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Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]

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Publication date
2003

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Citation for published version (APA):
van Royen, N. (2003). *Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]*.

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INTRODUCTION AND OUTLINE:
MECHANISMS AND MODULATION OF
COLLATERAL ARTERY DEVELOPMENT

Introduction

Current therapeutic approaches to obstructive coronary or peripheral artery disease entail pharmacological reductions in oxygen demand in tissue with inadequate vascular supply or mechanical or interventional restoration of blood flow. Pharmacologically induced reductions in energy needs are however associated with diminished function. Conversely, mechanical revascularization carries a finite morbidity and mortality, as well as the risk for recurrent vascular obstruction. Alternate therapeutic approaches would therefore be desirable in patients with obstructive vascular disease. Accordingly, investigations have focused on the development of "pharmacological bypasses" as potential alternatives.

Forms of vessel growth

There are different forms of vessel growth. One is vasculogenesis, that is the formation of a primary plexus of vessels by angioblasts. Whether vasculogenesis can occur in adulthood has remained under discussion. The concept of stem cells leading to growth of new vessels in instances of arterial obstruction is an attractive one, but proof of its existence is still lacking. This in part is because of the lack of a uniform detection system for endothelial progenitor cells believed to induce vasculogenesis in adulthood. Knowledge on the origin of these cells is therefore still lacking. Further, it seems unlikely at present that vasculogenesis contributes significantly to restoring blood flow to tissue subtended by obstructed or occluded arteries.

Two other forms of vessel growth, angiogenesis and arteriogenesis, have been shown to occur after birth. Angiogenesis refers to the sprouting of endothelial cells, leading to formation of new capillary networks. Angiogenesis is an integral component of processes like inflammation, wound healing, tumor growth and atherosclerosis. In occlusive arterial disease, angiogenesis occurs in the presence of ischemia. Ischemia leads to expression of hypoxia inducible factor-1 (HIF-1) and subsequently vascular endothelial growth factor (VEGF), a pro-angiogenic factor. Arteriogenesis, as another form of "vessel growth," refers to the transformation of pre-existing collateral arteriolar pathways into large conductance arteries. This process is independent of ischemia but is related to increased shear forces as a consequence of increased flow through pre-existing collateral arterioles due to an increase in the pressure gradient following occlusion of a major artery. Thus, in case of severe ischemic arterial disease, angiogenesis and arteriogenesis can occur simultaneously. However, both are distinctly different processes with different modulators and different outcomes (Table 1). The current review will focus on arteriogenesis as the most effective form of vessel growth for restoring or improving perfusion of tissue affected by an arterial occlusion [1].

Arteriogenesis: a built-in natural self-defence system

"Coronary vessels describe a circular course to ensure a better general distribution, and encircle and surround the base of the heart. From such an origin they are able to go off respectively to opposite regions of the heart, yet around the extremities they

come together again and here and there communicate by anastomoses. As a result fluid injected into one of them spreads at one and the same time through both. There is everywhere an equally great need of vital heat and nourishment, so deficiency of these is very fully guarded against by such anastomoses." [2] This concise description and initial proof of functional collateral arteries by the English anatomist Richard Lower dates back to 1669. Since then, numerous studies have confirmed Lower's observations. Later, Fulton demonstrated that the presence of such collateral arteries in the human heart depended on a history of prior coronary artery disease [3]. Other studies have shown that collateral arteries can protect against myocardial infarction, cardiogenic shock and death when the coronary artery becomes occluded [4,5,6]. However, as known from the clinical setting, the built-in natural defence system of collateral vessels does not invariably and fully protect against myocardial infarction. In fact, coronary artery disease remains the leading cause of death in developed countries. Therefore, investigations have focused on approaches for stimulating or augmenting the natural process of arteriogenesis.

Mechanisms of arteriogenesis: interplay between shear stress and circulating monocytes

Arteriogenesis has long been known to be an ischemia-independent process because clinical observations had demonstrated development of collateral arteries in regions far distant from regions of ischemia. More recent experimental studies confirmed these early clinical observations. For example, ligation of the femoral artery in rabbits was followed by a marked arteriogenic response in absence of a raise in ischemia markers like ADP, AMP or lactate [7-9].

The arterial diameter is known to increase in response to an increase in wall shear stress, that ultimately leads to a normalization of wall shear stress [10]. Thus, the most plausible mechanism of arteriogenesis is the hypothesis of a shear stress induced vessel growth. Upon closure of a major artery, flow redistributes over pre-existing collateral pathways (see figure 1 and 2). Increased flow through these collaterals is accompanied by increased shear forces, leading to activation of the normally quiescent endothelial cells in the pre-existing collateral arterioles. This in turn is followed by release of factors like monocyte chemoattractant protein-1 (MCP-1) [11] or transforming growth factor-beta (TGF-ß) [12] and an upregulation of endothelial receptors for circulating monocytes [13]. The final steps of this process are the docking of monocytes to the endothelium of the pre-existent collateral arterioles, their perivascular accumulation in the form of macrophages and their production of factors like MCP-1, basic fibroblast growth factor (bFGF), tumor necrosis factor alpha (TNFα), matrix metalloproteinases (MMPs) and other factors [14]. Synthesis and release of these factors is delicately regulated with distinct roles for each factor. MCP-1 production for example leads to attraction of more monocytes/macrophages to the site. GM-CSF (granulocyte macrophage-colony stimulating factor) production increases the life-span and thus the functionality of the perivascular macrophages. TGF-ß production induces synthesis of several other factors by the perivascular monocytes like platelet derived growth factor (PDGF) and bFGF. PDGF and bFGF

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in turn are directly mitotic for endothelial- and smooth muscle cells. TNFα creates the inflammatory milieu needed for arteriogenesis. Finally, the MMPs lead to degradation of tissue thereby creating the space needed for the growing vessels. It is emphasised that this complex interplay of various factors regulating arteriogenesis is only beginning to become unravelled and, hence, far from being fully understood. Generally, about two days after an arterial occlusion, mitotic indices of vascular cells begin to rise. In some histological sections of such vessels, more than 50% of the cells of the pre-existing collateral arterioles stain positive for proliferation of markers as compared to normal quiescent vessels, where mitotic indices approach 0%. The increase in mitotic indices leads to a rapid natural response to arterial obstruction and restoration of perfusion starting within the one week (see figure 3). Thereafter, the pre-existing collateral arterioles transform into large conductance arteries with an as much as 20-fold increase in their diameter. However, under ideal conditions in healthy young animals the maximum achieved restoration of blood flow approaches only about 50% of normal. In patients, the natural arteriogenic response may be more variable. In some patients, occluded coronary arteries are completely compensated for by collateral arteries while in other patients development of collateral arteries is only marginal and inadequate. This then provides the rationale for therapeutically stimulating and enhancing the natural process of arteriogenesis as an important new treatment option in many patients.

Experimental data on stimulation of arteriogenesis
As was highlighted above, arteriogenesis entails the following steps: Increased shear stress and endothelial activation, monocyte adhesion and transmigration, production of growth factors by peri-vascular macrophages and finally, transformation of small pre-existing collaterals into large conductance arteries (see Figure 1-3).
Theoretically, stimulation of arteriogenesis can be directed at any of these steps. Thus arteriogenic therapies can act via 1. alteration of shear stress and activation of endothelium, 2. the monocytic pathway, or 3. direct stimulation of endothelial and smooth muscle cell proliferation.
1. Exercise increases cardiac output and thus the energy requirements of the myocardium. Coronary flow increases and thus raises the shear stress along the arterial branches of the coronary circulation [15]. The higher shear stress prompting endothelial activation might be the mechanism accounting for the well documented improvement of the exercise training. However, other mechanisms like improved oxygen metabolism and changes in rheologic blood factors could be alternate explanations [16]. The latter possibility is supported by several studies that failed to show an improvement of tissue perfusion upon exercise training. Thus, definitive proof of the arteriogenic potential of exercise training is still lacking.
2. Stimulation of arteriogenesis via monocytic pathways is well supported by experimental findings. The first substance found to increase arteriogenesis via a monocytic pathway was MCP-1 [17]. Continuous intra-arterial infusion of MCP-1 was shown to produce an about sevenfold increase in the magnitude of the arteriogenic response to femoral artery ligation in rabbits. Further, the beneficial effect of MCP-

1 can be abolished by antibodies using one of the endothelial receptors for monocytes, intercellular adhesion molecule-1 (ICAM-1) which provides proof of the mode of action of MCP-1 via the monocytic pathway. Subsequently two other substances, granulocyte macrophage colony stimulating factor (GM-CSF) and TGF-ß were found to enhance arteriogenesis via the monocytic pathway. GM-CSF inhibits apoptosis of monocytes/macrophages and increases the mean life-span of these cells. The function of TGF-ß during barteriogenesis is probably a dual one. One is that it may increase monocytic transendothelial migration and a second one is that it induces the expression of growth factors like b-FGF and PDGF by monocytes/macrophages.

3. bFGF is a mitogen for both endothelial cells and vascular smooth muscle cells [18] and its beneficial effect on flow restoration upon arterial occlusion was demonstrated in several experimental studies [19]. However, these promising results thus far have not been reproduced in the clinical trials on b-FGF, although it is likely that more information on the clinical effects of b-FGF will soon become available from the TRAFFIC trial.

	Angiogenesis	**Arteriogenesis**
Substrate:	Capillary sprouting	Arteriolar remodelling
Driving force:	Ischemia	Shear stress
Involved Factors:	VEGF	MCP-1, bFGF
Target Cells:	Endothelial Cells	EC's & SMC's
Result:	Capillary networks	Collateral arteries

Table 1: Angiogenesis versus arteriogenesis

Where to go?
It seems likely that the therapeutic use of only a single growth factor is insufficient for augmenting the stimulation of the naturally occurring process of arteriogenesis. This is because arteriogenesis entails a cascade of events, depending on a series of factors with each exerting a different effect. Monocytes are multifunctional cells capable of producing all of these factors. Hence, intervening therapeutically with the monocytic pathway holds promise for a successful arteriogenic strategy. However, several issues need to be resolved before an arteriogenic strategy, acting via a monocytic pathway, will become clinically applicable and effective.

Mimicking the clinical situation
In experimental models, arteriogenesis is studied directly in relation to the time of an acute arterial occlusion. Such models are unlikely to fully represent the clinical situation with a slowly progressive arterial occlusion as part of the progression of atherosclerotic disease. Experimental models employing ameroid constrictors may mimic more closely this process although the occlusion still markedly differs from the time frame of months or even years in humans. It is important to bear this in

mind because the effects of arteriogenic substances on mature collateral vessels are unknown. In addition, patients enrolled in arteriogenic trials might suffer from hyperlipidemia. Whether this influences the process of arteriogenesis and the arteriogenic response to exogenously applied substances also needs to be determined in the experimental setting.

Another difference between the clinical and the animal experimental setting is the size of the species. Data on arteriogenic substances were mostly derived from small sized animal models. It remains unknown whether results from smaller species can directly be extrapolated to larger sized species as for example humans. Although the rate of the cell cycle is independent of the species, the number of mitosis required for the development of a functional human collateral artery is much greater in humans than in mice.

Potential side-effects of arteriogenic therapy

Folkman et al. showed that inhibition of angiogenesis by TNP-470 or Endostatin caused a reduction in atherosclerotic plaque growth in ApoE deficient mice, showing a direct role of angiogenesis in the progression of atherosclerotic plaques [20]. Although arteriogenesis is not directly involved in atherosclerotic plaque formation, arteriosclerosis and arteriogenesis share many aspects like invasion of monocytes, the inflammatory environment, elastolysis, migration and proliferation of smooth muscle cells and upregulation of adhesion molecules. It is possible that different arteriogenic factors differently affect atherosclerosis. While MCP-1 is believed to be associated with plaque formation, although definite proof is still lacking, GM-CSF was shown to be anti-atherogenic. The role of TGF-ß during atherogenesis still remains controversial. Before using any of these substances in clinical trials potential detrimental effects on atherosclerosis must be excluded.

How can arteriogenic substances be delivered?

The risk of possible side effects can largely be diminished by delivering the arteriogenic agents locally. In addition, arteriogenic growth factors, like angiogenic growth factors, were shown to be most effective when administered continuously and intra-arterially [19]. Thus, most ideally, arteriogenic substances are delivered locally through arteries, over prolonged periods and without washout to adjacent territories or organs. Gene transfer to vascular wall cells might be an option to circumvent the delivery problem. A stable transfection will result in a continuous intra-arterial delivery without any exogenous instrumentation (except for the transfection catheter). Other options include the use of slow-releasing stents or sustained-release microcapsules. The monocytes themselves might also be used as carriers of arteriogenic agents to the proliferating arteries. Isolated monocytes can be loaded ex-vivo with fluorescent microspheres and be re-infused into the circulation. We were able to show that such loaded and re-infused monocytes preferentially accumulate at sites of active arteriogenesis [21].

Endpoints for arteriogenic therapies

For proof of concept of therapeutic arteriogenesis, it will be important to define clinical endpoints. Treadmill exercise time and quality of life scores for example are, at the current developmental stage of therapeutic arteriogenesis, of little significance. The aim of any arteriogenic strategy is the restoration of tissue perfusion via growth of large collateral conductance arteries. Restoration of perfusion is achieved by normalizing the resistance of the vascular bed that supplies the tissue at risk. Therefore, three parameters are particularly useful for assessing the efficacy of arteriogenic therapies.

1. The angiographic appearance of collateral vessels: In the experimental setting, angiographic appearance of collateral vessels is a useful measure for detecting the outgrowth of pre-existent collateral arterioles that are still below the threshold of X-ray angiography (approximately 50 μm) towards collateral conductance arteries that measure between 100 μm and 2 mm in diameter. However, there may be patients with already full recruitment of the collateral circulation so that stimulation of arteriogenesis might not lead to an increase in the number of visible collateral arteries but rather to a slight increase in vessel diameter of the already visible collateral arteries. Even mild increases in diameter can significantly affect the resistance to flow as it is a function of the fourth power of the radius of the vessel. However, subtle diameter changes might be difficult to detect so that angiography will be of limited value in clinical studies. Use of the Rentrop score might to some extent overcome this limitation. It better reflects the haemodynamic functionality of the collateral circulation because it accounts for filling of the collateral arteries and the collateral-dependent coronary arteries.

2. Resistance of the collateral circulation: Resistance is calculated from the ratio of pressure difference and flow over a vascular bed. Such combined measurements of flow and pressure are feasible with guidewires, equipped with miniaturized flow and pressure sensors. These guidewires have become available in recent years and were shown to accurately document collateral vascular resistance [22].

3. Myocardial tissue perfusion: Any successful arteriogenic strategy increases perfusion of the collateral-dependent region. This in fact is the ultimate goal of any therapeutic arteriogenesis trial, that is restoration of perfusion and supply of oxygen leading to a decrease in clinical symptoms and an increase in physical activity. Most studies performed thus far on stimulating growth of the coronary collateral circulation employed an improvement in regional tissue perfusion as endpoint. Myocardial perfusion can be evaluated with echocardiography, Magnetic Resonance Imaging (MRI) or radionuclide perfusion imaging. Of these techniques, positron emission tomography (PET) and single photon emission computed tomography (SPECT) are standard approaches for evaluating regional myocardial perfusion. SPECT imaging with ^{201}Tl or ^{99m}Tc labeled flow agents has been well established as a means for the non-invasive detection of ischemic heart disease. Recently, Hendel et al. reported a beneficial effect of rhVEGF administration as documented by a reduction of perfusion defects using SPECT-imaging [23]. However, whether SPECT perfusion imaging can specifically detect changes in collateral flow remains

unknown. There may be two shortcomings of SPECT for evaluation of therapeutic arteriogenesis. First, it is unclear whether resting or stress perfusion images are more accurate for detecting arteriogenesis. As mentioned earlier, the aim of arteriogenic therapy is the decrease of resistance of the vasculature supplying the area at risk. Resistance is calculated from the ratio of the pressure difference over myocardial blood flow. Detection of changes in resistance requires a stress hyperemic challenge of the coronary circulation. Evaluating myocardial perfusion at rest is therefore limited in detecting changes in collateral flow. On the other hand, stress imaging may be associated with a coronary steal causing a decline in blood flow in the collateral dependent myocardium. Secondly, collateral vascular growth in humans preferentially targets the subendocardium while visualization of subendocardium perfusion by both SPECT and PET is limited [3]. Thus, although PET and SPECT are the best validated non-invasive perfusion imaging techniques, some issues need to be clarified before these techniques can be implemented in human trials on therapeutic arteriogenesis. A new promising concept in radionuclide imaging is the use of specific imaging targets. Virtually every adhesion molecule, receptor or protein involved in arteriogenesis could potentially be imaged with a specific radiolabeled antibody. Such imaging might result in highly sensitive and accurate detection of arteriogenesis in vivo.

Conclusion

Of the three known forms of vessel growth, arteriogenesis is the most efficient in the restoration of flow upon arterial occlusion. From experimental studies it is known that arteriogenesis can be stimulated via a monocytic pathway. Monocytes/macrophages are conceivably the most effective mediators of arteriogenesis since they are capable of producing the required arteriogenic factors. However, additional experiments are required that are designed to better reflect the anatomic substrate in humans with long-lasting atherosclerotic disease, hyperlipidemia etc.. Moreover, issues need to be resolved regarding potential side-effects, delivery techniques and imaging tools.

Outline of the thesis

The outline of this thesis is depicted in figure 4. Chapter 2 concerns a detailed review, describing the mechanisms of arteriogenesis, the differences between angiogenesis, vasculogenesis and arteriogenesis as well as some future perspectives for therapeutic arteriogenesis. In chapters 3 and 4 basic mechanisms of collateral artery development and the role of specific pathways of arteriogenesis have been studied in genetic knockout mice. This includes the TNF-α and the CD44 pathway. Chapters 5 to 7 are studies on the pharmacological modulation of collateral artery growth via the exogenous application of MCP-1, GM-CSF and TGF-ß. Chapters 8 and 9 describe a newly developed large animal model of arteriogenesis and the effects of MCP-1 using this model. Chapter 10 and 11 concern the effects of MCP-1 under conditions of hyperlipidemia as well as the interactions with the development of atherosclerosis. Finally in Chapter 12, the design of the START-trial is presented. In this trial, a total of 40 patients with peripheral artery disease will be treated with GM-CSF.
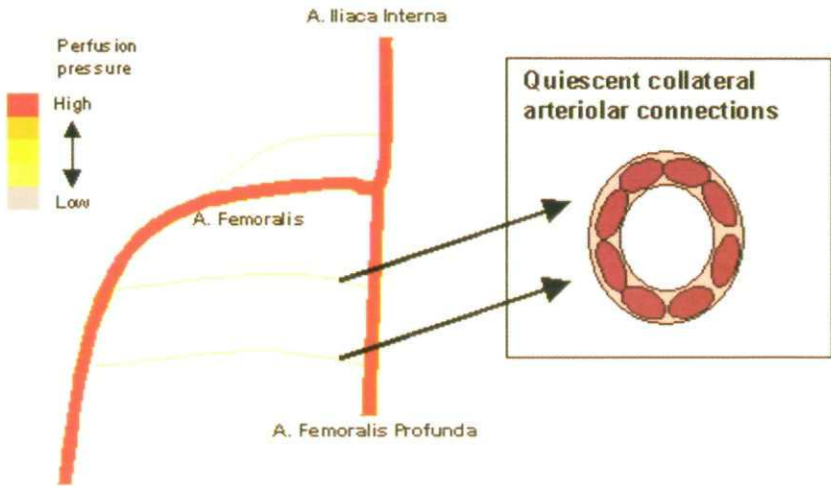
Figure 1. Pre-existing collateral arteriolar connections are present in the normal hindlimb circulation but flow is preferentially directed through the patent femoral artery.
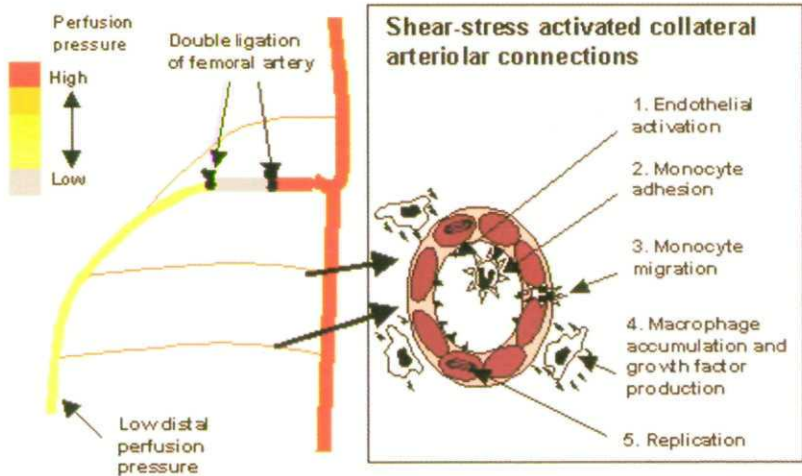

Figure 2. Pre-existing collateral arteriolar connections are recruited in case of acute occlusion of the femoral artery. Thereupon, the endothelium is activated and the process of arteriogenesis is initiated.
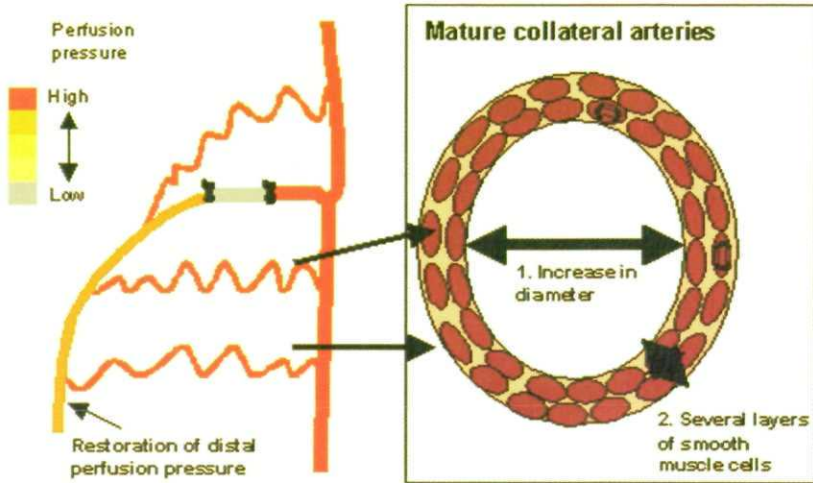
Figure 3. In time, large collateral conductance arteries are formed, possessing several layers of smooth muscle cells. The diameter of these arteries can be up to 20-fold as large as compared to the original diameter.
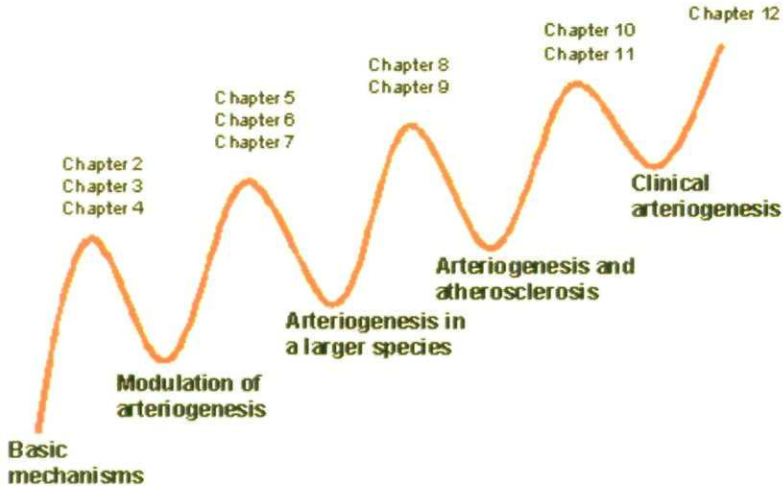

Figure 4. Outline of this thesis.

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