue samples (2 per slice) were collected from each perfusion territory. All samples were weighed and digested with proteinase K as previously described [10] after addition of an internal standard reference microsphere label (carmine, $1.5*10^4$/sample). Microspheres were counted by flow cytometry for subsequent calculation of tissue perfusion as previously described[12].

Analysis of tissue perfusion

Each sample was separately analyzed and classified as belonging to either the LAD or the RCA dependent perfusion territory based on the respective fluorescent microsphere labels. Only samples with a minimum count of 400 blue microspheres/g tissue were considered to be part of the original LCX perfusion territory and were used for collateral conductance calculation.

Calculation of collateral dependent flow at 2 weeks

For the analysis of collateral dependent flow directly after complete LCX occlusion, the LAD and the RCA perfusion territory samples were used as reference. The number of the respective microsphere label (i.e. orange and yellow-green) in each LCX tissue sample ($m_{baseline}$) and in the respective samples from LAD and RCA area were counted as described above. The LAD and RCA samples were then averaged per gram tissue and multiplied by the total weight of the respective perfusion territory, equaling the total number of microspheres injected (m_{flow}).



Supplemental figure 1. Sample collection after microsphere perfusion measurements from apical to basal
The left ventricle distal to the stent was cut into six slices. After identification of the different perfusion beds, the LCX area was divided into 92 samples, 46 from the LAD dependent territory (L1-L46) and 46 from the RCA dependent territory (R1-R46).

Coronary flow (CF) was calculated from the coronary diameter at the site of microsphere infusion and the corresponding average peak flow velocity (APV): CF[ml/min]=π*(diameter/2)^2*(APV/2). Baseline collateral dependent LCX perfusion was calculated as follows: perfusion [ml*min^{-1}*g^{-1}]=(CF*$m_{baseline}$*15000)*(m_{flow}*m_{ref}*$weight_{sample}$)$^{-1}$, where CF is coronary flow, $m_{baseline}$ is number of microspheres in tissue, m_{flow} is total number of microspheres injected, and m_{ref} is number of reference microspheres analyzed[13].

Collateral conductance calculation

Collateral conductances were calculated from the pressure/flow curves resulting from the different pressure/flow levels applied during the hemodynamic measurements at termination. Collateral dependent flow in the LCX area was calculated from the number of the respectively labeled microspheres in the tissue samples (m_{sample}) and in the blood flow reference samples (mblood) and the number of reference microspheres ($m_{samplerefr}$ $m_{bloodref}$):
perfusion [ml*min-1*g-1]=(m_{sample}*$m_{bloodref}$*V_{blood})*(t*m_{blood}* $m_{samplerefr}$*$weight_{sample}$)$^{-1}$, where m_{sample} is number of microspheres in tissue, $m_{bloodref}$ is number of reference microspheres in blood flow reference sample, V_{blood} is blood flow reference volume, t is duration of blood flow reference collection, mblood is number of microspheres in blood flow reference sample, and $m_{samplerefr}$ is number of reference microspheres in tissue.
The perfusion rates were then correlated to the corresponding pressure gradient between source vessel (i.e. LAD or RCA, $p_{proximal}$) and LCX (p_{distal}) and conductance was calculated from the slope of the resulting curve.

Immunohistochemistry

Collateral arteries were identified as arterial connections between to vascular territories, consisting of a stem, mid-zone and re-entry component after filling them with a bismuth-based contrast agent 28 days after the initial stent placement. They were macroscopically isolated and samples were embedded in paraffin and cut in 7µm thick sections. Blocking was done with 10% normal horse and 10% normal goat serum. Subsequently, endogenous biotin was blocked with the Dako Cytomation Biotin Blocking system according to manufacturer's protocol. Staining was performed after antibody retrieval by boiling the slides in 10 mM citrate buffer, pH 6.0 for 20 minutes. The following antibodies were used: α-SMA (mouse clone 1A4, 1:1500, 1 hour; Sigma-Aldrich, St. Louis, USA), polyclonal KI-67(rabbit, 1:25, overnight; Abcam, Cambridge, UK), horse-anti mouse biotinylated (1:750, 1 hour; Vector laboratories Inc., Burlingame, USA), Streptavidin-HRP (1:1000, 1 hour; Southern Biotech, Birmingham, USA) and Powervision anti-Rabbit-AP (Immunologic, 1 hour, Duiven, the Netherlands). Subsequently, sections were incubated in amino-9-ethylcarbazole (AEC) for 7 minutes and Fast Blue (Sigma chemical, St Louis, USA) for 5 minutes before being embedded.

Statistical analysis

All results are presented as mean ± standard error of means (SEM). Comparison between groups was performed with SPSS 15.0 (SPSS Inc., Chicago, IL) using Student's t-test. P-values <0.05 were considered statistically significant.

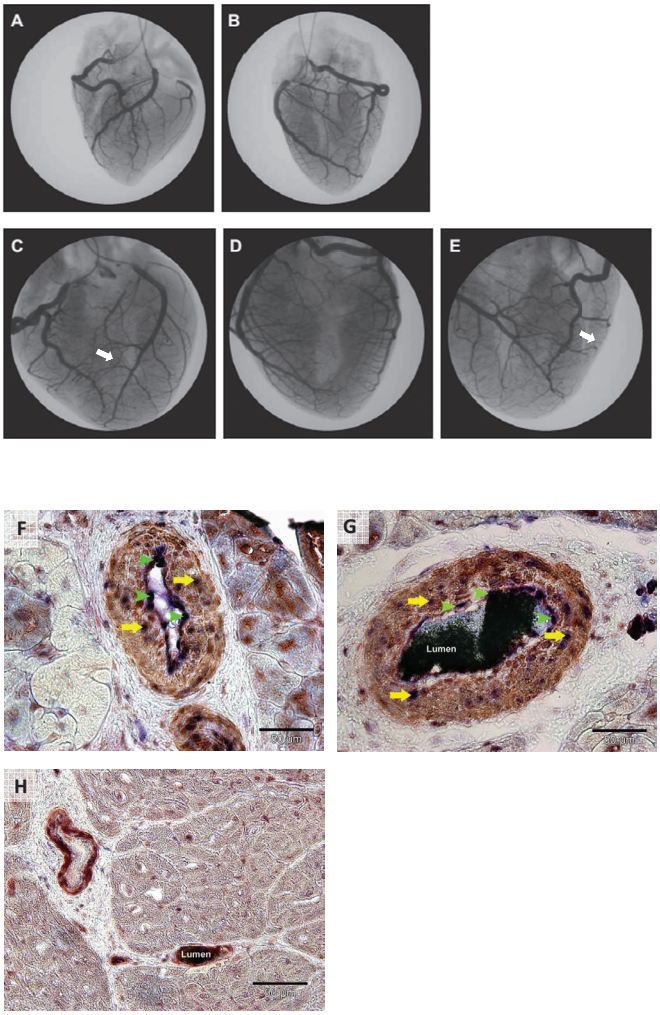


Figure 2.
(A-B) Post-mortem angiograms after acute LCX occlusion showed rapid filling of the distal LCX via pre-existent collateral connections. One of the small pre-existing connections (anastomosis) is clearly visible in the enlargement in the right bottom corner. (C-E) Angiography 28 days after stenosis induction showed increased vascular density and collateralization, indicating collateral growth. For proper comparison, the same highly standardized protocol was used on both time points (acute occlusion and day28 after stenosis). The white arrows indicate macroscopically visible collateral arteries (midzone).
(F-G) Histological sections of pig coronary collateral arteries after filling of the vasculature with bismuth-based contrast agent (lumen). Immunohistochemical staining for Ki-67 revealed vascular smooth muscle cell (yellow arrows) and endothelial cell proliferation (green arrowheads), indicating the remodeling of these vessels. Representative pictures of 5 sampling sites in two pig hearts. (H) The same staining protocol was used on pig heart tissue which was collected on day 0. Similar regions were selected for tissue collection. No KI-67 positive cells were found in the arterial wall.

RESULTS

Three animals died during stenosis induction due to acute in-stent thrombosis. A further 2 animals did not undergo collateral conductance measurements at 4 weeks as complete stent occlusion was evident during the 2 weeks surgery. Instead, the hearts were explanted and post-mortem angiography was performed as described below.

Pre-existence of coronary collateral anastomoses in the pig heart

Four additional pig hearts were collected for visualization of the pre-existent collateral network. The LCX was ligated and LAD and RCA were cannulated for injection of post-mortem contrast agent according to Fulton [14]. The heart was submerged in 37°C water and the coronaries were perfused for 8 min at 80mmHg. Despite the high viscosity of the agent, the distal LCX was quickly filled retrogradely, indicating the presence of pre-existent collateral anastomoses originating from LAD and RCA (figure 2 A, B).

Four weeks after stenosis induction, angiographic collateral density increased (figure 3 C-E). Several collateral arteries were followed macroscopically from stem to re-entry [15] and carefully excised. Immunohistochemical staining for Ki-67 confirmed active smooth muscle cell proliferation within these vessels (figure 2F, G).

CFI and FFR after stent implantation

During stenosis induction, arterial blood pressure and distal perfusion pressure were continuously monitored and recorded. The mean pressure derived collateral flow index (CFI) in healthy animals was 0.18 ± 0.03, indicating the presence of a recruitable collateral network.

After balloon retraction, fractional flow reserve measurements (FFR) under maximal vasodilatation confirmed the hemodynamic relevance of the applied coronary stenosis (FFR: 0.70 ± 0.03).

Baseline collateral perfusion

Two weeks after stenosis induction, the LCX stent was completely occluded with a stainless steel ball as described above. After initiation of the experiment all stents were still patent but two, which were excluded for the perfusion measurement at week 4. After metal sphere placement many of the animals showed TIMI 1 flow in the distal LCX due to the extensive anti-coagulation. Therefore all animals received protamine to allow coagulation and solid stent occlusion. Baseline collateral perfusion of the original LCX territory was assessed by injection differently labeled fluorescent microspheres into LAD and RCA during adenosine induced vasodilatation (LAD derived collateral perfusion: 0.43 ± 0.07 $ml*min^{-1}*g^{-1}$; RCA derived collateral perfusion: 0.37 ± 0.11 $ml*min^{-1}*g^{-1}$).

Collateral conductance

Before induction of the model and at the end of the observation period, i.e. after 3 and 4 weeks, collateral conductance measurements were performed and the hearts were explanted for quantification of myocardial fluorescent microsphere content. TTC (Triphenyl Tetrazolium Chloride) staining showed minor to no myocardial infarction, indicating myocardial protection via the collateral network at the time of complete LCX occlusion (figure 3).

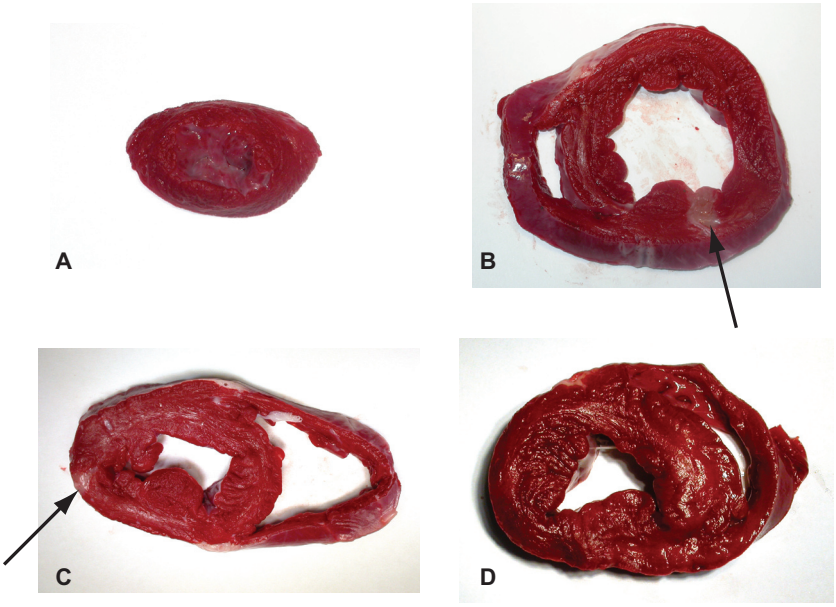


Figure 3.
Triphenyl Tetrazolium Chloride stainings after stenosis induction and subsequent total occlusion from different animals. Despite complete LCX occlusion, only minor to no myocardial infarctions (arrows) were observed. If present, infarctions were confined to the basis, resulting from the occlusion of coronary side branches by the ePTFE membrane.

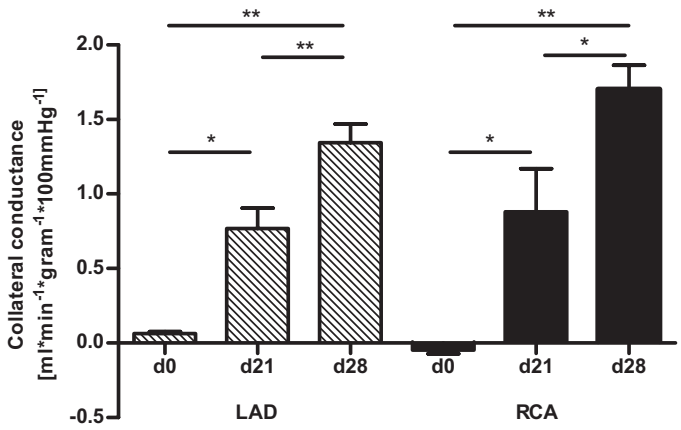


Figure 4.
Collateral conductance at baseline and after 21 and 28 days after stenosis induction and occlusion at day 14, separated for LAD and RCA derived collateral arteries. Compared to baseline collateral conductance increased significantly during the step wise occlusion of the LCX. Both LAD and RCA supplied collaterals showed active growth, indicated by the significant increase in collateral conductance between day 21 and 28 (p<0.05; ** p<0.01).*

Collateral conductance measurements further confirmed active collateral artery growth as collateral conductance significantly increased from close to zero after acute occlusion (collateral conductance [ml*min^{-1}*g^{-1}*100mmHg^{-1}]: LAD 0.06 ± 0.01, RCA -0.05 ± 0.03) to active flow in week in week 3 after stenosis induction. Between week 3 and 4, collateral artery growth continued, resulting in a further increase in collateral conductance ([ml*min^{-1}*g^{-1}*100mmHg^{-1}]: LAD 3 weeks: 0.77 ± 0.14, LAD 4 weeks: 1.35 ± 0.12, $p<0.01$; RCA 3 weeks: 0.88 ± 0.29, RCA 4 weeks: 1.70 ± 0.16, $p<0.05$) (figure 4).

DISCUSSION

The pig resembles the human in many aspects. Its size, weight, metabolism and particular its anatomy renders it the most human-like non-primate cardiovascular animal model. The pig as a model for coronary collateral growth has been controversially discussed since the groundbreaking experiments by Schaper and colleagues showing that the pig coronary circulation at baseline is less extensive than in e.g. dogs [2, 3, 16-18]. Hence, the pig has become a standard pre-clinical model to study acute myocardial infarction after acute coronary occlusion. However, various studies have shown that despite these findings, coronary collaterals can be recruited in the pig when given sufficient time [4-8, 10, 11]. In our model we also showed that pre-existing anastomoses must be present in the healthy pig heart. Selective infusion of contrast agent in the LAD and RCA resulted in filling of the LCX, even if there was no time to remodel any collateral arteries. The observation that the collateral conductance at baseline was close to zero is probably due to the edema occurring after acute ligation of the LCX, disabling any perfusion of the pre-existing anastomoses during the myocardial ischemia that is present.

Ideally, recruitment of collateral arteries occurs under circumstances that do not lead to massive myocardial infarction. This can be achieved by reducing the patent coronary artery lumen to such a degree that the stenosis becomes significant and the dormant collateral anastomoses are recruited, but that the downstream tissue perfusion is sufficient to prevent severe ischemia and tissue necrosis. Several techniques have been developed and used to reach this aim. One possibility is to place a ligature around the coronary artery without completely obstructing the vessel lumen, leading to a reproducible lumen stenosis directly after the procedure but not to complete occlusion. Another common technique is the ameroid constrictor implantation, inducing gradual occlusion within approximately 3 weeks after placement [19-21]. This results in the growth of coronary collateral arteries, which functionally almost resembles the well developed coronary collateral circulation in dogs, indicating the potential for coronary collateral artery growth in pigs [5, 6, 8, 11].

One of the major drawbacks of the ameroid constrictor and the coronary ligature technique is the need to dissect the target coronary artery, which requires open chest surgery once for the implantation and a second time for complete occlusion for the ligature procedure. As previously shown by Kupatt and colleagues, this can be circumvented by percutaneous placement of a coated reduction stent (~75% lumen stenosis), making open chest surgery unnecessary [17, 22]. Comparable to the ameroid constrictor implantation, the reduction stent leads to complete coronary occlusion in pigs within a few weeks after implantation into the LAD. While the time point of reaching hemodynamic relevance can only be estimated with the ameroid constrictor, the reduction stent directly induces a significant stenosis in most cases.

However, in both methods the moment of complete occlusion remains largely unknown. In our model, the actual lumen stenosis can be defined by fixing the appropriate ring to the stent prior to implantation. The size of the ring can easily be adjusted according to the vessel size. A hemodynamically relevant stenosis is caused immediately, defining the start of collateral recruitment by changing hemodynamic forces in the pre-existent anastomoses. Two weeks later, complete occlusion is achieved in a percutaneous fashion, again eluding open chest surgery, which indicates the moment that the pressure gradient and hence the driving force for arteriogenesis reach a maximum. In addition, the step-wise stenosis and occlusion process resembles typical thrombotic events in acute myocardial infarction in the presence of significant atherosclerotic lesions.

Using the described protocol of stenosis creation by stent implantation followed by total occlusion 2 weeks later, we observed only minor myocardial infarctions. Moreover, immunohistochemical stainings in angiographically identified pig coronary collateral arteries for the proliferation marker Ki-67 confirmed proliferative activity. Quantification of this index of proliferation could be useful in future applications of the model when comparing treatment strategies. Together, these observations further support the functional importance of the collateral network in the pig heart.

Another important advantage of this newly developed protocol is the possibility to separately address collateral function for different source vessels, i.e. LAD and RCA in the current study. Mostly, microspheres for blood flow determination are injected into either the left atrium or ventricle. This way, no distinction can be made between different sources. By applying the described collateral conductance measurements, efficacy testing of local treatments of 1 arterial bed, with the other serving as an intra-individual control can be realized. Furthermore, variations in coronary anatomy can be more easily taken into account. In the current study, collateral conductance from both vascular beds significantly increased between 3 and 4 weeks after stenosis induction indicating the ongoing growth of the collateral network. While collateral dependent LCX perfusion under vasodilatation with adenosine was similar for LAD and RCA derived collaterals, the conductance of RCA fed collaterals was higher than that of the vessels supplied by the LAD, both at 3 and 4 weeks after stenosis induction. However, the tissue mass supported by LAD derived collaterals was larger than the RCA dependent territory, translating into a comparable total conductance of both collateral networks. Although our model yields microsphere-based flow measurement using the gold standard method for flow measurements, this method does not yield flow peak velocities of all the distal coronary arteries during vasodilation. This unfortunately makes a valid calculation of microvascular resistence indices not possible in our model 23, which was designed primary for the assessment of collateral artery growth. The repeated infusion of larger amounts of microspheres could theoretically influence vessel growth by causing micro-infarctions. However, we did not encounter any negative side effects of the infusions in our model. No arrhythmias or histological micro infarctions were observed, suggesting that application of the microspheres (15μm) in the coronary arteries did not significantly compromise the flow in the microcirculation.

Our porcine model of coronary collateral artery growth more closely resembles the clinical course of coronary disease in humans by mimicking the step wise reduction in the arterial lumen of coronary vessels. However, this is achieved by an interventional approach and not by the natural progression of atherosclerotic disease. Atherosclerotic pigs are available, but the implementation of these pigs in our model at the current stage was not feasible due to the

extensive time periods. We therefore chose not to include this race in our current experiments. However, this limitation needs to be considered when comparing our model the clinical situation in human patients.

In summary, we here describe a new, minimally invasive model for coronary collateral artery growth in pigs following a pre-defined schedule of hemodynamic relevant LCX stenosis and subsequent complete occlusion, further allowing the separate assessment of collateral function from different source vessels, i.e. LAD and RCA. This model can therefore provide important insights into the anatomical variability of collateral supply and can further be used to evaluate the efficacy of local pro-angiogenic and pro-arteriogenic treatments.

Acknowledgements

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Submitted

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8

COX-2 inhibition reduces coronary collateral perfusion

ABSTRACT

Rationale Collateral artery growth can prevent serious ischemic complications in case of an arterial occlusion. Collateral artery formation requires a local inflammatory process that can be influenced by medication. Cyclooxygenase-2 inhibitors (COX-2i) are anti-inflammatory drugs inhibiting the rate limiting conversion of arachidonic acid to prostaglandins. COX-2 inhibition has been associated with the occurrence of cardiovascular events but its effect on collateral artery perfusion is unknown.

Methods In 24 pigs, collateral artery formation was induced by a stepwise occlusion at day 0 and 14 of the left circumflex artery (LCx) with a minimal invasive stent technique. Twelve pigs were treated daily with the selective COX-2i Celecoxib 400mg (day -2 till 28) and 12 animals served as controls. Fluorescent microspheres were infused in 8 pigs/ group during each intervention (day 0, 14 and 28) to determine collateral perfusion. Macrophage infiltration in the tissue was assessed in 4 pigs/ group. Cardiac function was invasively measured at 28 days by pressure-volume loops.

Results Daily treatment with Celecoxib selectively inhibited COX-2 function reflected by reduced Prostaglandin E_2 synthesis (control vs. Celecoxib) (pg/ml) 794.1± 229.4 vs. 186.3± 58.5, p=0.043, but not that of COX-1 dependent Tromboxane B_2 synthesis (pg/ml) 780.8± 210.1 vs. 689.5± 225.4, p=ns. Stepwise occlusion of the LCx induced collateral growth, sufficient to prevent myocardial infarction in all pigs. Celecoxib treatment resulted in a significant decrease in the collateral perfusion (day 28, COX-2i vs. control, [$ml*min^{-1}*g^{-1}*100mmHg^{-1}$]; from left anterior descending artery to LCx region: 0.55±0.08 vs. 1.35±0.12, p<0.0001; from right coronary artery to LCx region: 1.13±0.21 vs. 1.70±0.16, p=0.03) The reduced collateral perfusion had no effect on cardiac function in this model. No differences were found between the groups in blood monocyte counts, nor the amount of perivascular macrophages.

Conclusion The COX-2i Celecoxib decreases perfusion restoration in the pig heart, pointing to another serious side effect of COX-2i treatment.

INTRODUCTION

Cyclooxygenase (COX) is the rate limiting enzyme in the conversion of arachidonic acid to prostaglandin. The two most important isoforms of cyclooxygenase are COX-1 and COX-2. The first is constitutively expressed on cells and mediates many physiological responses and homeostasis. COX-2 is considered to be induced and upregulated by pathological conditions, such as inflammatory diseases. Selective COX-2 inhibitors came to the market several years ago as novel analgetic drugs. Although designed to reduce treatment related (gastro-intestinal) complications, it became apparent that the drugs gave rise to of the increase of cardiovascular events in users of COX-2 inhibitors [1].

An important factor that determines the outcome of cardiovascular disease, is the ability to restore the perfusion to a region at danger of ischemia. The most efficient way is by the growth of collateral arteries. This process, called arteriogenesis, comprises of the active remodeling of dormant interconnecting vessels into functional arteries capable of redirecting large quantities of blood. A well developed collateral network has shown to be beneficial for the cardiac function after myocardial infarction on short [2] and long term [3], and can also prevent cardiovascular events [4]. Arteriogenesis is considered to be an inflammatory process [5], that can be influenced by medication that alters inflammatory responses and hemodynamics [6]. COX inhibition reduces inflammation by diminishing prostaglandin levels and can therefore potentially influence collateral growth negatively [7]. The effect of selective COX-2 inhibition on collateral artery perfusion, however, has not yet been studied. We hypothesized that selective COX-2 inhibition, by the clinically prescribed drug Celecoxib, reduces collateral perfusion. Because many of the complications due to the use of COX-2 inhibitors were related to myocardial damage [8], we studied the effect of COX-2 inhibition on collateral growth in the heart using a porcine model.

METHODS

Animals

All animal experiments were executed after obtaining the appropriate approval from the animal ethical committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals published by The US National Institute of Health* (NIH Publication No. 85-23, revised 1996). Twenty -four Dalland Landrace pigs (body weight 65±2 kg) were purchased from van Beek (Lelystad, the Netherlands) and housed in the animal facility at the University Medical Center Utrecht. All animals received a daily dose of 400mg amoidarone starting 10 day pre-operative and 75mg clopidogrel from day -3 till day 10. Before stent placement, a single dose of 500mg aspirin was given intravenously to all animals to prevent acute stent thrombosis.

Anesthesia and analgesia

Prior to the operation, the animals received intramuscular premedication (10 mg/kg ketamin, 0.4 mg/kg midazolam, 0.5 mg atropine), followed by intravenous induction of anesthesia with 4 mg/kg thiopental, antibiotic prophylaxis (amoxicillin/clavulanic acid) and intubation. Throughout the surgical procedures, animals were mechanically ventilated and anesthesia was given intravenously with 0.5 mg/kg/h midazolam, 2.5 µg/kg/h sufentanil and 0.1 mg/kg/h

pancuronium to keep the animals sedated throughout the experiment. During the procedure all animals received metoprolol (5mg i.v., immediately after start anesthesia) to reduce heart rate and heparin (100 IE/kg i.v.) to prevent coagulation. After the operations, a fentanyl plaster (25ug) was applied as pain relief.

Study design

The animals were randomized into 2 groups of 12 pigs. Group 1 received COX-2 inhibition by Celecoxib 400mg/ day throughout the whole experiment (day -2 till day 28). Group 2 served as a control and did not receive any Celecoxib. To initiate collateral growth in the heart we used a porcine model of gradual coronary artery occlusion [9]. This model follows a pre-defined schedule of hemodynamic relevant left circumflex (LCX) coronary artery stenosis and subsequent complete occlusion and is described below in short.

At day 0, a tapered stent was placed in the left circumflex artery (LCX) to create a significant arterial lumen reduction. Fluorescent microspheres (Fluospheres, 15µm, Molecular Probes Inc., Eugene, Oregon) were infused under maximal vasodilatation (10mg/ml/min adenosine i.v.) in each of the coronary arteries to mark the perfusion territories at baseline.

After two weeks, the tapered stent was sealed off by placing a metal sphere in front of the narrow opening. Again, fluorescent microspheres (different colors) were infused under maximal vasodilatation in the right coronary artery (RCA) and left anterior descending artery (LAD).

One month after the initial stent placement a hemodynamic measurement was performed to assess the functionality of the collateral arteries (8 animals/group). During the measurement, different colors of microspheres were infused in the RCA and LAD under controlled pressure/ flow levels. A pressure sensor placed distally from the occluded stent measured the distal perfusion pressure.

At the end of the experiment, the heart was removed and the ventricle was cut into 6 even slices. Before they were processed for analysis of microsphere counts, a myocardial viability staining was performed with 1% triphenyltetrazolium chloride (TTC) in 0.9% potassium chloride (NaCl) at 37˚C. The slices were then cut, weight and digested. Microspheres were counted by flow cytometry (FC500, Beckman Coulter, Miami, FL, USA), to calculate the collateral conductance from the RCA to the LCX region and from the LAD to the former LCX region afterwards.

Four animals per group did not receive the hemodynamic measurements and were used for post-mortem angiograms and histology. These animals underwent the same protocol, but did not receive any TTC staining or microsphere infusion.

Cardiac function

Cardiac function was assessed with a conduction catheter placed in the left ventricle (n= 8 COX-2i, n= 11 control). The recorded pressure and volume ratios were displayed and acquired at a 250 Hz sampling rate with a Leycom CFL-512 (CD Leycom, Zoetermeer, the Netherlands). All measurements were done during temporary arrest of ventilation to prevent breathing artifacts. The catheter was calibrated with 10% saline infusion. Cardiac stress was acquired by subjecting the animals to intravenous dobutamine (2.5µg/kg/min, n= 7 COX-2i, n=4 control). Analysis of the pressure- volume loops was performed using custom software as previously described [10].

Blood collection for cell counts and stimulation

During all experiments blood was collected immediately after arterial access and 5 minutes after the intervention (either stent placement or occlusion). Heparin was used as an anti-coagulant. White blood cell counts were measured with a automated cell counter (Cell Dyn 1800, Abbott Laboratories, Illinois, USA). Baseline samples (first operation, before stent placement) were used for stimulation with 100µg/ml LPS (E. Coli 055-B5, Sigma-Aldrich, St. Louis, USA) in a 37˚C and 5% CO_2 environment. The following day, samples were taken from the stove and centrifuged at 300G for 5 minutes. The plasma was collected and stored at -20˚C until use.

Prostaglandin ELISAs

The LPS stimulated plasma samples were thawed at room temperature. A Prostaglandin E_2 (PGE_2) ELISA (Assay Designs Inc., Ann Arbor, USA) was performed according to manufacturer's protocol. Thromboxane B_2 (TXB_2), the stable variant of Thromboxane A_2 (TXA_2) and a downstream target of COX-1, was measured using a TXB_2 ELISA kit (Assay Designs Inc, Ann Arbor, USA). All samples were measured in duplicates (n= 7 COX-2i, n= 8 control).

Immunohistochemistry

Two weeks after the occlusion of the LCX, the accumulation of macrophages in the tissue was assessed in both Celecoxib treated (n=4) and control (n=4) group. The hearts were explanted and the LAD and RCA were cannulated for injection of post-mortem contrast agent, according to Fulton [11]. Collateral arteries were identified as arterial connections between two vascular territories, consisting of a stem, mid-zone and re-entry component after filling them with a bismuth-based contrast agent, Per animal, three regions containing the collateral arteries were macroscopically isolated and snap frozen in liquid nitrogen. All samples were embedded in Tissuetek (Sakura, Zoeterwoude, the Netherlands) and cut in 5µm thick sections. The slides were fixated in ice cold aceton after which the arterioles were stained by αSMA-FITC (Sigma-aldrich, Zwijdrecht, the Netherlands; 1:300 in PBS with 1% BSA, 30 minutes at room temperature). Next, acid phosphatase was applied for 30 minutes at 37 degrees (dark) to detect the macrophages and the sections were counterstained with heamatoxiline for 5 seconds. All sections were inspected under a microscope (Olympus BX60) and pictures were taken (Olympus DP71) at a 200x magnification. Macrophages accumulating in the perivascular tissue were counted by a blinded observer in at least 15 tissue sections/ animal.

Statistics

All statistics were performed using SPSS 15.0 (SPSS Inc.). Mann Whitney U test was used to compare differences between two groups. The values were presented as mean ± SEM and p-values < 0.05 were regarded as significant.

Daily dose of Celecoxib resulted in specific COX-2 inhibition

To assess whether daily treatment with Celecoxib resulted in a specific inhibition of COX-2 isoform without affecting COX-1, we tested the prostaglandin E_2 and thromboxane B_2 (the stable variant of thromboxane A_2) levels in treated and non-treated animals. COX-2 inhibitors suppress the biosynthesis of prostaglandin E_2 without concomitant inhibition of thromboxane A2, which derives predominantly from platelet COX-1 [12]. Treated animals showed significantly less prostaglandin E_2 production compared to non-treated animals (186.3± 58.5 versus 794.1± 229.4, p=0.043), while thromboxane levels were not influenced (689.5± 225.4 versus 780.8± 210.1, p=ns) (figure 1). This indicates that Celecoxib specifically inhibited the COX-2 isoform in our pigs.

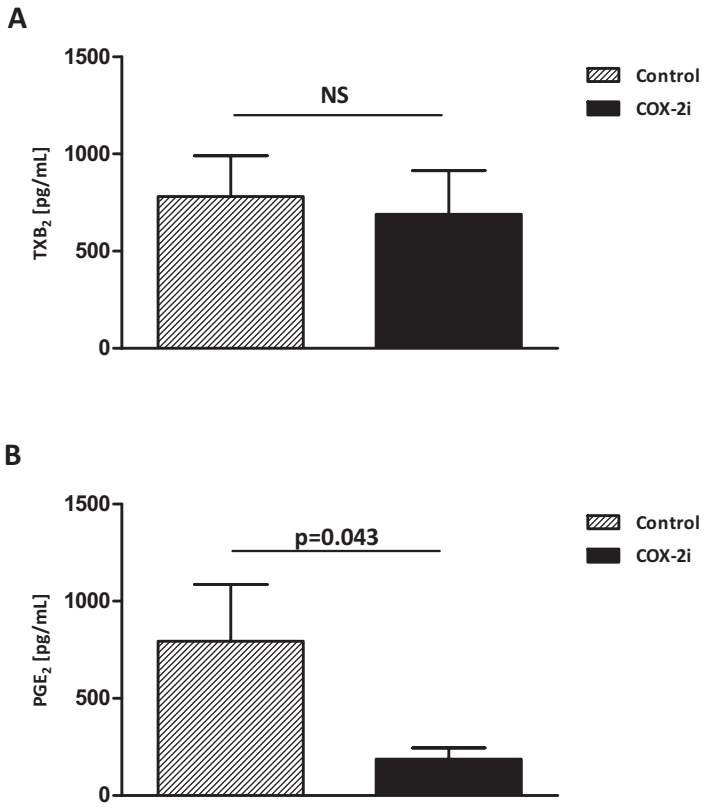

Figure 1. Celecoxib treatment results in a specific prostaglandin E2 reduction without effecting tromboxane levels

Prostaglandin E_2 and Tromboxane B_2 were measured in pig plasma. Whole blood samples of 7 Celecoxib (COX-2i) treated and 8 control pigs were stimulated with 10ug LPS for 24 hours. An ELISA was used to quantify both products and all samples were measured in duplicates. Significantly less COX-2 induced prostaglandin E_2 was observed in animals treated with the COX-2 inhibitor (p= 0.043), while the COX-1 related thomboxane levels were similar in all animals.

The stepwise occlusion of the LCX artery resulted in coronary collateral growth

The placement of the stent and its occlusion did not lead to significant myocardial infarction downstream of the stent as previously described [9]. No differences were differences were found between Celecoxib treated and the control group.

Celecoxib inhibits collateral artery perfusion in the heart

Celecoxib treatment animals significantly reduced collateral conductance at 4 weeks compared to the control group (collateral conductance in [ml*min^{-1}*gram^{-1}*100mmHg^{-1}] for Celecoxib treated versus control: LAD to LCX region 0.55±0.08 versus 1.35±0.12, p<0.0001. RCA to LCX region 1.13±0.21 versus 1.70±0.16, p=0.03) (figure 2).

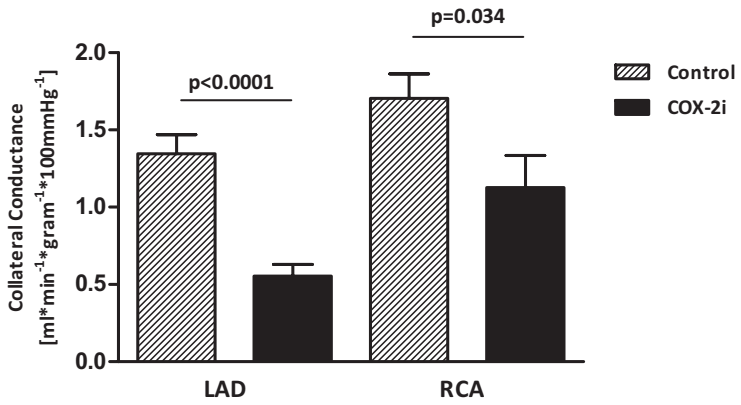

Figure2. Celecoxib treatment results in a reduction in collateral conductance
After stepwise occlusion of the left circumflex artery (LCX), the collateral conductance was measured at day 28, separated for LAD and RCA derived collateral arteries. The Celecoxib treated group (COX-2i) (n=8) show a decreased conduction for both LAD (p<0.0001) and RCA (p= 0.034) supplied collaterals, when compared to the control group (n=8).

Cardiac function did not differ between treatment groups

To assess whether the deterioration of collateral perfusion had an effect on cardiac function, we performed pressure- volume loop recordings at day 28 (table 1) with 2.5µg/kg/min dobutamine and without dobutamine. No differences in cardiac function were observed between both groups at 4 weeks, even after challenging the heart with dobutamine.

Monocyte/ macrophages numbers in blood and tissue did not differ between the two groups

Since COX-2 inhibitors are well known anti-inflammatory drugs, we investigated whether the chronic treatment with Celecoxib influenced the number of monocytes in the blood or macrophages in the tissue. These cells are key players in collateral artery growth and any decline in number or function directly influences arteriogenesis negatively. Throughout the experiment, blood leukocyte number and blood monocyte numbers did not differ between the groups (Table 2). Also the extravasation of monocytes into the perivascular tissue was not different between Celecoxib treated animals and controls 4 weeks after ischemia induction (Table 2).

Table 1. Cardiac function did not differ between Celecoxib treated and control animals

	No dobutamine		Dobutamine (2.5µg/kg/min)	
Parameter	**Control**	**Celecoxib treated**	**Control**	**Celecoxib treated**
HR (beats/min)	64.5 ±3.8	65.2 ±5.0	69.0± 8.8	70.0 ± 4.2
EDP (mmHg)	10.7 ±1.3	21.5 ±6.1	15.1± 7.3	18.8± 7.7
dP/dtmax (mmHg/sec)	1460.9 ±112.5	1270.7 ±98.6	3378.4± 602.6	3018.6± 257.4
dP/dtmin (mmHg/sec)	-1428.7 ±118.6	-1304.1 ±111.9	-1853.5± 387.1	-1718.3± 126.5
EDV (ml)	137.3 ±9.45	123.59 ±8.4	150.5± 29.7	138.9± 11.8
ESV (ml)	72.1 ±7.9	60.1 ±5.8	85.9± 37.3	64.3± 10.3
EF (%)	50.2 ±2.9	53.2 ±2.5	50.1± 10.0	57.5± 4.5
SW (ml*mmHg)	5463.5 ±413.2	5426.3 ±696.2	7094.4±798.5	8016.5±623.3
Tau (ms)	52.4 ±2.5	55.0 ±2.23	53.7± 3.4	57.2± 4.1
Ees (mmHg/ml)	1.45± 0.4	2.11± 0.48	4.4± 1.9	3.4± 0.6

Cardiac function was assessed with a pressure-volume catheter, 28 days after starting the left circumflex artery occlusion. The data is presented as mean ± SEM. HR= heart rate; EDP= end-diastolic pressure; dP/dtmax= measure of contractility, dP/dtmin= measure of relaxation, EDV= end- diastolic volume; ESV= end- systolic volume; EF= ejection fraction; SW= stroke work; Tau= relaxation constant; Ees= end-systolic elastance. Control: n=11 ; COX-2i: n=8. There were no significant differences between the two groups

Table 2. Monocyte numbers are not affected by treatment with Celecoxib

	Control	Celecoxib treated
Monocytes/mL blood		
Day 0:	1.22 ±0.06	1.38 ±0.05
Baseline (x10^6)	1.41 ±0.07	1.48 ±0.08
After arterial narrowing (x10^6)		
Day 14:		
Before arterial occlusion (x10^6)	1.17 ±0.11	1.16 ±0.08
After arterial occlusion (x10^6)	1.34 ±0.14	1.41 ±1.1
Day 28:		
Before measurement collateral function (x10^6)	1.11 ±0.10	1.19 ±0.13
Macrophages/collateral artery		
Day 28 (perivascular space)	1.99 ±0.36	1.83 ±0.28

During collateral artery growth, the number of monocytes in blood and tissue were assessed (n=8 control versus n= 8 Celecoxib treated). At each intervention blood was drawn and counted on an automated cell counter. At day 0 collateral growth was initiated by creating a significant arterial lumen narrowing. At day 14 collateral growth is already present and an additional stimulus is given by totally occluding the artery. At day 28 collateral function was assessed. In both control and Celecoxib treated animals, the monocytes followed the same pattern and no significant differences were observed.

Macrophages were counted in histologic specimens, collected at 4 weeks after the initiation of the experiment. In each pig (n= 4 per groups) three sites of collateral growth were sampled. The macrophages were stained with acid phosphatase. Positive cells, containing a nucleus and situated in the perivascular space, were counted by a blinded observer in at least 15 tissue sections/ animal. No significant differences were found between the control and the Celecoxib treated group.

DISCUSSION

Collateral arteries growth is an important protective mechanism in patients suffering from atherosclerotic disease, since this process can prevent the occurrence of cardiovascular events and improve outcome [13]. The rate in which collateral growth takes place depends on the recruitment of inflammatory cells to the pre-existing collateral connections. Any substance modulating this process can potentially affect collateral artery growth. In the present study, we investigated whether the treatment with the COX-2 inhibitor Celecoxib altered coronary collateral artery perfusion recovery. We showed that the chronic use of Celecoxib resulted in a reduction in collateral dependent perfusion in the porcine heart.

Celecoxib is one of the few selective COX-2 inhibitors still on the market today. Most of its direct competitors were retracted after serious safety concerns were raised [1, 8]. The main problem with all selective COX-2 inhibitors (including Celecoxib) was the increased occurrence of cardiovascular complications, even in low risk patients. In search for the responsible mechanism, studies focused on the possible pro-thrombotic [14-16], pro-arteriogenic [17, 18] or hypertensive properties [19] of these drugs. The present study shows that COX-2 inhibitors deteriorate coronary collateral perfusion. As a result, their normal protective function is reduced, that might increase the chance of having a cardiovascular complication.

The translation of experimental data into the patient's setting is a major problem in arteriogenesis research, as large discrepancies are found between the results in animal models and men [20]. Furthermore, previous studies have demonstrated that the choice of model is very important when studying COX-2 inhibition in the heart, since the outcome can be fundamentally different when another species is studied. After myocardial infarction or Doxorubicin treatment, COX-2 inhibition in mice preserved myocardial dimensions and improved function [21-23], while the COX-2 inhibition in pigs had a detrimental effect [24]. We therefore choose the model most similar to the human situation, a porcine model for coronary collateral growth, and used a clinical dose of Celecoxib, 400mg/day.

In the pigs, the Celecoxib treatment resulted in a selective inhibition of COX-2, while COX-1 was unaffected. It has been postulated that pig hearts do not exhibit networks of pre-capillary arterioles and were therefore not capable of proper collateral growth [25]. Porcine models, were therefore regarded as unsuitable for arteriogenesis research. Previous studies, however, showed that pre-existing connections are present in the porcine heart [9, 26, 27]. Furthermore these connections are able to grow into functional collateral arteries in response to gradual lumen loss, thereby closely mimicking the process in the human heart.

Even though the collateral conductance in the Celecoxib treated group was about 50% of that in control animals, cardiac function was not significantly different from that in control animals. Also during cardiac stress, induced by the dobutamine, the collateral perfusion in the Celecoxib group was sufficient to prevent loss of the myocardial function. It is, however, possible that 28 days of gradual occlusion did not provide enough time for adverse cardiac remodeling and an effect of the reduced collateral conductance by Celecoxib on cardiac function will become evident during a longer follow-up. On the other hand we can also not exclude the possibility that a catch up in perfusion may occur in the treated group at longer durations of follow up.

Since COX-2 inhibitors belong to the family of anti-inflammatory drugs, we investigated whether the treatment with Celecoxib had an effect on collateral growth by altering monocyte number

or function. Monocytes have been identified as important players in collateral artery growth[28-34]. Monocytes and macrophages are a potent source of COX-2 [35]. Treatment with Celecoxib, however, did not cause any change in pattern, regarding the number of monocytes circulating the blood in response to the stepwise lumen narrowing. Even more important, no differences in the amount of macrophages present around developing collateral arteries were seen at 4 weeks. At this time point, there still is active collateral growth in this model, since the conductance increases significantly between week 3 and 4 [9]. Nevertheless, it is possible that Celecoxib does affect the speed in which monocytes extravasate in the early phase of growth and any influence on macrophage counts would only be visible at an earlier time point. This is supported by a paper by Tajima and colleagues, who show that only the early migration of cultured RAW264.7 macrophages deteriorates by the addition of the selective COX-2 inhibitor CAY10404 [36].

Some studies have reported that the chronic use of Celecoxib results in a mild increase of the blood pressure [19]. Hypertension can affect collateral artery growth by altering the hemodynamic forces on the blood vessel wall [37]. However, in a previous study in pigs using a double dose of Celecoxib, no effect of blood pressure was seen [24]. It is therefore not likely that any change in blood pressure is responsible for the decline in collateral artery growth.

In conclusion, chronic COX-2i treatment reduces coronary collateral perfusion in a large animal model without a direct effect on cardiac function. This can have serious implications for the patients taking these drugs as the reduction in arteriogenesis might lead to more cardiovascular events and a worse outcome.

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9

Summary and discussion

The aim of the work described in this thesis was to further elucidate the mechanisms involved in arteriogenesis. Furthermore, we focused on providing tools that could contribute to a better translation of experimental data from bench to bedside.

The thesis starts with a short review of the current knowledge on the effects of classic cardiovascular risk factors on collateral artery growth (**chapter 2**). Collateral artery growth mostly coincides with the presence of one or more cardiovascular risk factors. Risk factors can affect collateral artery growth directly and indirectly, by altering hemodynamic forces or influencing cellular function and proliferation. Interestingly, their presence does not necessarily have a negative effect on arteriogenesis. Controversy still exists on the role of each individual risk factor and how it contributes to or hampers collateral growth. Most evidence indicates that elderly and hypercholesterolemic patients are most likely to have a poor collateral circulation. For diabetes, obesity and hypertension, the present data are not yet conclusive. Smoking does not seem to negatively influence arteriogenesis. The treatment of the risk factors also has an effect on collateral growth. In particular statins and ACE inhibitors are positively related with collateral growth and can improve their function and thereby clinical outcome.

In chapters 3- 5, we explore the role of the innate immune system in collateral artery growth. The innate immune system classically responds non-specifically to the exposure of pathogen associated molecular patterns (PAMPs) [1]. Within the innate immune system, Toll Like Receptors (TLR) play a major role. TLRs do not only recognize PAMPs, but also endogenous ligands

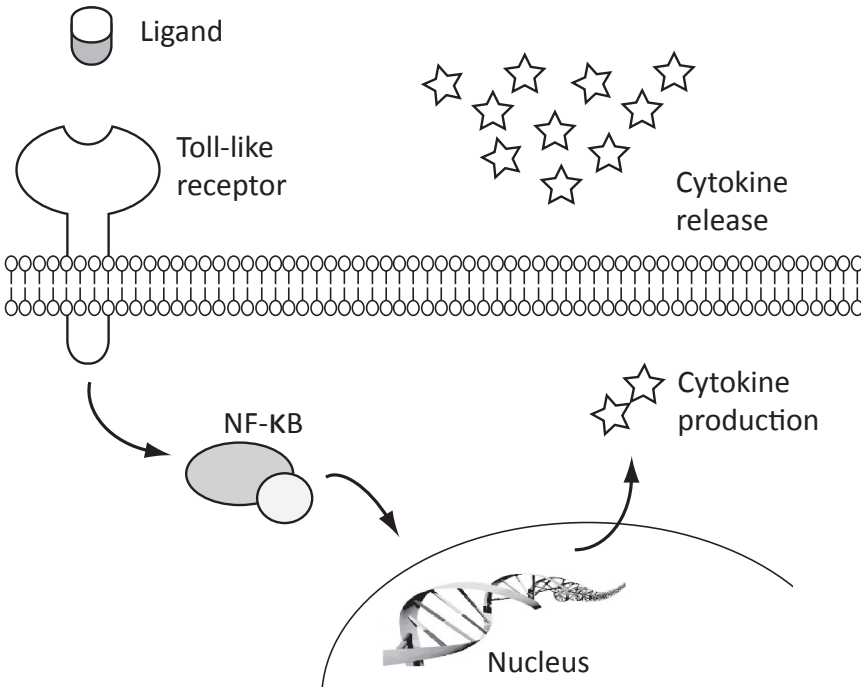

Figure 1. Schematic overview of TLR cascade on the cellular surface.

produced in response to stress or injury, like for instance Extra Domain A of alternatively spliced fibronectin (EDA)[2]. This indicates that the tasks of the innate immune system are much more extensive than protection from the outside world only and also plays a role in the internal homeostasis[3]. A simplified scheme of TLR activation is presented in figure 1.

Chapter 3 provides genetic evidence for the causal involvement of TLR 2 and TLR 4 in collateral artery growth. In mice lacking either TLR 2 or TLR 4, arteriogenesis was severely impaired. Without functional TLR 2 or TLR 4, the infiltration of monocytes into the perivascular space of the developing collateral arteries was decreased. In normal (wild type) mice, these infiltrating leukocytes display high TLR expression, whereas the endothelium lining the collaterals only stained weak. The importance of the leukocytic TLR expression in collateral artery growth was further demonstrated by the fact that the attenuation in collateral growth could be rescued by transplanting wild type bone marrow into the TLR deficient mice strains.

Chapter 4 confirms the importance of the circulating leukocytes in arteriogenesis. In this chapter we focused on the regulation of collateral artery growth by NF-κB. NF-κB can be activated by TLR 2 and TLR 4 signaling, but is also activated in response to changes in the shear stress acting on endothelial cells that occur during collateral development. NF-κB consists of several subunits, in which the p50 subunit functions as a brake on the gene transcription. In mice that genetically lack NF-κB p50 (NF-κB p50 -/-) we showed that, after arterial ligation, more monocytes infiltrate the perivascular space of developing collaterals. This resulted in more collateral growth. This improvement was also seen when wild type mice were transplanted with NF-κB p50-/- bone marrow. Vice versa, in NF-κB p50-/- mice with wild type blood cells, arteriogenesis was not increased. This demonstrates that the NF-κB p50 in bone marrow derived cells regulates arteriogenesis.

In **chapter 5,** we show that Extra Domain A of fibronectin (EDA) is upregulated at the site of collateral artery growth. EDA is a potential ligand of TLR2 and 4, but also has a role in e.g. cell adhesion. In EDA-/- mice, perfusion restoration after an arterial occlusion was impaired. Lower levels of circulating monocytes and a decrease in the number of macrophage around developing collateral arteries were likely to be responsible for the decrease in perfusion recovery. This study demonstrated for the first time an causal role for EDA in arteriogenesis. However, whether this is via TLR binding, or due to other roles of EDA, still has to be elucidated.

In **chapter 6,** we show that old mice (> 18 months old; normal lifespan mouse 1-2 years) have a significantly reduced perfusion recovery after arterial ligation, indicating a decline in collateral artery growth at high age. Even though old mice exhibited more circulating monocytes and higher cytokine levels they still expressed less macrophages round their developing collateral arteries. To test whether the circulating cells were responsible for this decline we rejuvenated the cells in the circulation by transplanting young bone marrow into the old mice. Unfortunately, young cells didn't improve the collateral growth. In reverse, the transplantation of old bone marrow in young mice significantly hampered collateral growth. When challenging these old monocytes, only intracellular adhesion molecule-1 (ICAM-1) seemed to be less reactive. Since ICAM-1 plays an important role in arteriogenesis[4], this could be a contributing factor in the worse outcome in old mice.

In **chapter 7,** we present a new model for coronary collateral artery growth, which more closely reflects the human situation. Most studies on collateral artery growth are executed in small animals (mice, rats, rabbits), limiting the technical possibilities and thereby the feasibility of

more specialized models. Especially coronary artery disease models have proven to be difficult and peripheral artery disease models were therefore often preferred. The acute occlusion of the femoral or iliac artery serves a good basis for mechanistic research into peripheral collateral growth, however clinical studies often focus on coronary disease patients. In these patients the arterial lumen narrowing occurs in a very different pattern than in peripheral disease, following a more step wise progression. In this chapter we describe a new method which enables successful stimulation of coronary collateral growth in the porcine heart. We used a minimally invasive approach, following a pre-defined schedule of hemodynamic relevant left circumflex artery (LCX) stenosis and subsequent complete occlusion. In this model collateral function can be assessed separately for the different source vessels, i.e. left anterior descending artery (LAD) and right coronary artery (RCA). It therefore provides important insights into the anatomical variability of collateral supply. Furthermore it can be used to evaluate the efficacy of local pro-arteriogenic treatments.

In **chapter 8,** we used the newly developed model to test whether the clinically available selective COX-2 inhibitor, Celecoxib, had an effect on coronary collateral artery growth. Previous reports indicated negative cardiovascular side effects of COX-2 inhibitors. We show that the chronic treatment with a selective COX-2 inhibitor reduces collateral artery growth in the heart after 4 weeks, thereby potentially contributing to the increased cardiovascular events in patients.

DISCUSSION AND FUTURE PERSPECTIVES

Arteriogenesis, or the growth of collateral arteries, is the most important process in restoring blood flow to jeopardized tissue after an arterial occlusion. However, the spontaneous growth of these arteries is very often insufficient to fully compensate for the perfusion loss. Therapeutic stimulation of the process could therefore be of great clinical benefit. Previous studies have demonstrated the involvement of inflammatory processes in arteriogenesis. The obtained knowledge on pro-arteriogenic factors has lead to some clinical trials. So far, none of the compounds succeeded without giving serious side effects, mainly due to the simultaneous progression of the underlying atherosclerosis.

The innate immune system contributes to the growth of collateral arteries. The expression of toll like receptor 2 and 4 on bone marrow derived cells has a regulatory role in arteriogenesis. Arteriogenesis could be promoted by activating this pathway. Toll like receptor signaling is, however, not specific to arteriogenesis alone. Although TLRs are classically known for their importance in the first line of defense against invading pathogens, they are also involved in various aspects of cardiovascular disease. When considering pro-TLR therapies, there are a few other roles of TLRs that we have to be taken into account.

TLRs are expressed in atherosclerotic lesions [5]. In mouse models, the stimulation of TLRs showed an association with neo-intima formation [6] and vascular remodeling [7]. In mice expressing a non functional form of TLR 4, plaque formation was reduced and less outward remodeling was seen [7], pointing to the functional involvement of TLR 4 in the progression of the atherosclerotic plaques. When a plaque progresses or destabilizes to such an extent that it causes ischemia downstream, TLR also play their part in the post-infarction damage. The absence of the TLR 2 or 4 receptor in mice resulted in less ischemia-reperfusion injury in mouse

organs [8-12]. This indicates that the activation of TLRs during this process leads to activation of pathways that are detrimental for the outcome. Inhibition of the TLR 2 receptor with an antagonizing drug can also prevent this in mice [13]. The results were so promising that the results are now repeated in pigs, so that a translation to the clinic can be made more easily.

Most studies concerning TLRs in cardiovascular disease come up with targets that are directed against their action. It therefore seems unlikely that promoting their function for more collateral formation will lead to a successful therapeutic target. However, this is not certain. Although TLR activation causes an inflammatory response which has the potential to aggravate atherosclerotic plaques, this does not need to happen when interventuional strategies are considered with the objective to stimulate arteriogenesis. The impact of TLR signaling in atherosclerosis seems to be more related to vascular cells rather than myeloid cells. The transplantation of TLR 2 or TLR 4 deficient bone marrow in an atherosclerosis prone mice did not prevent atherosclerosis formation [14], while total deficiency of TLR 2 or TLR 4 did reduce the plaque formation [15, 16]. Furthermore, the expression of endothelial TLRs during arthrosclerosis has shown to be confined only to the low shear regions and not at other sites of the vascular system [14]. Thus, the high shear which occurs in the developing collateral arteries might even dampen the vascular TLR expression. So although TLR activation play a role in the progression of both arteriogenesis and atherosclerosis, there are some apposing effects. This could potentially make TLR signaling an interesting target for the interference with collateral artery remodeling after all.

Now it is known that arteriogenesis could be regulated by influencing the circulating cells, the question remains how to target them in the most effective, easy and safe way. Direct receptor activation by systemically applying an agonist is relatively easy and has previously proven to be effective. However, the activated cells circulate through the whole body, including the plaque prone parts of the arterial system, without a specific call to home at the site the arteriogenesis should take place. Progression of atherosclerotic plaques could than easily occur, especially when treating for longer periods. Only until a receptor is found that is specifically involved in arteriogenesis and not in atherosclerosis progression, this option offers a successful treatment route and could find its way into the clinic. In case of the TLR signaling it is unknown if the TLR ligated circulated cells indeed play a role in atherosclerosis or if the stimulatory effects are fully attributed to resident cells.

Although we do know that the circulating cells play a key role, we actually do not precisely know how they become activated during arteriogenesis. It is most likely done by the production of a local ligand which is upregulated in response to the changes in shear or cyclic stretch. This ligand is then locally released into the bloodstream, where it can activate the, so important, circulating cells. We know that these ligands (for instance MCP-1, but possibly also EDA of fibronectin) are being produced in order to have an arteriogenic response, but so far, it seems that the reaction of the circulating cells upon ligand binding is the limiting factor for the extent and speed of the process. Nevertheless, stimulation of local ligand production could also be a way to enhance the process of arteriogenesis. A possible advantage of this method is that the circulating cells are most activated locally, and immediately stick to the vessel wall, leaving less activated cells circulating the whole body. Furthermore, a chemotactic gradient could enhance the homing of the circulating cells to the site of collateral growth, instead of other part of the body. Unfortunately, stimulating the production of local ligands is rather tough to establish and technically challenging. Local infusion of the ligand of choice by a intra- arterial catheter does

not seem work, as the study by *van Rooyen at al* proved that this still leads to systemic activation of the cells and therefore systemic effects [17]. Whether this problem also occurs when one chooses other ways to enhance local production of the endogenous ligand, still has to be investigated.

ARTERIOGENESIS: FROM BENCH TO THE BEDSIDE

In arteriogenesis research we aim not only on unraveling the processes involved, but also focus on a translation from bench to bedside. Animal models represent a very valuable tool in the identification and understanding of molecular mechanisms underlying collateral growth. However, they are mostly designed to study the role of one specific factor. Because arteriogenesis comprises of many non specific steps, such as endothelial activation and leukocyte recruitment, it can be easily influenced by other processes in the human body that take place simultaneously. The best known 'confounder' during collateral growth is atherosclerosis, but not only the presence of the atherosclerosis itself, but also its risk factors and medication, all influence the natural growth of collaterals and the response to a pro-arteriogenic therapy. As mentioned previously, many therapeutic strategies failed in clinical studies while they proved to be successful in experimental settings. There are different explanations that could be considered to understand the failure of clinical interventional studies with the objective to improve collateral formation. In the present thesis we executed studies with an attempt to better understand the translational capacities of our animal models. For instance we studied if and how age influences the arteriogenesis process.

Indeed, it remains the question whether the simulation of circulating cells, which is so successful in enhancing collateral growth in animal models, actually increases arteriogenesis in patients, which are mostly of old age. Old mice do not benefit as much for improving the inflammatory potential of the circulating cells, since the aging of the resident cells already limits the capacity to remodel the collateral arteries. Although more research is needed before any definite conclusions can be drawn, it suggests now that future therapies that focus on enhancing the potential of circulating cells alone, are less likely to be successful. Future, pre-clinical studies should therefore not only identify a potential candidate, but also validate it in the right models, taken the underlying disease, the right organ system and time into account.

We also established a new animal model that allowed us to study gradual occlusive disease in relation to collateral formation. In the clinical situation most patients will have suffered from different degrees of arterial narrowing before an occlusion is evident. In currently available animal models only acute ischemia in a healthy subject is studied. By introducing this new model, which more closely resembles the normal evolution of collateral growth, we hope to further improve the translation from bench to bedside. This eventually will lead to the discovery of a safe and effective drug to improve collateral growth.

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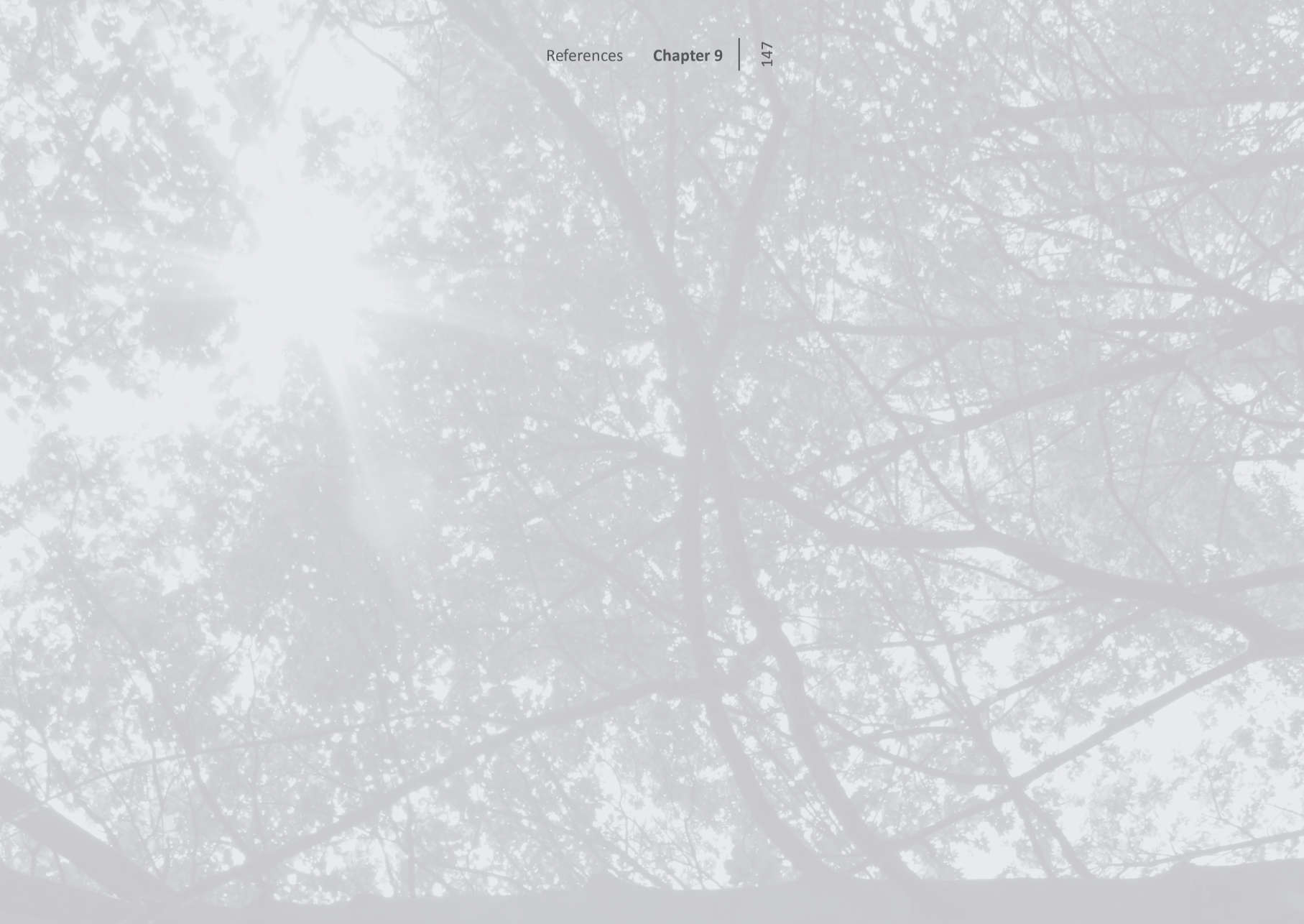

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Samenvatting

Slagaders zijn de bloedvaten die zuurstofrijk bloed naar de organen toe brengen. Wanneer een slagader verstopt raakt kan er onvoldoende bloed passeren. Dit kan ernstige gevolgen hebben voor organen zoals het hart, omdat deze dan niet meer voorzien worden van zuurstof en voedingsstoffen. Als dit gebeurt in vaten die het hart van bloed moeten voorzien, leidt dit in eerste instantie tot pijn op de borst, een verminderde hartfunctie en in het ergste geval zelfs tot het afsterven van het weefsel (een infarct). Het menselijk lichaam heeft een aantal oplossingen om weefselschade te voorkomen; een verminderde bloedtoevoer naar een orgaan kan door middel van de groei van bloedvaten hersteld worden.
Tijdens het leven zijn er twee belangrijke vormen van vaatgroei: *angiogenese* en *arteriogenese*. Angiogenese is het ontspruiten van nieuwe haarvaatjes op plaatsen waar een zuurstof tekort heerst. Cellen die te weinig zuurstof krijgen, geven stress signalen die angiogenese stimuleren. Dit is een belangrijk proces dat onder andere tijdens wondgenezing en lichaamsgroei plaatsvindt. Alhoewel haarvaten een zeer belangrijke rol spelen in het herstel van de doorbloeding van weefsels, zijn ze door hun kleine diameter niet in staat grote hoeveelheden bloed tegelijk te vervoeren. Voor het herstel van grote doorbloedingsstoornissen is arteriogenese van belang, oftewel de vorming van slagaders (arteriën). Slagaders hebben een grotere diameter dan haarvaatjes en zijn hierdoor in staat grote hoeveelheden bloed te transporteren. In tegenstelling tot angiogenese ontspruiten de arteriën zich niet, maar ontwikkelend ze zich uit vanaf de geboorte klaarliggende vaatverbindingen. Op het moment dat een slagader verstopt raakt, worden deze ongebruikte vaatverbindingen aangesproken om als omleiding te dienen en organen van bloed te voorzien. In de eerste instantie zijn deze echter niet groot genoeg om voldoende bloed te vervoeren. Er treed een proces in gang waarbij het vat groter en steviger wordt en in staat is te functioneren als omleidende slagader (collateraal). Deze grote vaten kunnen hierdoor aanzienlijke hoeveelheden bloed te vervoeren en hierdoor ernstig leed voorkomen,in tegenstelling tot de haarvaten, die tijdens angiogenese ontstaan. Hierdoor functioneren ze als natuurlijke 'bypasses' en kunnen ze weefselschade en daaruit voortkomende ziekte vermijden.
Het proces van arteriogenese is helaas niet feilloos; de groei van de collateralen vindt niet bij iedereen in voldoende mate plaats. Een goede collateraalvorming werkt beschermend tegen de effecten van slagaderafsluitingen en verbeterd bijvoorbeeld de prognose na een hartaanval. Helaas is er op het moment nog veel onbekend over het mechanisme dat aan collateraalvorming ten grondslag ligt enis er nog geen medicamenteuze therapie beschikbaar om dit proces te stimuleren. Het doel van het werk beschreven in dit proefschrift is het ophelderen van de mechanismen die een rol spelen in de vorming van collateralen.
In dit proefschrift worden onderwerpen besproken, die op verschillende wijzen het proces van arteriogenese bestrijken. Hieronder volgt een korte samenvatting van de onderwerpen die besproken worden in de verschillende hoofdstukken.

Hoofdstuk 2: Slagaderverstoppingen worden vaak veroorzaakt door verkalking in een proces dat ook wel arteriosclerose genoemd wordt. Slagaderverkalking leidt tot hart-en vaatziekten, een veelvoorkomende ziekte die veel slachtoffers eist in Nederland. Collaterale vaatgroei kan de gevolgen van slagaderverkalking tegengaan in mensen die lijden aan hart- en vaatziekten . Het ontstaan van hart- en vaatziekten wordt voor een belangrijk deel bepaald door de aanwezigheid van risicofactoren voor deze ziekte. In hoofdstuk 2 van dit proefschrift wordt een overzicht gegeven van de huidige kennis over de effecten die bekende risicofactoren voor

hart- en vaatziekten hebben op collaterale vaatgroei. De verschillende risicofactoren voor hart- en vaatziekten beïnvloeden collaterale vaatgroei elk op hun eigen manier. Zo zijn er aanwijzingen dat hoge leeftijd en hoog cholesterol een negatief effect op de collateraalvorming te hebben. Echter, ondanks het feit dat roken de kans op hart- en vaatziekten sterk verhoogt, beïnvloedt het dit collaterale vaatgroei niet negatief, .

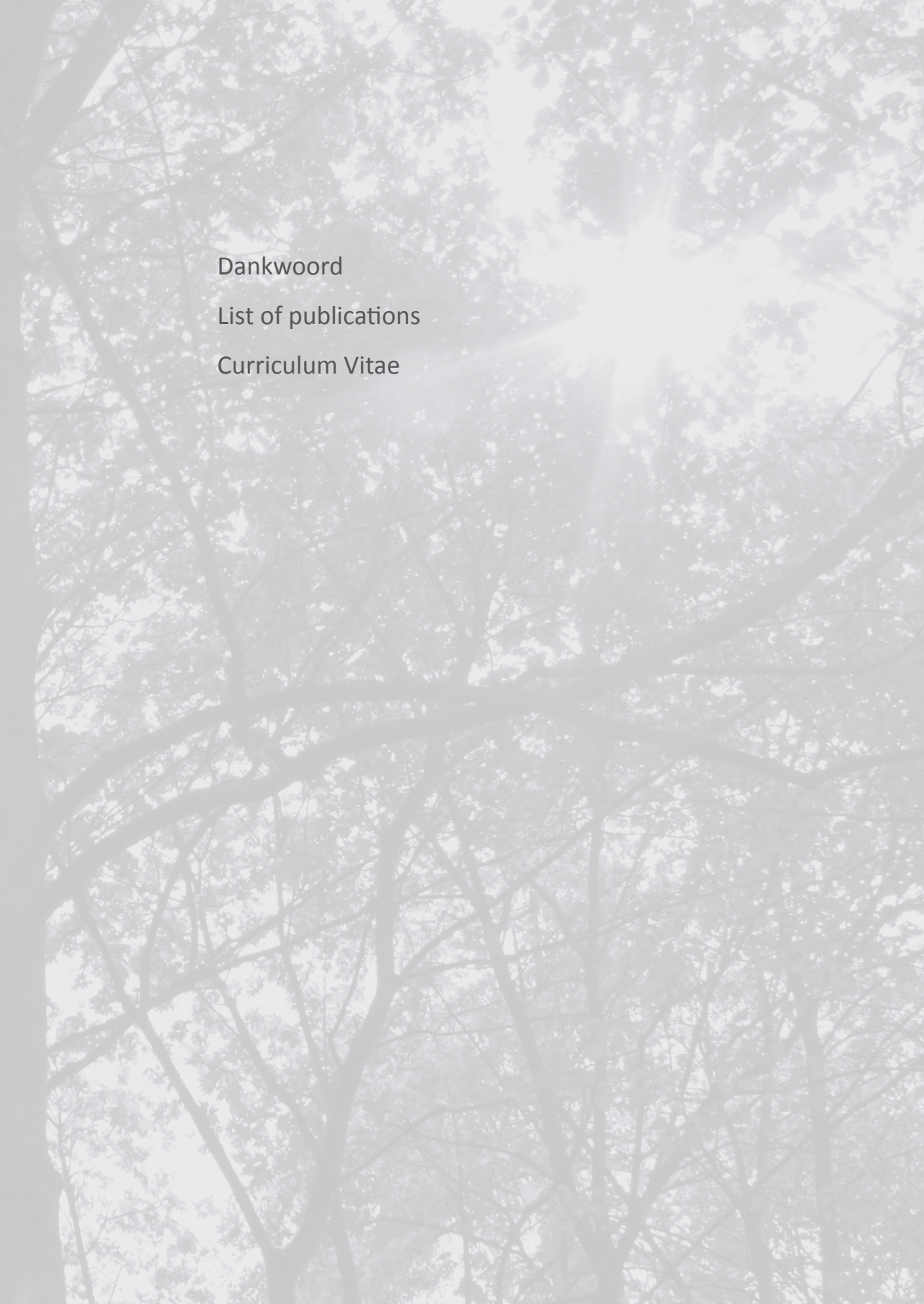
Hoofdstuk 3 tot 5: Ontsteking speelt een belangrijke rol in collateraal vorming. Alhoewel ontsteking vaak geassocieerd wordt met ziekte en pijn, vaak tijdens infecties, kan ontsteking ook een gunstige rol spelen. In de hoofdstukken 3 - 5 is onderzocht of ontsteking die in gang wordt gezet door het aangeboren immuun systeem een rol speelt bij collateraalvorming. Het aangeboren immuun systeem is het deel van onze afweer dat ons als eerste beschermd tegen schadelijke invloeden van buitenaf. Het herkent algemene onderdelen van bacteriën, schimmels en virussen en is dus niet specifiek gericht tegen één ziekteverwekker. Wanneer dit systeem geactiveerd wordt, worden witte bloedcellen actief en treed er een ontstekingsreactie op. Het aangeboren immuunsysteem reageert echter niet alleen op schadelijke invloeden van buitenaf, maar ook op producten die vrijkomen na het ontstaan van schade in ons lijf. Deze kunnen bijvoorbeeld vrijkomen wanneer een orgaan onvoldoende bloed ontvangt doordeverstopping van een bloedvat. In dit proefschrift wordt duidelijk dat collaterale vaatgroei baat heeft (of: gestimuleerd wordt door) bij een reactie van het aangeboren immuunsysteem. Hierbij blijkt het goed functioneren van de ontstekingcellen in het bloed en niet zozeer de locale ontsteking in het weefsel het meest bepalend voor de mate van vaatgroei.

Hoofdstuk 6: In dit hoofdstuk is de relatie tussen of collateraalvorming en leeftijd onderzocht. Het voorkomen van hart- en vaatziekte neemt toe met het toenemen van de leeftijd. Oude mensen zullen dus vaker last hebben van slagaderafsluitingen dan jonge mensen en daardoor meer behoefte hebben aan een goede collaterale vaatgroei. Uit ons onderzoek komt naar voren dat het hebben van hogere leeftijd de vaatgroei negatief beïnvloed: oudere muizen zijn slechter in staat om nieuwe vaatomleidingen te vormen dan jonge muizen. In eerdere hoofdstukken hebben wij beschreven dat circulerende witte bloedcellen bijdragen aan arteriogenese. Daarom is in verdere experimenten geprobeerd vaatvorming in oude dieren te bespoedigen door witte bloedcellen van jonge dieren aan te bieden. Helaas blijkt deze strategie niet in staat om voldoende vaatherstel te geven. De uitkomsten van deze experimenten suggereren dat ook lokale factoren die voortkomen uit een weefsel dat onvoldoende bloedtoevoer ontvangt een rol spelen. Het kan zijn dat deze 'noodsignalen' minder aanwezig zijn op hogere leeftijd, of dat de communicatie tussen de witte bloedcellen de weefselsignalen niet meer goed verlopen. Verder onderzoek is nodig om te bepalen in welke mate deze lokale factoren en de ontstekingsreacties van witte bloedcellen aan arteriogenese bijdragen, en of deze twee met elkaar in relatie staan.

Hoofdstuk 7 en 8: In deze hoofdstukken presenteren we een nieuw groot proefdier model om onderzoek te doen naar collateraalvorming in het hart. Tot op heden worden met name kleine proefdieren (muizen) gebruikt voor onderzoek naar collateraalvorming. Hierbij werden veelbelovende resultaten behaald met het stimuleren van collateraalvorming in de achterpoten. Echter, de vertaalslag naar arteriogenese rondom het hart van de mens bleek onsuccesvol. Wij denken dat dit komt doordat dat het onderzoek in de kleine proefdieren vele technische beperkingen heeft en hierdoor te ver staat van de medische praktijk. Zo kan in kleine proefdieren het vat niet geleidelijk afgesloten worden, zoals dat meestal tijdens slagaderverkalking in de mens plaatsvindt. Daarnaast is onderzoek naar collateraalvorming in het muizenhart praktisch

niet uitvoerbaar vanwege het kleine formaat van de vaatjes in muizenhartjes. Het hart van een varken is zeer vergelijkbaar met dat van een mens, in grootte en opbouw. Om deze redenen hebben wij een nieuw experimenteel model ontwikkeld in varkens waarbij we geleidelijk een slagader in het hart weten af te sluiten. Als gevolg hiervan ontwikkelen de varkens collaterale vaten in het hart, die de effecten van de toegebrachte verstopping ten dele teniet doen. De functie van deze vaten kunnen wij zeer nauwkeurig meten. Dit biedt de mogelijkheid om de effecten van nieuwe therapieën voor hart- en vaatziekten op arteriogenesein het hartte kunnen evalueren, voordat er klinische studies in mensen mee gedaan worden.

In conclusie levert dit proefschrift nieuwe inzichten en vragen op met betrekking to het proces van collateraalvorming. Ook biedt het een aantal praktische mogelijkheden aan: een diermodel om arteriogenese in hart van varkens te bestuderen zal de vertaling van vindingen uit fundamenteel cardiovasculair onderzoek in de richting van de patiënt in de kliniek bevorderen. Verder onderzoek is nodig om vast te stellen welke lokale factoren in beschadigd weefsel arteriogenese stimuleren en hoe deze communiceren met de cellen in het bloed. Daarnaast dient zich een therapeutische mogelijkheid aan: stimulatie van het aangeboren immuunsysteem ter bevordering van arteriogenese lijkt een veelbelovend therapeutisch doelwit in de behandeling van hart en vaatziekten.



Dankwoord

List of publications

Curriculum Vitae

DANKWOORD

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Arteriogenesis Requires Toll-Like Receptor 2 and 4 Expression in Bone-Marrow Derived Cells
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Absence of Extra Domain A of Fibronectin diminishes perfusion restoration after arterial occlusion
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In preparation

CURRICULUM VITAE

Daphne de Groot was born on August 29, 1982 in Hoorn, the Netherlands. After graduating from secondary school in 2000 (Goois Lyceum, Bussum), she studied Medicine at the University Medical Center Utrecht. During her medical training, she completed a scientific internship at the Department of Experimental Cardiology. She obtained her medical degree in 2006, followed by four months of voluntary work as a medical doctor at Kitwe Central Hospital in Zambia. After returning, she started as a PhD candidate at the Department of Experimental Cardiology at the University Medical Center Utrecht, supervised by prof. dr. G. Pasterkamp, prof. dr. D.P.V. de Kleijn and dr. I.E. Hoefer. As of januari 2011, she is working at the Groene Hart Medical Center as a resident at the Department of Surgery, led by dr. R.F. Schmitz.