Growth factor-induced therapeutic angiogenesis and arteriogenesis in the heart—gene therapy

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Abstract

Myocardial ischemia is one of the most promising targets of gene therapy. Although several growth factors and delivery approaches have yielded positive results in preclinical studies, first clinical studies have shown little or no real clinical benefit to the patients. It is likely that less than optimal gene therapy approaches have been used so far, and more thorough preclinical studies are needed in order to establish safe, efficient pro-angiogenic therapy. Growth factor, gene transfer vector, delivery method and target microenvironment need to be chosen based on the therapeutic target. It has become apparent that induction of large collateral arteries in the myocardium may need a different approach than rapid growth of neovasculature around infarction scar. Large animal models are necessary in the determination of optimal therapeutic agent, dose and clinically relevant delivery strategy.

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1. Introduction

Collateral vessel growth by the induction of the expression of growth factors, such as vascular endothelial growth factor (VEGF), by shear stress is a natural process occurring in human myocardium undergoing vessel occlusion [1]. Gradual occlusion of the vessel redirects blood flow into quiescent collateral vessels and increased shear stress induces enlargement of these vessels with thickening of the smooth muscle layer surrounding the vessels [2]. Individual capacity to grow these channels is highly variable, and requires a slow course of disease progression. Impaired blood flow in the tissue downstream from the occlusion creates a hypoxic environment, which induces expression of several growth factors, such as VEGF and fibroblast growth factors (FGFs). Expression of these growth factors in turn triggers a process termed angiogenesis, growth of capillary level vessels. The processes of naturally occurring angiogenesis and arteriogenesis are, however, impaired by old age [3], high cholesterol levels [4], diabetes [5] and endothelial dysfunction, i.e., the same attributes often associated with patients who are no longer suitable candidates for conventional revascularization techniques. Angiogenesis in response to exogenous angiogenic cytokines and growth factors is, however, at least partially able to override these phenomena, thus supporting the usefulness of gene therapy approach [6]. The goal of growth factor gene therapy is to induce local growth of blood vessels with techniques suitable for most patients [7].

Both collateral growth and angiogenesis are targets for gene therapy in the myocardium. Induction of collateral growth can be beneficial both in severe, multivessel arterial occlusive disease as a preventive treatment and after acute occlusion of the vessel to improve the function of the conducting vessel [8]. Angiogenesis increases local tissue
2. Mechanisms of blood vessel growth

Growth of blood vessels has different mechanisms depending on the goal, initial stimulus and tissue. In developing embryo, coronary vessels are formed by vasculogenesis, a process in which the course and even the caliber of proximal and distal arteries are governed in the absence of blood flow [9]. Although growth of the blood vessels in adults mainly follows the same basic mechanisms, data obtained in different tissue environments should be applied to myocardium with caution [10].

In adult myocardium, arterial remodelling occurs mainly in response to alterations in blood flow and shear stress against the vascular wall. Diameter of an artery determines the velocity of blood flow in it, and any deviation from that relationship results either in growth or regression of the vessel. Collateral vessels form thin-walled, microvascular anastomoses that connect coronary arteries at all levels. Once a stenosis forms in the conducting coronary artery, or a major artery is acutely occluded, blood flow takes the path of the lowest resistance via the collateral vessels into the periphery. Blood flow, hydrostatic pressure and shear stress against the wall of the pre-existing collateral vessels increase rapidly leading to activation of the endothelium and up-regulation of vascular growth factors [11]. When vessels enlarge and develop a fully formed media by proliferation and remodelling in response to these growth factors, a biological bypass is formed to compensate for the decreased flow in the conducting vessel [12]. Collateralization takes from weeks to months to be completed, and despite the rapid growth and increase in the diameter, collaterals never reach the conductance of the coronary vessel they replace.

Myocardium extracts normally 70% of oxygen from the circulating blood leaving little capacity to increase oxygen uptake. Myocardium is also highly dependent on aerobic metabolism. Immediate dilatation of the quiescent, pre-existing collaterals can normally replace up to one half of the flow required to support the tissue downstream. Areas completely deprived of blood flow will undergo necrosis and form fibrous scar tissue in the healing process, whereas tissue receiving insufficient blood flow turns into a power save mode of hibernating, viable myocardium. Extent of tissue damage and, thus, restoration of heart function and even survival of the patient are therefore dependent on the rapid restoration of blood flow.Gene therapy approaches aim to overexpress factors naturally up-regulated during growth of collaterals and thereby speed up the arteriogenesis process. Another, less explored possibility is to prepare the collateral network for ischemic insult by enhancing collateral vessel growth in myocardium prior to complete occlusion of coronaries. Such approach may be beneficial for patients with severe, wide-spread coronary artery disease. Long-term, low-level expression of growth factors would probably produce an optimal, sustained response (Fig. 1).

Collateral vessel growth takes place proximal to the occlusion site in the normoxic myocardium. Prevailing theory suggests that normoxic tissues are largely unresponsive to angiogenic stimuli. When the goal is to induce arteriogenesis in the myocardium, it is therefore essential to use growth factors that are also potent in normoxic tissues and gene therapy vectors that produce a sufficient gene transfer efficiency in healthy tissue. As growth of collateral vessels is a combination of endothelial changes, smooth muscle cell proliferation and remodelling of connective tissue, growth factors used should be able to affect, either directly or indirectly, a variety of cell types from endothelial cells to smooth muscle cells and maybe also fibroblasts.

Angiogenesis, growth and enlargement of capillary vessels occur naturally in response to ischemia. Scar formed after myocardial infarction is surrounded by hibernating but viable myocardium, and a network of neovessels is formed within days to nourish the endangered tissue. Angiogenesis starts with proliferation and migration of endothelial cells that spread out from the pre-existing capillaries to form new vascular sprouts. Newly formed vessels are susceptible to regression until pericyte coverage is obtained. Such intermediate stage, a plasticity window, is required for the vascular network to be trimmed to respond to the metabolic...
needs of the tissue [13]. Growth of excessive capillary network increases local tissue perfusion, but in order to compensate the flow in the occluded vessel territory and sustain cardiac function, larger vessels need to be grown from upstream of the ischemic area. Angiogenesis in the ischemic zone lowers the local resistance of the capillary bed and results in increased flow in the arteries upstream with the same pressure [14].

Rapid, efficient overexpression of growth factors in the peri-infarct zone is a promising gene therapy approach. Patients that have undergone acute myocardial infarction often require invasive treatment, and gene transfer could be performed without additional interventions either independently or in combination with intravascular revascularization strategies or with bypass surgery.

Capillary network in the infarct zone requires flow from the larger arteries upstream from the ischemic area, and large collateral arteries require a functional capillary network for effective exchange of oxygen and nutrients. Although angiogenesis and arteriogenesis can exist as independent processes and have several distinct features, both processes of vessel growth are connected also in natural response to decreased flow in the myocardium. Several growth factors, including VEGFs, FGFs, PDGFs, MCP-1 and GM-CSF, have been shown to induce both angiogenesis and arteriogenesis [7]. In addition, both processes require proliferation and migration of the same cell types, mainly endothelial cells and smooth muscle cells. As both processes are required simultaneously for optimal clinical outcome, similarities are important clues in search for optimal therapeutic genes.

3. What to deliver?

Gene therapy approaches usually aim to locally overexpress one of the growth factors naturally participating in the process of vascular growth and repair [7,15] (Fig. 2). Whereas growth of collateral vessels is likely governed mostly by hemodynamic factors, angiogenesis process in the heart is initiated by hypoxia and induced by growth factors secreted by the surrounding cells. One therapeutic strategy is to overexpress hypoxia inducible regulators of gene expression like hypoxia inducible factor-1alpha (HIF-1α). The aim of such approaches is to mimic natural responses to ischemic stress, and to produce a natural cascade of growth factors downstream from the hypoxic switch. One of the main downstream targets of HIF1-α, VEGF was found in infarcted human myocardium years before its characterization and naming [16]. VEGF is the most potent angiogenic growth factor known, capable of producing vasodilation, increased vascular permeability, proliferation and migration of endothelial and likely also smooth muscle cells [17]. Endothelial cells proliferate and migrate to form vascular sprouts, connect with other blood vessels and eventually form a lumen [18]. Thin-walled endothelial tubes are at this point dependent on continuous growth factor expression and prone to regression [19,20]. Newly formed vascular network is trimmed to respond to needs of surrounding tissue probably by means of oxygen tension, negative regulators of angiogenesis and by blood flow [13]. Sustained expression of growth factors such as VEGF at this stage is alone sufficient to prevent vessel regression and promote stabilization of the vessels [21].

The next step of vessel growth is maturation of capillaries, acquisition of smooth muscle cell coat and deposition of extracellular matrix. Angiopoietins display a consistent expression pattern in angiogenic processes throughout the body. Angiopoietin-2 (Ang-2) destabilizes vessels by promoting extracellular matrix degradation and by loosening connections between endothelial cells and is expressed at the sites of angiogenesis and vessel regression [22]. The function of Ang-2 seems to be dependent on the presence or absence of VEGF. When expressed alone, loosening of endothelial cell connections induces apoptosis, but the presence of VEGF provides required survival signals for endothelial cells, and disattachment leads to cell proliferation and migration [23]. Overexpression of Ang-2 alone has been inefficient to trigger angiogenesis in in vivo experiments [24,25]. In contrast, angiopoietin-1 (Ang-1) is expressed in the quiescent state and has been suggested to reduce vascular permeability and stabilize vessels both by establishing endothelial cell connections and by recruiting periendothelial cells [26–29]. Surprisingly, although the role of Ang-1 is suggested to be more stabilizing and related to the quiescent state, several studies have shown that Ang-1 has also both angiogenic and arteriogenic properties when overexpressed alone or in combination with VEGF [30,31]. Hallmark of vessel maturation, recruitment of perivascular cells, has been shown to be largely dependent on yet another family of growth factors, platelet-derived growth factors (PDGFs) [32]. Although overexpression of PDGFs alone may not be an optimal stimulus for vessel growth, successful combination approaches with VEGF have been published [33] (Fig. 3).

Although the initiator of collateral growth seems to be increased flow and shear stress, the result is activation of the endothelium and production of several growth factors [11,12]. One of the most widely studied molecules, monocyte chemoattractant protein-1 (MCP-1), exerts its functions by increasing monocyte infiltration into the growing vessels. After they have reached the site of collateral growth, macrophages start to release a variety of growth factors to simulate cell proliferation [34]. Increased shear stress induces also directly expression of several growth factors via shear stress responsive element, for example VEGFs, FGFs and PDGFs. Several angiogenic proteins have also shown to be potent inducers of arteriogenesis although their role in natural process of collateral growth is unclear. Ang-1, FGF-4, placental growth factor (PIGF), hepatocyte growth factor (HGF)
and a large number of other growth factors have improved collateral vessel growth in animal models of hindlimb and myocardial ischemia [25,35,36].

A wide variety of growth factors participate in the growth of blood vessels in the heart and overexpression of one factor may not produce an optimal response. It is therefore likely that a combination of growth factors or a factor capable of up-regulating other factors should be used. Recently, adenoviral overexpression of Hif1-α was shown to up-regulate the expression of multiple angiogenic growth factors in vivo [37]. Some growth factors have been shown to display species-specific actions and should therefore be tested in more than one species before use in human applications.

Fig. 2. Variety of growth factors participating in the angiogenesis process. Most gene therapy approaches aim to overexpress one of the growth factors which are naturally up-regulated during the angiogenesis process. While normal human myocardium does not produce VEGF (A), cells surrounding a myocardial infarct zone stain positive for VEGF (B). Capillaries in normal human myocardium (C) and angiogenesis in the edge of infarct scar (D). VEGF overexpression in the pig myocardium by adenoviral gene transfer (F) induces dramatic capillary enlargement in the pig myocardium 6 days after gene transfer (H) while VEGF expression is weak (E) and capillaries in the control gene (AdLacZ)-transduced myocardium remain unchanged (G).
Another possibility to improve both angiogenesis and arteriogenesis in the ischemic tissue is to inhibit endogenous anti-angiogenic processes and negative feedback loops such as up-regulation of Thrombospondin-1 by VEGF [38]. Inhibition of endogenous Hif1-α inactivation improved angiogenesis in the ischemic limb [39].

4. Where to deliver?

Where and how the gene therapy should be delivered depends on the therapeutic goal and on the properties of the growth factor itself. Growth of capillary vessels is desired in the infarct edge area and collateral growth in the healthy tissue extending from the patent coronary vessel to the hibernating, jeopardized myocardium (Fig. 4).

Also, the microenvironment for transduction and the target cells for the produced growth factor should be considered [40]. Growth factors exert their functions via specific receptors expressed on the target cells, and therapeutic effect can only be achieved when both ligands and receptors are able to interact. Neither vectors nor produced proteins will efficiently penetrate intact basement membrane, much less will they penetrate larger caliber vessel wall with multiple smooth muscle cell (SMC) layers. Growth factors intended to act on arterial endothelium should therefore be available in the vessel lumen, as well as factors acting on mural cells in the tissue surrounding the vasculature. Also the solubility of the growth factor determines the bioavailability of the ligand. A growth factor that binds efficiently to heparan proteoglycans on cell surface will stay in the vicinity of the producing cells whereas a soluble growth factor will diffuse further and cover a larger tissue area [41].

Whereas local delivery of a growth factor in a wrong microenvironment is inefficient, too widespread overexpression of the growth factor may have serious side effects. Expression can be directed to the right tissue compartment by using a proper delivery method but localized gene transfer gives only a rough estimate of the transduced area and it is difficult to restrict expression to a specific cell type. Tissue- or cell-specific targeting of gene delivery vectors has been developed to improve myocardial specificity of the gene transfer [42].

Fig. 3. Growth factors for gene therapy from nature’s own angiogenesis tool box. Angiogenesis is a complex, multi-step process in which every step has its own mixture of growth factors. Almost every single one of them has been studied for its potency to orchestrate the whole process of vessel growth. The natural trigger for angiogenesis is hypoxia. Hypoxia induces expression of variety of growth factors including VEGF and Ang-2. Pericytes detach from the capillaries, basement membrane is degraded by matrix metalloproteineses (MMPs) and plasma proteins extravasate to form a protein-rich matrix in the interstitial space. Growth factors up-regulated by hypoxia and released from the extracellular matrix induce proliferation and migration of endothelial cells and pericytes. Excessive neovessel network is then trimmed to respond to the metabolic needs of the tissue. Vessels that receive insufficient blood flow regress, while others with sufficient flow mature, require pericyte coverage and deposit a basement membrane.

Fig. 4. Therapeutic goal determines gene delivery strategy and microenvironment. Collateral growth is needed upstream and around the occlusion site in the normoxic myocardium. Process requires proliferation of smooth muscle cells around pre-existing collaterals while effects on the endothelium are secondary to the increase in the blood flow. Angiogenesis, on the other hand, is needed in the periphery around the infarction scar and growth factors are mainly required to stimulate proliferation and migration of endothelial cells.
5. How to deliver?

A wide variety of delivery methods have been introduced to achieve therapeutic angiogenesis in the myocardium ranging from systemic delivery of growth factors via mouse tail vein to highly sophisticated, local delivery methods in large animal models. Majority of the models require invasive procedures, such as thoracotomy, which themselves cause damage and therefore start repair processes and induce expression of endogenous growth factors in young, healthy animals. More importantly, highly invasive, stressful gene delivery methods are not suitable for very sick patients most urgently in need for alternative therapies.

Growth factors or gene transfer vectors can be injected into coronary arteries via regular angiography catheters. Intracoronary (IC) approach is relatively simple, does not require specialized equipment or specially trained physicians and does not require additional invasive procedures when done in combination with angiography. Usually the aim of IC approaches is transduction of endothelial cells. Although beneficial therapeutic effects have been reported in animal models, several studies have been published showing the low clinical efficacy of this method [7]. Intravenous and intra-arterial approaches are possible without the need for specialized equipment. As intravenous delivery of VEGF recombinant protein has been shown to be ineffective [43], efficacy of the intracoronary delivery depends on the first-pass effect [44]. Endothelium is an effective barrier for both proteins and viral particles. When tissue distribution of bFGF was studied after IC and i.v. administrations, 0.88% and 0.26% of $^{125}$I-labelled bFGF were found in the myocardium, respectively [45].

Therapeutic agents can also be delivered to the tissue surrounding the vessels. In the intrapericardial approach, vector is delivered into the pericardium surrounding the heart. Although good transduction efficiency can be achieved in the peri- and epicardium, no therapeutic effect on collateral perfusion has been observed [46]. Although high concentration of therapeutic protein is produced in the pericardial fluid, proteins may not penetrate into the myocardium and reach the growing collateral vessels. More localized delivery can be achieved with a collar placed around the treated vessel or with direct injection with a needle catheter [47]. Even high local protein concentration around the vessel leads mainly to angiogenic response in the adventitia of the vessels and does not penetrate deeper into the vessel wall [48].

Intramyocardial gene transfer allows vector delivery directly to the target area. Direct intramyocardial injections can be combined with traditional surgical procedures. Percutaneous techniques such as NOGA and Stiletto injection catheters for direct, intramyocardial injections are suitable for patients too compromised to undergo invasive surgical procedures and allow local delivery of the growth factors [49,50]. Furthermore, techniques for the evaluation of therapeutic effects and potential side effects essential for clinical trials are readily available in large animal models. In large animal models, techniques developed for human use are applicable and can therefore be more directly transferred to clinical trials.

6. How to deliver? Growing selection of gene transfer vectors

One therapeutic goal should be to determine the vector used for gene transfer. Treatment after acute myocardial infarction probably requires a different time course of gene expression than treatment of chronic myocardial ischemia. Also, damaged myocardium after infarction forms a different microenvironment than relatively healthy myocardium preconditioned for decreasing oxygen tension during slow progression of arterial occlusive disease in chronic ischemia.

Growth factors can be delivered into vascular tissues as recombinant proteins either directly or using substances that slowly release protein into the tissue. The main limitation of the protein approach is short half-life of growth factors in the tissue, as growth of blood vessels requires the presence of angiogenic stimulus until a steady state of the vessel structure is achieved [51] or flow is increased to a level sufficient to prevent vessel regression. Genes encoding growth factors can be delivered either nonvirally using plasmid constructs or using viral vectors. Plasmids are easy to produce and safe, but their main drawback in myocardium is a very low transduction efficiency [52]. Transduction efficiency of plasmid constructs can be significantly improved with liposomes [53].

Availability of viral vectors is increasing as new viruses are developed for gene therapy, and already established vectors are modified to improve their properties. An ideal viral vector for gene transfer would have high transduction efficiency, low toxicity, no immunogenicity, capacity to carry large gene constructs, selective gene expression in desired cell type and possibility to regulate gene expression. Such vector has not yet been created, and gene transfer vector has to be chosen based on what are the most important qualities for the therapeutic application.

Salvage of local tissue perfusion after myocardial infarction requires quick, high transduction efficiency. Adenovirus is one of the most widely used and studied gene transfer vectors. It has high transduction efficiency, relatively high transgene capacity and it can be produced with high titers. Transgene expression is transient and lasts 2–3 weeks in large mammals with normal immune system, time frame sufficient to build capillary network and increase tissue perfusion.

Growth of collateral vessels and arterialization of neovessels require longer transgene expression, from several weeks up to months. This can be achieved using episomal vectors or vectors that integrate into the host cell genome. Adeno-associated viruses (AAV) have lower
transduction efficiency than adenoviruses but have natural tropism to muscle tissue and transgene expression lasting up to a year after gene transfer [54]. DNA-capacity of AAVs is sufficient for most growth factors, but the maximal capacity (<5 kb) limits the possibility to develop regulated gene expression systems. Lentiviruses have higher capacity for transgenes and they are also capable of transducing both quiescent and proliferating cells. To overcome the safety issues arising from integration into the host cell genome, several regulated expression systems have been developed, but the main limitations for their use in cardiovascular approaches are low transduction efficiency and low titers of virus preparations.

7. How much to deliver?

As with all drugs, gene therapy approaches also have a therapeutic window, a concentration at which they produce an optimal therapeutic response and minimal side effects. In addition to variation in response to a given concentration of a growth factor, patients may also display considerable variation in the transduction efficiency and level of protein production. The challenge with viral delivery approaches is thus to determine the correct viral dose that produces an optimal amount and the target tissue distribution of the growth factor in a given tissue environment.

In conventional pharmacological approaches, drugs are usually distributed systemically. One of the main advantages of gene therapy is the possibility to deliver therapeutic proteins locally. On the other hand, it makes determination of correct dose and delivery more challenging. Whereas concentration of a drug in the blood stream can easily be determined, it takes far more preclinical experience in large animal models to be able to determine correct viral dose to achieve an optimal local protein concentration [40]. Spreading of the virus solution in the target tissue, transduction efficiency, level of protein production by the transduced cells and the solubility of the growth factor are all equally important determinants of the resulting therapeutic effect.

A vast majority of preclinical gene therapy experiments have been done in mouse models. Although mouse models are invaluable for transgenic and gene knock-out experiments and in understanding the biological function of growth factors, information derived from small animal models should be applied to human therapies with caution [7]. Viral loads and resulting protein concentrations achieved in mouse experiments are not necessarily applicable for human use. For example, injection of 1.0×10^{11} viral particles [55] or a volume of 3 ml [56] into tail vein of a mouse would respond to 2.5×10^{14} viral particles and a volume of 7 l in man. Also, the small tissue mass may complicate the determination of both efficacy and correct dose of a growth factor. It is easy to cover the entire myocardium of a mouse with a few injections, while covering the hundreds of times larger heart of a pig (or a man) is a much harder task. Also as trivial a detail as the needle track caused by the intramuscular injection has a whole different proportions in mouse than in man. For example, a 28-G needle causes approximately a 500-μm-wide, necrotic needle track in the muscle tissue in which inflammatory cells and damaged myocytes act as sources of endogenous growth factors. That needle track is 1/8 of the width of the left ventricle muscle of a mouse, but only 1/200 of the width of the left ventricle in man (Fig. 5).

8. What are the risks?

Side effects of gene therapy may be caused by the delivery process, the vector carrying the transgene or the gene product itself. Since one of the main goals of gene therapy is to find treatment strategies feasible for no option patients, delivery of the gene therapy vector itself should be as noninvasive and low risk procedure as possible. Surgical approaches and long-term or repeated infusions are therefore less likely to be relevant clinically feasible delivery strategies for myocardial angiogenesis.

Both viral and nonviral gene delivery vectors can cause adverse effects in vivo. High doses of naked plasmids have been reported to cause necrosis and inflammation [57]. Most viruses produce an immune reaction which may lead to a range of clinical symptoms, from transient rise in body temperature to septic shock. Vectors integrating into the host genome may activate oncogenes, interfere with
normal gene transcription or even promote mutations in the host genome [7].

Side effects of angiogenic growth factors in large animal models and man are poorly documented, as invasive approaches in small animal models have masked side effects specific for angiogenic molecules in the heart. Also, follow-up times of these studies have been quite short. One of the main concerns in angiogenic gene therapy is excessive vessel growth in the transduced tissue, so-called hemangioma or glomeruloid body formation [58]. Such structures will, however, usually undergo blood flow-dependent remodeling and normalization of the vascular architecture [59]. Systemic expression of the growth factors may produce harmful side effects in the distant organs, i.e., promote tumor growth, retinopathy or arthritis. Regulation of gene expression by tissue specific promoters or vectors regulated by conditions like hypoxia may help to limit angiogenic effects to target tissues [60]. Although vascular permeability and subsequent edema are known side effects of VEGFs, only recently its manifestation in the heart, pericardial effusion, was documented as a dose-limiting side effect [52]. Thus, comprehensive evaluation of potential side effects is needed in large animal models to determine the optimal dose and delivery strategy for each vector and gene combination.

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