

Gene Therapy Approaches for the Prevention of Restenosis

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Abstract: Experimental gene-therapeutic approaches for the prevention of restenosis after balloon angioplasty are the major source of our insight into pathways operative in the process of vascular renarrowing. We now understand that thrombosis and inflammation are the key mechanisms triggering vascular "healing" in response to injury and know a multitude of potential gene-therapeutic strategies to interfere with appositional thrombus formation, proliferation and migration of vascular smooth muscle cells, lesional recruitment of inflammatory cells or excess deposition of extracellular matrix. Thus far, the major limitation for clinical anti-restenotic gene therapy are concerns about the safety and efficacy of vector systems in use for the local overexpression of transgenes, which in turn is one of the most attractive advantages of gene therapy compared to systemic drug therapy. Here, we review the molecular mechanisms operative in postangioplasty restenosis by highlighting their respective gene therapeutic approaches and the current viral and non-viral vector systems.

Keywords: Gene therapy, restenosis, balloon angioplasty, inflammation, smooth muscle cell proliferation, adenovirus.

INTRODUCTION

Restenosis limits the benefit of percutaneous angioplasty by various degrees that range from 10%-70% depending on the vascular site and type of intervention. Of approximately 1 million patients who underwent coronary intervention worldwide in 1999, instant-restenosis developed in approximately 250.000 [1]. In addition to the variability depending on the vascular bed of intervention, the incidence and the extent of restenosis is determined by the individual cardiovascular risk profile and the composition of the atherosclerotic plaque. In particular, the level of the proinflammatory parameter C-reactive protein (CRP) both in blood [2] as well as within the plaque [3], was shown to be an independent predictor of restenosis.

Although the mechanisms leading to vascular reocclusion are still poorly understood, elastic recoil, periinterventional arterial thrombosis and inflammation are known as key determinants of the vigourosity of the vascular response to injury [4]. Proliferation and migration of vascular smooth muscle cells (VSMC), excess deposition of extracellular matrix and adventitial scarring with subsequent vessel constriction [5] further contribute to late lumen loss [6].

While stent implantation significantly improved early patency rates due to mechanical inhibition of the elastic recoil, restenosis due to intimal hyperplasia was not significantly affected before the era of drug-eluting stents [7]. However, stenting required adaptation of the periinterventional antithrombotic regimen, which now consists of heparin, aspirin and clopidogrel in order to prevent reocclusion due to subacute stent thrombosis. Although improved antithrombotic therapy with inhibitors of

platelet aggregation directed against the glycoprotein Iib/IIIa-receptor passivated the vessel wall, it did not reduce the rate of restenosis significantly. Today, rapamycin-coated stents, which release the cytostatic compound into their close environment, have shown the most promising results in both, coronary [8] and peripheral sites [9] of intervention and have largely overcome the use of primary brachytherapy. While high local drug concentrations can be achieved through the elution of drugs from polymer coats of such stents, catheter-based gene therapy at the time of angioplasty would achieve this goal in situations where stent placement is not possible or desirable.

POTENTIAL INDICATIONS FOR VASCULAR GENE THERAPY

In addition to restenosis after balloon injury, the prevention of reocclusion in saphenous vein grafts represents another potential field for gene therapeutic approaches as the time of injury and the kinetics of the vascular response are known. Both forms of vascular restenosis are fundamentally different from the process of atherosclerosis, although proliferative and inflammatory phenomena were observed in all these forms of vascular remodeling [10].

Spontaneous formation of subintimal fatty streaks consisting of lipid laden macrophages is considered the primary morphological change in atherosclerosis [11]. Mechanical forces, such as turbulent flow and shear stress as well as the endothelial barrier regulating mononuclear infiltration through the expression of selective adhesion molecules play a central role in the initiation phase. Accumulation of cholesterol esters and oxidized lipoproteins by the scavenger receptor on monocytic cells leads to foam cell formation of macrophages - one of the key players in regulating plaque stability and arteriothrombosis, which contributes to the progression of the disease.

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In contrast, graft sclerosis of saphenous veins that are used as bypass material combines the adaptive response to altered hemodynamic stimuli with the response to vascular injury. The extent of the resulting neointimal lesion is related to the degree of vascular injury caused by surgical manipulation and ischemia from occlusion of the vasa vasorum [12, 13]. In addition, the vein must adapt to the increase in intraluminal pressure within the arterial circulation, resulting in a thickening of the vessel wall.

GENE THERAPY

Vector Systems for Gene Transfer

Since each vector has its advantages and disadvantages, one of the challenges of gene therapy is the choice of the optimal system for the introduction of genes into the particular tissue. Essentially, gene transfer vehicles can be grouped into viral and non-viral, with an emerging class of constructs that incorporate features of both and therefore, represent chimeras between the two; they are often referred to as virosomes.

One of the preferred viral vectors for gene transfer into vascular cells is recombinant adenovirus; this is, first, due to the ability of adenovirus, in contrast to retrovirus, to transduce non-dividing cells; second, the high efficiency of uptake of the virus by endothelial cells due to the expression of appropriate integrins ($\alpha v\beta 5$) that function as viral receptors on these cells; and third, in the context of restenosis, transgene expression is regarded to be important only for the initial phase after injury, and adenovirus-directed gene expression is limited to a period of 2-3 weeks, since adenoviral DNA does not integrate into the host cell genome, a feature that has turned out to be an important advantage over retroviral systems. In contrast, the drawbacks of adenovirus that include inactivation by serum, are less relevant in this context, due to the local delivery through balloon catheters that generate a local serum-free environment at the site of administration.

Another emerging viral gene delivery system is herpes simplex based vectors. In an *in vivo* rabbit model system, such an engineered vector has been shown to infect all vascular layers without prior injury to the endothelium, and exhibit no systemic toxicity. Moreover, it can be eliminated, if necessary, by administration of the antiviral drug acyclovir [14].

The third vector system that may be well suited is AAV (adeno-associated virus). AAV vectors transduce nondividing cells, integrate into the host genome, and have been shown in a rabbit carotid artery model to provide transgene expression for at least 28 days [15]. The advantage of AAV lies in its site-specific integration mainly into a locus at 19q 13.3-qter, which would be an important feature for gene therapy, since it avoids integration-mediated inactivation of vital genes such as anti-proliferative genes, however, conventional AAV vectors have lost this property.

Non-viral systems include liposomes of varying composition, such as cationic and anionic liposomes, polyplexes, and dendrimers. They offer the advantage of biosafety, but are generally less efficient gene transfer vehicles. Liposomes usually consist of a mixture of lipids

forming bilayers that resemble many features, of the cell membrane. Anionic and neutral liposomes are less toxic than cationic liposomes and are more compatible with biological fluids, however, lack endosome disruptive mechanisms and their efficiency is usually low. Canonic liposomes bind easily to and condense DNA, an important advantage, but their efficiency depends on the route of administration. Dendrimers are chemically distinct and consist of, e.g. polyamidoamine polymers with a defined spherical structure. In addition, polyethylenimine, a polycation, has been successfully used *in vitro* and *in vivo*. Various attempts have been made to incorporate into liposomes viral and non-viral proteins with the aim of enhancing transfection efficiency, but also to achieve cell type specificity. A recent example of a virosome is the HJV-AVE (hcmagglutinating virus of Japan-artificial viral envelope) composition, which was developed by combining liposomes with fusion proteins derived from HVJ [16]. Immunoliposomes contain antibodies that direct the liposome towards tissue-specific markers.

Principles of Inhibiting Gene Function

Several mechanistically distinct gene therapy approaches have been undertaken to interfere with the expression of genes, first, expression can be diminished by the introduction of nucleic acid constructs that interfere with mRNA stability, such as antisense oligonucleotides, hammerhead ribozymes, and, more recently, siRNA (for targeting, e.g. PDGF and its receptors). Second, genes with inhibitory function, either naturally occurring inhibitors or artificially created dominant-negative (dn) forms, can be overexpressed. Examples include inhibitors of the cell cycle such as dn c-Myb, a mutated p21/WAF/CIP1, or the cyclin-dependent kinase inhibitor p57Kip2; inhibition of NF- κ B, a main regulator of the inflammatory response through expression of its inhibitor I κ B α ; inhibition of tissue factor by tissue factor pathway inhibitor (TFPI); of TGF β through expression of a soluble TGF β receptor (decoy receptor); and a dn mutant of MCP-1, a chemoattractant for monocytes [17]. Third, the regulation of a target gene can be influenced on the level of transcription, either by decoy oligonucleotides, which are either short double-stranded oligonucleotides or dumb-bell shaped, circular oligonucleotides that represent transcription factor binding sites, and thus compete for binding of a specific transcription factor that is relevant for the respective gene [18, 19], or by the expression of artificial transcription factors. The latter can be engineered based on combinations of distinct zinc fingers to bind to almost any given sequence in the promoter region to modulate the expression of a specific gene. Last not least, genes can be overexpressed that have a beneficial influence on various aspects of vessel wall physiology, e.g. nitric oxide synthase, sodium oxide dismutase, or inhibitors of metalloproteinases (TIMP). Most affiliates of this arsenal have already been used for gene therapy studies, including restenosis.

Molecular Mechanisms of Restenosis: Genes to Targets

Angioplasty of a stenotic atherosclerotic artery results in fracturing of the atherosclerotic plaque, injury of the inner layers of the vessel and stretching of the vessel wall. It evokes, or at least boosts, an existing inflammatory reaction, and induces smooth muscle cell replication, migration and

matrix deposition. If not counteracted by stent placement, recoil forces add to these reactions. In addition, restructuring of cells and matrix within the artery wall results in tissue shrinkage and a change in vessel wall geometry. At later stages, monocyte infiltration occurs and contributes further to a vicious circle of cytokine production, coagulation and inflammation.

The precise molecular mechanisms underlying neointima formation after injury are only partially understood. This is largely due to heterogeneity in the experimental systems, which include rats, (knockout) mice, rabbits or pigs, as well as the sites under investigation (e.g. carotid or iliac arteries); their relation to the situation in humans should be considered with caution, as well as to the situation where an atherosclerotic lesion already exists. One of the best-studied models is the rat carotid artery injury model (for review, see [20]). The responses following balloon angioplasty have been grouped into four "waves". First, SMC proliferation occurs, probably in response to liberation of growth factors from dying SMC that can be completely accounted for by release of bFGF, but not PDGF [21, 22]. Angiotensin II may also contribute at this stage. Second, SMC migration takes place, and in contrast to the initial phase, this is now strongly dependent of PDGF, with additional contributions of TGF β , bFGF and Angiotensin II. Once these cells have arrived in the intima, they start to replicate; however, it is controversial whether this second wave of replication (referred to as the "third wave" in the context of neointima formation) requires the same or distinct growth factors as the initial one. Finally, the proliferative response can be further stimulated by tagain) TGF β , bFGF, and Angiotensin II. In addition, loss of growth inhibitory properties could contribute significantly to proliferation. Several lines of evidence, including the use of L-arginine and L-NMMA, which are agonists and antagonists of NO production, respectively, suggest the involvement of NO at this stage. However, in other species including humans, thrombus formation and leukocyte infiltration are prominent after vascular injury and often precede SMC proliferation and migration.

During SMC migration, the extracellular matrix (ECM) needs to be degraded, e.g. through the action of plasmin, which is in turn regulated by PAI-I. PAI-I also plays a role in activating TGF β from its inactive precursor, which leads to the expression of ECM genes at later stages. In addition, PAI-I, together with tissue factor, is a main regulator of coagulation, and both are expressed upon inflammation. Tissue factor and its associated proteases (factor VIIa, Xa) also trigger thrombotic mechanisms, e.g. mitogenic and chemotactic effects on SMC through eliciting a proinflammatory response [23]. The various crosstalks between these molecules with their partially overlapping functions in inflammation, coagulation, and ECM synthesis may give an impression of the complexity of the molecular mechanisms underlying the restenotic process, and may, due to our limited knowledge, still be a simplification of the real situation.

Gene Therapy Approaches for the Prevention of Restenosis

One of the most striking points when reviewing the literature on gene therapy experiments in the context of restenosis is the multitude of genes and constructs that have been tested, and even more surprising that in most studies

significant effects have been observed. This high diversity of potential targets may reflect the complexity of the disease, but still important knowledge of these molecules ("drivers or passengers") is lacking. From what is known about the molecular mechanisms of restenosis, researchers have come up with approaches that can be grouped essentially into five classes of genes, namely those involved in SMC proliferation (DNA synthesis and cell cycle control, growth factors), inflammation, coagulation, monocyte function, and apoptosis.

Interfering with proliferation has been the most widely used strategy. It appears straightforward, since SMC proliferation is an essential component of the disease, however, migration of SMC may contribute significantly: although the approach may look quite non-specific at first glance, specificity is achieved through local application of the antiproliferative gene, and major side effects are not expected. The first studies, done in the early 90s, have employed the herpes simplex virus thymidine kinase (HSV-TK) gene, mostly through recombinant adenoviral transfer [24, 25]. HSV-TK metabolizes the drug ganciclovir to a nucleoside analog that inhibits DNA synthesis in those cells expressing the transgene; however, since the metabolite is diffusible, the advantage of the system lies in the inhibition of also the surrounding cells ("bystander effect") that makes up for the sometimes rather limited transduction efficiency. HSV-TK gene transfer, followed by 2 weeks of systemic ganciclovir treatment, has significantly reduced injury-induced SMC accumulation [24].

Following these early experiments, several other antiproliferative strategies have been tested; First, interfering with cell cycle regulatory mechanisms by introduction of the retinoblastoma (Rb) gene [26-28], of the cyclin/cyclin-dependent kinase inhibitor p21 [29], the growth-arrest homeobox gene Gax [30], cytosine deaminase [31], and several others (see Table 1). Second, interference with growth factors that are known to act on SMC has been investigated. This includes the generation of dn forms of growth factor receptors, or the introduction of antisense constructs. Yukawa *et al.* reported growth suppression of vascular SMCs *in vivo* using adenovirus-mediated gene transfer of a truncated form of fibroblast growth factor receptor [32]; Cohen-Sacks encapsulated antisense phosphorothioate oligonucleotides directed against the PDGF β -R in biodegradable polymeric nanospheres and successfully inhibited restenosis in a rat carotid artery restenosis model [33]; hammerhead ribozymes directed against PDGFA inhibited smooth muscle cell proliferation and neointima formation in a similar model [34]. The same group has used hammerhead ribozymes also to target TGF β [35], which is mitogenic for SMC but also stimulates extracellular matrix deposition, a hallmark of the restenotic process, and reported diminished angiotensin II stimulated DNA synthesis in human SMC. A different approach to inhibit TGF β , namely through expression of a soluble TGF-beta type II receptor, was undertaken in porcine coronary arteries [36]. In accordance, Nabel *et al.* noted that expression of TGF β stimulated fibrocellular hyperplasia in porcine arteries [37].

Table 1. Molecular Targets and Transgenes

Genetic construct	Function	Reference
IISV-TK	cell cycle	[24,25]
Rb	cell cycle	[26-28]
p21	cell cycle	[29]
gax	cell cycle	[30]
cytosine deaminase	cell cycle	[31]
G1	cell cycle	[57, 58]
c-Myc	cell cycle	[59, 60]
p16-p27 chimeras	cell cycle	[61-63]
p21/WAF/CIP	cell cycle	[64]
E2F	cell cycle	[65, 18]
p57Kip2	cell cycle	[66]
cyclin B1	cell cycle	[67]
CDC2 kinase	cell cycle	[67]
ras	signal transduction	[68, 69]
Gbeta/gamma	signal transduction	[70]
NOS	signal transduction vascular tone	[53, 71]
FGFR1	growth factor/receptor	[32]
PDGF-A/PDGFβR	growth factor/receptor	[33, 34]
activin A	growth factor/receptor	[72]
VEGF	growth factor/receptor	[73-75]
HGF	growth factor/receptor	[76]
TGFβ/TGFβR	growth factor/receptor extracell. matrix	[35, 36]
TIMP	extracell. matrix	[77, 78]
u-PAR	extracell. matrix haemostasis	[39]
PAI-I	haemostasis	[38]
TFPI	haemostasis	[16]
MCP-1	chemoattractant	[17, 51, 52]
NF-κB	transcription factor	[47, 48]
AP-1	transcription factor	[19, 79]
SOD	antioxidant	[80]
FasL/p35	anti-apoptotic	[50]
tissue kallikrein	vascular tone, etc.	[81, 82]
cecropin A	antibacterial	[83]

The contribution of the PAI-I/uPAR system to the restenotic process has been a matter of debate. PAI-I has a major function in thrombolysis, but also acts in controlling extracellular matrix (PCM) degradation. SMC migration requires controlled proteolytic degradation of the ECM surrounding the cell. Elevated plasma levels of PAI-I are associated with myocardial infarction, atherosclerosis, and restenosis. PAI-I is increased in atherosclerotic arteries and failed vein grafts. However, data generated in PAI-I knockout mice suggest that PA I-1 may decrease lesion formation after arterial injury, and that PAI-I gene transfer would prevent restenosis. DeYoung *et al* have used adenovirus-mediated overexpression of PAI-I in a rat carotid balloon injury model and found initial retardation, but after 14 days significantly increased neointima formation [38]. A different approach to study the contribution of the PAI-I/uPAR system has utilized adenovirus-mediated expression of an artificial plasmin inhibitor (ATF, BPT1), demonstrating inhibition of rat carotid artery neointima formation, presumably through inhibition of SMC migration [39]. These results would suggest that activation of the plasmin system increases neointima formation; for a detailed discussion of the role of PAI-I in restenosis and related diseases, see [40]. Recently, the transcription factor NAK-1 was identified as a main regulator of PAI-I, and both proteins are expressed in arteriosclerotic lesions [41]. A dn form of NAK-1 increased lesion formation in a murine carotid artery ligation model [42]. The identification of this novel regulator of PAI-I will also allow new insights into the mechanisms of restenosis.

To investigate the contribution of coagulation to the restenotic process, several groups including ourshave inhibited tissue factor using tissue factor pathway inhibitor (TFPI). Already in 1997, Oltrona *et al.* administered recombinant TFPI to the carotid arteries of minipigs after repeated balloon hyperinflation, and noted significantly diminished stenosis when given within 24 hours after injury [43]. Expression of TFPI using HVJ-AVE liposomes resulted in significantly diminished neointima formation in a rabbit iliac artery model after balloon injury [16]. In our studies using adenovirus-mediated TFPI overexpression in a, rabbit hypercholesterolemic balloon injury model, we could show reduced neointima formation that was due to both coagulation-dependent and -independent mechanisms. e.g. reduced matrix metalloproteinase-2 and -9 expression, and diminished lesional macrophage infiltration (Kopp *et al.* submitted).

The narrow time frame of effectiveness of recombinant TFPI observed in the experiments of Oltrona *et al.* further supports the notion that early events, such as an inflammatory reaction occurring at, or briefly after injury trigger, or at least contribute significantly, to the restenotic process. We have therefore, tested inhibition of NF-κB, a transcription factor acting very early as a key regulator of the inflammatory reaction for its involvement in restenosis. In endothelial cells, NF-κB controls the expression of several pro-inflammatory genes, including cytokines (IL-1, IL-6, IL-8, MCP-I), adhesion molecules (E-selectin, ICAM-1, VCAM-1), and many others (e.g. tissue factor, iNOS, COX-2). In addition, NF-κB has been shown to counteract apoptosis through expression of anti-apoptotic genes, and that it is required for the survival of SMC *in vitro* [44]. Pharmacological inhibition of NF-κB by the antioxidant

pyrrolidine dithiocarbamate has resulted in reduced neointima formation in a rat carotid artery model, including diminished expression of iNOS and COX-2 [45]. Moreover, in clinical studies, probucol as well as AGI-I067, a metabolically stable modification thereof, reduced restenosis after PCI, however, both agents may act also through other mechanisms than inhibition of NF- κ B [46]. In a rabbit iliac artery model, overexpression of I κ B α , a specific inhibitor of NF- κ B using recombinant adenovirus, resulted in reduced ICAM-1 and MCP-1 expression, as well as diminished recruitment of macrophages into the wounded area as assayed after 8 days. In addition, expression of inhibitor of apoptosis proteins was reduced and the percentage of apoptotic cells increased compared to control-treated contralateral vessels. Animals examined 5 weeks after treatment exhibited a significantly reduced degree of lumen narrowing due to positive remodeling [47]. Similar results were observed when overexpression of I κ B α was combined with stents [48].

Apoptosis is a regulatory mechanism of major importance not only during embryonic development but also for several patho-physiological processes, and is closely linked to proliferation. Driving SMC into apoptosis during the process of proliferation and migration was therefore, an apparent possibility that was consequently investigated by several groups. Transduction of rabbit iliac arteries with recombinant adenoviral vectors for FasL reduced neointima formation, which occurred through killing of Fas expressing neighbouring SMC by FasL transduced cells [49]; this system has recently been further refined by co-expression of p35 [50]. The advantage of this approach is the specificity of the Fas/FasL system for SMC, which does not affect endothelial cells and therefore, is expected not to interfere with re-endothelialization.

Infiltration and activation of monocytes is an important attribute of the restenotic process. To test whether monocyte infiltration is dependent on MCP-1, Mori *et al.* generated a dn form of MCP-1. After introduction into the skeletal muscle of hypercholesterolemic rabbits followed by balloon injury, they observed diminished monocyte infiltration in the injured arterial wall and attenuated development of neointimal hyperplasia [51, 52]. Likewise, a mutant MCP-1 suppressed neointimal hyperplasia in the carotid arteries of rats, rabbits and monkeys following balloon injury [17].

Several other genes that do not fall into one of the above mentioned categories have been assayed for their involvement in the restenotic process and its prevention, one of the more prominent ones being iNOS [53]. The majority of these approaches was successful, as reported. Still, it will be a challenge to dissect which strategies are the most promising ones to follow for clinical studies, of which some have already been conducted. This includes the PREVENT single-center randomized trial that uses ex-vivo introduction of E2F decoys into human vascular bypass grafts, resulting to fewer graft occlusions, revisions, or critical stenoses at 12 months after operation [54]. Furthermore, the KAT (Kuopio Angiogenesis Trial) and AGENT (Angiogenic Gene Therapy) studies that appear to aim at formation of new blood vessels rather than prevention of restenosis *per se* should be mentioned. They utilized intracoronary transfer of

adenoviral constructs for VEGF and FGF, respectively. Both studies did not show increase of lumen diameter, but, in accordance with the concept of new blood vessel formation, improvement of other parameters, such as myocardial perfusion and exercise time [55, 56].

CONCLUSIONS

Restenosis has been an attractive target disease for gene therapists, due to the accessibility of the site of treatment and to the possibility of localized delivery of the (viral or non-viral) vector. While clinical gene therapy is still in its infancy, and has been scooped in part by drug-eluting stents, a number of *in vivo* studies involving a wide variety of genetic inhibitors have begun to shape our understanding of the molecular mechanisms underlying the disease. This will hopefully lead to the rational design of even more improved therapeutic strategies to tackle the problems that are still remaining.

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