Gene therapy antiproliferative strategies against cardiovascular disease

Review Article

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List of abbreviations: apoE, apolipoprotein E; AP-1, activator protein-1; BrdU, 5-bromodeoxyuridine; CDK, cyclin-dependent kinase; CKI, CDK inhibitory protein; EC, endothelial cell; ERK, extracellular signal-regulated kinase; IVUS, intravascular ultrasound; JNK, c-jun NH2-terminal protein kinase; MAPK, mitogen-activated protein kinase; ODN, oligodeoxynucleotide; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; pRb, retinoblastoma protein; PTCA, percutaneous transluminal angioplasty; SAPK, stress-activated protein kinase; TGF-β, transforming growth factor-β; VSMC, vascular smooth muscle cell.

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Summary

Excessive cellular proliferation is thought to contribute to the pathogenesis of several forms of cardiovascular disease (e.g., atherosclerosis, restenosis after angioplasty, and vessel bypass graft failure). Therefore, candidate targets for the treatment of these disorders include cell cycle regulatory factors, such as cyclin-dependent kinases (CDKs), cyclins, CDK inhibitory proteins (CKIs), tumor suppressors, growth factors and their receptors, and transcription factors. Importantly, animal models of atherosclerosis have demonstrated an inverse correlation between neointimal cell proliferation and atheroma size, suggesting that excessive cell growth prevails at the onset of atherogenesis. Cell growth may also predominate at the onset of human atherosclerosis. Thus, given that affected humans often exhibit advanced atherosclerotic plaques when first diagnosed, the potential benefit of antiproliferative strategies for the treatment of atherosclerosis in clinic is doubtful. The antiproliferative approaches used so far in the setting of vascular obstructive disease have focused on restenosis and graft atherosclerosis, during which neointimal hyperplasia is spatially localized and develops over a short period of time (typically 2-12 months). Vascular interventions, both endovascular and open surgical, allow minimally invasive, easily monitored gene delivery. Thus, gene therapy strategies are emerging as an attractive approach for the treatment of vascular proliferative disease. In this review, we will discuss the use of gene therapy strategies against cellular proliferation in animal models and clinical trials of cardiovascular disease.

I. Introduction

Large-scale clinical trials conducted over the last decades have allowed the identification of independent risk factors that increase the prevalence and severity of atherosclerosis (e.g., hypercholesterolemia, hypertension, smoking). Cardiovascular risk factors initiate and perpetuate an inflammatory response within the injured arterial wall that promotes the development of atherosclerotic plaques (Ross, 1999; Lusis, 2000; Dzau et al, 2002; Steinberg, 2002) (Figure 1). Chemokines and cytokines secreted by leukocytes that accumulate within the injured arterial wall promote their own proliferation, as well as the growth and migration of the underlying vascular smooth muscle cells (VSMCs) (Figure 2). This inflammatory response also plays a critical role during restenosis after angioplasty and graft atherosclerosis. Thus, understanding the molecular mechanisms that control hyperplastic growth of vascular cells should help develop novel therapeutic strategies for the treatment of vascular obstructive disease.

Although arterial cell proliferation occurs in animal models during all phases of atherogenesis (Ross, 1999; Díez-Juan and Andrés, 2001; Cortés et al, 2002), studies with hyperlipidemic rabbits have shown an inverse correlation between atheroma size and cellular proliferation within the atheromatous plaque (Spraragen et al, 1962; McMillan and Stary, 1968; Rosenfeld and Ross, 1990). Experimental angioplasty is also characterized by
abundant proliferation of VSMCs, followed by the reestablishment of the quiescent phenotype, typically within 2-4 weeks (Bauters and Isner, 1997; Libby and Tanaka, 1997; Andrés, 1998). These animal studies suggest that vascular cell proliferation prevails at the onset of atherogenesis and restenosis.

Figure 1. Neointimal lesion development in response to cardiovascular risk factors and mechanical injury. Exposure of the arterial wall to cardiovascular risk factors and mechanical injury leads to endothelial damage. Recruitment of circulating leukocytes is promoted by the expression of adhesion molecules by the injured endothelial cells. Neointimal leukocytes release a plethora of cytokines and chemokines that initiate and perpetuate an inflammatory response, which activates signal transduction pathways and transcription factors that promote the hyperplastic growth of the lesion. Accumulation of noncellular material also contributes to atheroma development.

Figure 2. Early atherogenesis is associated with abundant cell proliferation within the arterial wall. Immunohistochemical analysis of aortic arch cross-section of male New Zealand rabbits fed control chow or a cholesterol-rich diet for 2 months. Animals were injected with 5-bromodeoxyuridine (BrdU) prior to sacrifice. Specimens were incubated with anti-BrdU and anti-RAM11 antibodies to monitor cell proliferation and to identify macrophages, respectively (Cortés et al., 2002). Arrowheads indicate the internal elastic lamina. Note lack of atherosclerosis and undetectable immunoreactivity for BrdU and RAM11 within the aortic arch of control rabbits. In contrast, prominent fatty streaks enriched in lipid-laden macrophages are seen in cholesterol-fed animals. Some macrophages are also detected within the media. Abundant BrdU immunoreactivity demonstrates a high proliferative activity, particularly within the atherosclerotic lesion. All photomicrographs are at the same magnification.
Expression of proliferation markers in human primary atheromatous plaques and restenotic lesions has been well documented (Essed et al., 1983; Gordon et al., 1990; Burriug, 1991; Nobuyoshi et al., 1991; Katsuda et al., 1993; Kearney et al., 1997; O’Brien et al., 1993, 2000; Rekhter and Gordon, 1995; Wei et al., 1997; Orekhov et al., 1998; Tanner et al., 1998; Veinot et al., 1998). However, controversy exists regarding the magnitude of the proliferative response, ranging from a very low index of cell proliferation (Gordon et al., 1990; Katsuda et al., 1993; O’Brien et al., 1993; 2000; Rekhter and Gordon, 1995; Veinot et al., 1998) to abundance of dividing cells (Essed et al., 1983; Nobuyoshi et al., 1991; Pickering et al., 1993; Kearney et al., 1997). Aside from methodological issues (e. g., differences in the fixatives used for tissue preservation, antigen accessibility, diversity of proliferation markers analyzed in these studies), some of the reported variance with regard to the issue of cell proliferation might relate to differences in the arteries being analyzed (i. e., peripheral, coronary and carotid arteries) and variance in the stage of atherosclerosis at the time of tissue harvesting (Isner, 1994).

The cell types that undergo cell proliferation within human atherosclerotic tissue include VSMCs, leukocytes and endothelial cells (ECs) (Gordon et al., 1990; Burriug, 1991; Katsuda et al., 1993; O’Brien et al., 1993; 2000; Rekhter and Gordon, 1995; Orekhov et al., 1998; Veinot et al., 1998). Histological examination in 20 patients undergoing antemortem coronary angioplasty revealed that the extent of intimal proliferation was significantly greater in lesions with evidence of medial or adventitial tears than in lesions with no or only intimal tears (Nobuyoshi et al., 1991). Human carotid artery primary atherosclerotic tissue retrieved by endarterectomy surgery displayed greater proliferative activity in the intimal lesion versus the underlying media (Rekhter and Gordon, 1995). Moreover, monocyte/macrophage proliferation predominated in the intima (46% versus 9.7% α-actin immunoreactive VSMCs, 14.3% ECs, 13.1% T lymphocytes), whereas VSMC proliferation prevailed in the media (44.4% versus 20% ECs, 13.0% monocyte/macrophages, and 14.3% T lymphocytes). It is also noteworthy that cell proliferation in human peripheral and coronary arteries is greater in restenotic versus primary lesions (O’Brien et al., 1993; 2000; Pickering et al., 1993). Furthermore, cultured VSMCs from human advanced primary stenosing disclosed lower proliferative capacity than cells from fresh restenosing lesions (Dartsch et al., 1990). Thus, similar to the situation in animal models, proliferation during human atherosclerosis and restenosis might peak at the onset of these pathologies and then progressively decline.

Cell cycle progression is controlled by several cyclin-dependent kinases (CDKs) that associate with regulatory cyclins (Morgan, 1995) (Figure 3). Active CDK/cyclin holoenzymes hyperphosphorylate the retinoblastoma protein (pRb) and the related pocket proteins p107 and p130 from mid G1 to mitosis. Phosphorylation of pRb and related pocket proteins contributes to the transactivation of genes with functional E2F-binding sites, including several growth and cell-cycle regulators (i.e., c-myc, pRb, cdc2, cyclin E, cyclin A), and genes encoding proteins that are required for nucleotide and DNA biosynthesis (i. e., DNA polymerase α, histone H2A, proliferating cell nuclear antigen, thymidine kinase) (Dyson, 1998; Lavia and Jansen-Durr, 1999; Stevaux and Dyson, 2002). Interaction of CDK/cyclins with CDK inhibitory proteins (CKIs) attenuates CDK activity and promotes growth arrest (Philipp-Staheli et al., 2001). CKIs of the Cip/Kip family (p21Cip1, p27Kip1 and p57Kip2) bind to and inhibit a wide spectrum of CDK/cyclin holoenzymes, while members of the Ink4 family (p16Ink4a, p15Ink4b, p18Ink4c, p19Ink4d) are specific for cyclin D-associated CDKs.

**Figure 3.** Control of mammalian cell cycle by CDK/cyclin holoenzyme and growth suppressors of the CKI family. Sequential activation of specific CDK/cyclin complexes leads to progression through the different phases of the cell cycle. Inhibitory proteins of the CKI family (Cip/Kip and Ink4) inhibit CDK/cyclin activity.
Mitogenic and antimitogenic stimuli affect the rates of synthesis and degradation of CKIs, as well as their redistribution among different CDK/cyclin pairs (Philipp-Staheli et al, 2001). For example, p27^Kip1^ promotes the assembly of CDK4/cyclin D complexes by binding to them, thus facilitating CDK2/cyclin E activation through G1/S phase.

VSMC proliferation in the balloon-injured rat carotid artery is associated with a temporally and spatially coordinated expression of CDKs and cyclins (Wei et al, 1997; Braun-Dullaeus et al, 2001). Importantly, augmented expression of these factors is associated with an increase in their kinase activity (Abe et al, 1994; Wei et al, 1997), demonstrating the assembly of functional CDK/cyclin holoenzymes in the injured arterial wall. Expression of CDK2 and cyclin E was also detected in human VSMCs within atherosclerotic and restenotic tissue (Kearney et al, 1997; Wei et al, 1997; Ihling et al, 1999), suggesting that induction of positive cell-cycle control genes is a hallmark of vascular proliferative disease in human patients.

In the following sections, we will discuss the use of gene therapy strategies targeting cellular proliferation in preclinical (Table 1) and clinical studies (Table 2) related to cardiovascular disease.

### Table 1: Attenuation of neointimal thickening by antiproliferative gene therapy approaches in animal models of vascular proliferative disease.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Target gene</th>
<th>Animal model</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>Antisense (ODN)</strong></td>
<td></td>
<td></td>
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<tr>
<td>CDK2</td>
<td>Balloon angioplasty (rat)</td>
<td>Abe et al, 1994; Morishita et al, 1994a</td>
<td></td>
</tr>
<tr>
<td>CDC2</td>
<td>Balloon angioplasty (rat)</td>
<td>Abe et al, 1994; Morishita et al, 1994b</td>
<td></td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>Balloon angioplasty (rat)</td>
<td>Morishita et al, 1994b</td>
<td></td>
</tr>
<tr>
<td>CDC2/PCNA</td>
<td>Graft arteriosclerosis (rabbit, rat)</td>
<td>Mann et al, 1995; Miniati et al, 2000</td>
<td></td>
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<tr>
<td>CDC2/PCNA</td>
<td>Balloon angioplasty (rat)</td>
<td>Morishita et al, 1993</td>
<td></td>
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<tr>
<td>CDK2</td>
<td>Graft arteriosclerosis (mouse)</td>
<td>Suzuki et al, 1997</td>
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<tr>
<td>c-myc *</td>
<td>Balloon angioplasty (pig, rat)</td>
<td>Simons et al, 1992; Gunn et al, 1997</td>
<td></td>
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<tr>
<td>c-myc *</td>
<td>Balloon angioplasty (rat, pig, rabbit)</td>
<td>Bennett et al, 1994a; Shi et al, 1994b; Kipshidze et al, 2001, 2002</td>
<td></td>
</tr>
<tr>
<td>c-myc *</td>
<td>Graft arteriosclerosis (pig)</td>
<td>Mannion et al, 1998</td>
<td></td>
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<tr>
<td>PDGFβ receptor</td>
<td>Balloon angioplasty (rat)</td>
<td>Cohen-Sacks et al, 2002</td>
<td></td>
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<tr>
<td><strong>Antisense (retrovirus)</strong></td>
<td>Cyclin G1</td>
<td>Balloon angioplasty (rat)</td>
<td>Zhu et al, 1997</td>
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<tr>
<td><strong>Ribozyme</strong></td>
<td>PCNA</td>
<td>Stent (pig)</td>
<td>Frimerman et al, 1999</td>
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<tr>
<td>TGF-β1</td>
<td>Balloon angioplasty (rat)</td>
<td>Yamamoto et al, 2000</td>
<td></td>
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<tr>
<td>PDGF-A</td>
<td>Balloon angioplasty (rat)</td>
<td>Kotani et al, 2003</td>
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<tr>
<td>12-lipoxygenase</td>
<td>Balloon angioplasty (rat)</td>
<td>Gu et al, 2001</td>
<td></td>
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<tr>
<td><strong>‘Decoy’ ODN</strong></td>
<td>E2F</td>
<td>Balloon angioplasty (rat, pig)</td>
<td>Morishita et al, 1995; Ahn et al, 2002a; Nakamura et al, 2002</td>
</tr>
<tr>
<td>E2F</td>
<td>Graft arteriosclerosis (rabbit, mouse, monkey)</td>
<td>Mann et al, 1997; Kawauchi et al, 2000; Ehsan et al, 2001</td>
<td></td>
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<tr>
<td>AP-1</td>
<td>Balloon angioplasty (rat, rabbit, minipig)</td>
<td>Ahn et al, 2002b; Buchwald et al, 2002; Kume et al, 2002</td>
<td></td>
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<tr>
<td><strong>Overexpression of growth suppressors</strong></td>
<td>p21^{Cip1}</td>
<td>Balloon angioplasty (rat, mouse, pig)</td>
<td>Chang et al, 1995a; Yang et al, 1996; Ueno et al, 1997a; Condorelli et al, 2001;</td>
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<tr>
<td>p21^{Cip1}</td>
<td>Graft arteriosclerosis (rabbit)</td>
<td>Bai et al, 1998</td>
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<tr>
<td>p27^{Kip1}</td>
<td>Balloon angioplasty (rat, pig)</td>
<td>Chen et al, 1997; Tanner et al, 2000</td>
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<tr>
<td>pRb</td>
<td>Balloon angioplasty (rat, pig)</td>
<td>Chang et al, 1995b; Smith et al, 1997b</td>
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<tr>
<td>RB2/p130</td>
<td>Balloon angioplasty (rat)</td>
<td>Claudio et al, 1999</td>
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<tr>
<td>p53</td>
<td>Balloon angioplasty (rabbit, rat)</td>
<td>Yonemitsu et al, 1998; Scheinman et al, 1999; Matsushita et al, 2000</td>
<td></td>
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<tr>
<td>GAX</td>
<td>Balloon angioplasty (rat, rabbit)</td>
<td>Maillard et al, 1997; Smith et al, 1997a; Perlman et al, 1999</td>
<td></td>
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<tr>
<td>GATA-6</td>
<td>Balloon angioplasty (rat)</td>
<td>Mano et al, 1999</td>
<td></td>
</tr>
<tr>
<td><strong>Overexpression of dominant-negative mutants</strong></td>
<td>RAS</td>
<td>Balloon angioplasty (rat)</td>
<td>Indolfi et al, 1995; Ueno et al, 1997b</td>
</tr>
<tr>
<td>ERK</td>
<td>Balloon angioplasty (rat)</td>
<td>Izumi et al, 2001</td>
<td></td>
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<tr>
<td>JNK</td>
<td>Balloon angioplasty (rat)</td>
<td>Izumi et al, 2001</td>
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</table>

* These inhibitory effects might be caused by a nonantisense mechanism (Burgess et al, 1995; Chavany et al, 1995; Guvakova et al, 1995; Villa et al, 1995; Wang et al, 1996).
II. Preclinical studies

Antiproliferative gene therapy strategies designed for the treatment of experimental cardiovascular disease include the following: 1) inactivation of positive cell cycle regulators (e. g., CDK/cyclins, protooncogenes, E2F, growth factors) by antisense approaches, ribozymes, and transcription factor ‘decoy’ strategies (Figure 4), 2) overexpression of negative regulators of cell growth (e. g., CKIs, p53, pRb, GAX, and GATA-6), and 3) overexpression of transdominant negative mutants of positive cell cycle regulators (e. g., Ras, mitogen-activated protein kinases).

Table 2: Gene therapy clinical trials for vascular proliferative disease based on cytostatic strategies.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Design</th>
<th>Strategy</th>
<th>Disease</th>
<th>Outcome</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREVENT I</td>
<td>Randomized, double-blinded, single center</td>
<td>E2F decoy ODN ex vivo transfection of vein graft</td>
<td>Autologous vein graft failure after peripheral artery bypass</td>
<td>70-74% decreases in the level of positive cell cycle regulators expressed by VSMCs in the vein, and reduction in primary graft failure</td>
<td>Mann et al, 1999</td>
</tr>
<tr>
<td>PREVENT II</td>
<td>Randomized multicenter, double-blinded, placebo-controlled</td>
<td>E2F decoy ODN ex vivo transfection of vein graft</td>
<td>Autologous vein graft failure after coronary artery bypass</td>
<td>Larger patency and inhibition neointimal thickening</td>
<td>Dzau et al, 2002</td>
</tr>
<tr>
<td>ITALICS</td>
<td>Randomized, placebo-controlled</td>
<td>c-myc antisense ODN delivery after stent implantation</td>
<td>In-stent coronary restenosis</td>
<td>No reduction in angiographic restenosis rate</td>
<td>Kutryk et al, 2002</td>
</tr>
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</table>

PREVENT: Project of ex-vivo vein graft engineering via transfection
ITALICS: Investigation by the thoraxcenter of antisense DNA using local delivery and IVUS after coronary stenting

Figure 4. Targeted gene inactivation by means of gene therapy strategies. Decoy approach by delivering a double-stranded ODN corresponding to the optimum DNA recognition sequence of the transcription factor of interest (TF) leads to attenuation of its interaction with the authentic cis-elements in cellular target genes, thus resulting in reduced gene transcription. Ribozymes inactivate the gene of interest by degrading their transcript. Antisense ODNs hybridize in a complementary fashion and stoicheometrically with the target mRNA, thus causing blockade of translation or synthesis of a truncated (inactive) protein.
A. Antisense approach

The gene of interest is inactivated by using a synthetic antisense oligodeoxynucleotide (ODN) that hybridizes in a complementary fashion and stoichiometrically with the target mRNA.

1. CDKs and cyclins

The efficacy of antisense ODN strategies targeting CDKs and cyclins to reduce neointimal lesion formation has been demonstrated in several animal models of balloon angioplasty. These studies include antisense oligodeoxynucleotides against CDK2 (Abe et al, 1994; Morishita et al, 1994a), CDC2 (Morishita et al, 1993; 1994b; Abe et al, 1994) and cyclin B1 (Morishita et al, 1994b). Interestingly, cotransfection of antisense ODN against CDC2 kinase and cyclin B1 resulted in further inhibition of neointima formation, as compared to blockade of either gene target alone (Morishita et al, 1994b). Of note, Morishita et al. (1993) reported sustained inhibition of neointima formation in the rat carotid balloon-injury model after a single intraluminal molecular delivery of combined CDC2 and proliferating cell nuclear antigen (PCNA) antisense ODNs, whereas this approach had no effect in the coronary arteries of pigs after balloon angioplasty (Robinson et al, 1997). Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibited VSMC proliferation and neointima formation after balloon angioplasty (Zhu et al, 1997). Attenuated graft atherosclerosis has been also observed upon inactivation of CDC2/PCNA (Mann et al, 1995; Miniati et al, 2000) and CDC2 (Suzuki et al, 1997) with antisense ODN.

2. Mitogen-responsive nuclear factors that promote cell growth

Several “immediate-early” genes (e.g., c-fos, c-jun, c-myc, c-myb, egr-1) are induced in serum-stimulated VSMCs, and their overexpression can promote VSMC proliferation in vitro (Castellot et al, 1985; Kindy and Sonenshein, 1986; Reilly et al, 1989; Brown et al, 1992; Campan et al, 1992; Rothman et al, 1994; Bennett et al, 1994b; Gorski and Walsh, 1995). VSMCs cultured from atheromatous plaques present higher levels of c-myc mRNA than in VSMCs from normal arteries (Parkes et al, 1991), and arterial injury induced the expression of several “immediate-early” gene (Lambert et al, 2001; Miano et al, 1990; 1993; Sylvester et al, 1998). Antisense ODNs against c-myc and c-myb reportedly inhibited in a sequence-specific manner both VSMC proliferation in vitro (Pukac et al, 1990; Brown et al, 1992; Ebbecke et al, 1992; Simons and Rosenberg, 1992; Biro et al, 1993; Shi et al, 1993; Bennett et al, 1994a; Shi et al, 1994a; Gunn et al, 1997), and neointima formation after angioplasty (Simons et al, 1992; Bennett et al, 1994a; Shi et al, 1994b; Gunn et al, 1997; Kipshidze et al, 2001, 2002) and vein grafting (Mannon et al, 1998) in vivo. However, these inhibitory effects may be mediated by a nonantisense mechanism (Burgess et al, 1995; Chavany et al, 1995; Guvakova et al, 1995; Villa et al, 1995; Wang et al, 1996).

It has been recently shown that nanospheres containing antisense ODN against PDGF receptor inhibit neointimal thickening in the rat carotid model of balloon angioplasty (Cohen-Sacks et al, 2002).

B. Ribozymes

Ribozymes represent a unique class of RNA molecules that catalytically cleave the specific target RNA, thus resulting in targeted gene inactivation. Su et al. (2000) designed a DNA-RNA chimeric hammerhead ribozyme targeted to human transforming growth factor-β1 (TGF-β1) that significantly inhibited angiotensin II-stimulated TGF-β1 mRNA and protein expression in human VSMCs, and efficiently inhibited the growth of these cells. Likewise, cleavage of the platelet-derived growth factor (PDGF) A-chain mRNA by hammerhead ribozyme attenuated human and rat VSMC growth in vitro (Hu et al, 2001a,b) and inhibited neointima formation in the rat carotid artery model of balloon injury (Kotani et al, 2003).

Studies using experimental models of angioplasty provided the first evidence that ribozymes might represent useful tools in cardiovascular therapy. Frimerman et al. (1999) reported the efficacy of chimeric hammerhead ribozyme to PCNA in reducing stent-induced stenosis in a porcine coronary model, and ribozyme strategy against TGF-β1 inhibited neointimal formation after balloon injury in the rat carotid artery model (Yamamoto et al, 2000). 12-Lipoxygenase products of arachidonate metabolism have growth and chemotactic effects in vascular smooth muscle cells, and ribozyme against this enzyme prevents intimal hyperplasia in balloon-injured rat carotid arteries (Gu et al, 2001).

C. Transcription factor ‘decoy’ strategies

This approach consists of delivering a double-stranded ODN corresponding to the optimum DNA target sequence of the transcription factor of interest, thus leading to the sequestration of the specific trans-acting factor and attenuation of its interaction with the authentic cis-elements in cellular target genes.

1. E2F

E2F participates in the transcriptional activation of genes encoding proteins that are required for nucleotide and DNA biosynthesis (e.g., DNA polymerase α, histone H2A, pcna, thymidine kinase) (Dyson, 1998; Lavia and Jansen-Durr, 1999) and in several growth and cell-cycle regulators (e.g., c-myc, pRb, cdc2, cyclin E, cyclin A).

Experimental neointimal thickening in balloon-injured arteries (Morishita et al, 1995; Nakamura et al, 2002), vein grafts (Mann et al, 1997; Ehsan et al, 2001), and cardiac allografts (Kawauchi et al, 2000) is prevented by the use of a synthetic ‘decoy’ ODN containing an E2F consensus binding site that inactivates the transcription factor E2F. Ahn et al. (2002a) developed a novel E2F
‘decoy’ ODN with a circular dumbbell structure (CD-E2F) and compared its properties with those of conventional phosphorothioated E2F ‘decoy’ ODN (PS-E2F). CD-E2F displayed more stability and stronger antiproliferative activity than PS-E2F when assayed in cultured VSMCs, and was more effective in inhibiting neointimal formation in vivo.

2. Activator protein-1 (AP-1)

Cell proliferation in the rat carotid artery model of angioplasty correlated with elevated expression and high DNA-binding activity of transcription factors of the AP-1 family (Miano et al, 1990; Miano et al, 1993; Hu et al, 1997; Sylvestre et al, 1998; Andrés et al, 2001). Under conditions of PDGF stimulation, AP-1 ‘decoy’ ODN delivery into cultured human VSMCs significantly reduced cell number and TGF-β1 production (Kume et al, 2002), and attenuated neointimal thickening when applied at the site of balloon angioplasty in rabbit carotid artery (Kume et al, 2002) and minipig coronary arteries (Buchwald et al, 2002). Circular dumbbell AP-1 ‘decoy’ ODN was more effective in inhibiting the proliferation of VSMCs in vitro and neointimal hyperplasia in vivo compared to conventional phosphorothioated AP-1 decoy ODN. (Ahn et al, 2002b).

D. Overexpression of growth suppressors

1. CKIs

The efficacy of CKIs in inhibiting CDK activity and cell cycle progression has been widely documented in a variety of normal and tumour cells in vitro. The first evidence that p21Cip1 and p27Kip1 may function as negative regulators of neointimal hyperplasia was suggested in animal studies showing the upregulation of these CKIs at late time points following balloon angioplasty, coinciding with the restoration of the quiescent phenotype after the initial proliferative wave (Chen et al, 1997; Tanner et al, 1998). The protective role of p27Kip1 against neointimal thickening has been rigorously demonstrated in several animal models of angioplasty (Chang et al, 1995a; Yang et al, 1996; Chen et al, 1997; Ueno et al, 1997a; Tanner et al, 2000; Condorelli et al, 2001). Overexpression of p21Cip1 also attenuated neointimal lesion formation in a rabbit model of vein grafting (Bai et al, 1998).

2. p53

p53 is a transcription factor that functions as a tumor suppressor displaying both antiproliferative and proapoptotic actions. These effects result from complex regulatory networks, including transcriptional activation of antiproliferative and proapoptotic genes (e. g., p21Cip1 and Bax, respectively), transcriptional repression of proproliferative and antiapoptotic genes (e. g., IGF-II and bcl-2, respectively), and direct protein-protein interactions (e. g., with helicases and caspases). Increased VSMC atherosclerotic plaques not undergoing proliferation. Concordant expression of TGF-β receptors I and II in virtually all cells positive for p27Kip1 within human atherogenicity (Castro et al, 2003).

Regarding human atherosclerosis, p53 is overexpressed in intimal and medial VSMCs towards basic fibroblast growth factor (bFGF or FGF2) (Olson et al, 2000). Intrinsinc differences in the regulation of p27Kip1 might also play an important role in creating variance in the proliferative and migratory capacity of VSMCs isolated from different vascular beds, which might in turn contribute to establishing regional variability in atherogenecity (Castro et al, 2003).

Tanner et al (1998) have reported more frequent expression of p27Kip1 and p21Cip1 within regions of human coronary atheromas not undergoing proliferation. Concordant expression of TGF-β receptors I and II in virtually all cells positive for p27Kip1 within human atherosclerotic plaques indicates that TGF-β1 present in these lesions may contribute to p27Kip1 upregulation (Ihling et al, 1999). Moreover, coexpression of p53 and p21Cip1 in human carotid atheroma plaque cells that revealed lack of proliferation markers suggests that induction of p21Cip1 may occur via transcriptional activation by p53 (Ihling et al, 1997).

Ectopic expression of p21Cip1 and p27Kip1, but not p16Ink4a, significantly reduced neointimal thickening in several animal models of angioplasty (Chang et al, 1995a; Yang et al, 1996; Chen et al, 1997; Ueno et al, 1997a; Tanner et al, 2000; Condorelli et al, 2001). Overexpression of p21Cip1 also attenuated neointimal lesion formation in a rat atherosclerosis model (Castro et al, 2003).
et al., 1995), and lack of proliferation markers in vascular cells coexpressing p53 and p21<sup>Cip1</sup> within advanced human atherosclerotic lesions suggests that transcriptional activation of the p21<sup>Cip1</sup> gene by p53 may be a protective mechanism against excessive vascular cell growth (Ihling et al., 1997).

p53 appears to play an important role in the pathogenesis of restenosis, as suggested by both animal and human studies. Transfection of antisense p53 ODN into rat intact carotid artery decreased p53 protein expression and resulted in a significant increase in neointimal lesion growth at 2 and 4 weeks after balloon-angioplasty (Matsushita et al., 2000). Evidence suggests that human cytomegalovirus (HCMV) infection contributes to the development of atherosclerosis and restenosis, and part of this effect may be due to increased VSMC proliferation and migration by inactivation of p53 (Speir et al., 1994; Zhou et al., 1996; 1999; Tanaka et al., 1999). It is also noteworthy that human VSMCs from restenosis or in-stent stenosis sites demonstrate normal or enhanced responses to p53 when compared to VSMCs from normal vessels (Scott et al., 2002). Moreover, p53 gene transfer effectively inhibited neointimal hyperplasia after experimental angioplasty (Yonemitsu et al., 1998; Scheinman et al., 1999; Matsushita et al., 2000), and in human saphenous vein (George et al., 2001).

3. pRb

The complex interplay between pRb and transcription factors of the E2F family plays a critical role in the control of cell growth (Stevaux and Dyson, 2002). E2F-dependent transactivation of genes required for cell cycle progression is prevented in quiescent cells due to the accumulation of hypophosphorylated pRb. Hyperphorylation of pRb by mitogenic stimuli leads to E2F activation and cell growth. Transfer of antisense pRb ODN into human VSMCs resulted in the induction of the proapoptotic factors bax and p53, and this was associated with increased number of apoptotic cells and a higher rate of DNA synthesis (Aoki et al., 1999). Inhibition of VSMC proliferation in vitro and attenuation of neointima formation after balloon angioplasty can be achieved by adenovirus-mediated transfer of several forms of pRb, including full-length constitutively active (nonphosphorylatable) and phosphorylation-competent pRb, and truncated versions of pRb (Chang et al., 1995b; Smith et al., 1997b). Similarly, adenoviral transfer of the pRb related protein RB2/p130 inhibited VSMC proliferation in vitro and prevented neointimal hyperplasia after experimental angioplasty (Claudio et al., 1999).

4. GATA-6

The GATA transcription factors play a critical role in the establishment of hematopoietic cell lineages and during the development of the cardiovascular system (Simon, 1995). GATA-6 is rapidly downregulated upon mitogen stimulation of quiescent VSMCs (Suzuki et al., 1996), and overexpression of GATA-6 induced p21<sup>Cip1</sup> expression and G1 cell cycle arrest (Perlman et al., 1998). Importantly, p21<sup>Cip1</sup>-null mouse embryonic fibroblasts were refractory to the GATA-6-induced growth inhibition (Perlman et al., 1998). The level of GATA-6 mRNA, protein, and DNA-binding activity is transiently downregulated at early time points after balloon angioplasty in the rat carotid artery, and reversal of GATA-6 downregulation by adenovirus-mediated GATA-6 gene transfer to the vessel wall inhibited intimal hyperplasia in this animal model (Mano et al., 1999).

5. GAX

Gax is a homeobox gene highly expressed in cultures of quiescent VSMCs, which is rapidly downregulated in vitro upon growth factor stimulation of VSMCs, and after balloon angioplasty in vivo (Gorski et al., 1993; Weir et al., 1995). Overexpression of GAX inhibited VSMC proliferation in vitro and attenuated neointimal thickening in balloon-injured rat carotid arteries in a p21<sup>Cip1</sup>-dependent manner (Smith et al., 1997a; Perlman et al., 1999). Percutaneous delivery of the Gax gene also inhibited vessel stenosis in a rabbit model of balloon angioplasty (Maillard et al., 1997).

E. Overexpression of transdominant negative mutants of positive cell cycle regulators.

1. Ras

Ras-dependent signaling plays an important role in mitogen-stimulated cell growth (Pronk and Bos, 1994). Ras is implicated in the activation of the G1 CDK/cyclin/E2F pathway (Winston et al., 1996; Aktas et al., 1997; Kerkhoff and Rapp, 1997; Leone et al., 1997; Lloyd et al., 1997; Peep et al., 1997; Zou et al., 1997) and is critical for the normal induction of cyclin A promoter activity and DNA synthesis in mitogen-stimulated VSMCs (Sylvester et al., 1998). Consistent with these findings, local delivery of transdominant negative mutants of Ras attenuated neointimal thickening after experimental balloon angioplasty (Indolfi et al., 1995; Ueno et al., 1997b).

2. Mitogen-activated protein kinases (MAPKs)

The MAPK pathway is critical in the transduction of proliferative signals in many mammalian tissues, including the cardiovascular system (Zou et al., 1998; Bogoyevitch, 2000). Several families of MAPKs have been described, including the stress-activated protein kinases/c-jun NH<sup>2</sup>-terminal protein kinases (SAPKs/JNKs), extracellular signal-regulated kinases (ERKs), and p38. JNKs and ERKs disclosed persistent hyperexpression and activation in atherosclerotic lesions of cholesterol-fed rabbits, suggesting that these factors play critical roles in initiating and perpetuating cell proliferation during the development of atherosclerosis (Hu et al., 2000; Metzler et al., 2000). Likewise, angioplasty in porcine and rat arteries led to the

III. Clinical studies

The antiproliferative approaches used so far for the treatment of cardiovascular disease have focused on restenosis and graft atherosclerosis, during which neointimal hyperplasia is rapid and localized. These disorders remain the major limitation of revascularization by percutaneous transluminal angioplasty (PTCA) and artery bypass surgery.

A. E2F ‘decoy’

Encouraging results of the E2F ‘decoy’ strategy in animal models of balloon angioplasty and graft atherosclerosis (see above) led to the initiation of the first Project of Ex-vivo Vein graft Engineering via Transfection (PREVENT I) (Mann et al, 1999). In this single-centre, randomized, controlled gene therapy trial, 41 patients undergoing bypass for the treatment of peripheral arterial occlusions were randomly assigned untreated (n=16), E2F-‘decoy’-ODN-treated (n=17), or scrambled-ODN-treated (n=8) human infragenual vein grafts. Ex vivo delivery of ODNs was achieved intraoperatively via pressure-mediated transfection. This procedure was associated with a 70-74% decrease in the level of PCNA and c-myc mRNA expressed by the VSMCs in the vein, and a statistically significant reduction in primary graft failure compared to control groups. Following to this pilot trial, a randomized, double-blinded, placebo controlled Phase IIb trial (PREVENT II) was carried out in patients undergoing coronary artery bypass surgery. The results of quantitative coronary angiography and intravascular ultrasound (IVUS) showed larger patency and inhibition of neointimal thickening in treated patients at 12 months after intervention (Dzau et al, 2002).

B. c-myc antisense ODN

Pharmacokinetics and clinical safety of ascending doses of c-myc antisense ODN (LR-3280) administered after PTCA was assessed by Roque et al. (2001a). Seventy eight patients were randomized to receive either standard care (n = 26) or standard care and escalating doses (1 to 24 mg) of LR-3280 (n = 52), administered into target vessel through a guiding catheter. The peak plasma concentrations of LR-3280 occurred at 1 minute and decreasing rapidly after approximately 1 hour, with little LR-3280 detected in the urine between 0-6 hours and 12-24 hours. The intracoronary administration of LR-3280 was well tolerated at doses up to 24 mg and produced no adverse effects in dilated coronary arteries, thus providing the basis for the evaluation of local delivery of c-myc antisense ODN for the prevention of human vasculoproliferative disease.

Kutryk et al. (2002) recently reported the results of the Investigation by the Thoraxcenter of Antisense DNA using Local delivery and IVUS after Coronary Stenting (ITALICS) trial. This randomized, placebo controlled study was designed to determine the efficacy of antisense ODN against c-myc in inhibiting in-stent restenosis. Eighty-five patients were randomly assigned to receive either c-myc antisense ODN or saline vehicle by intracoronary local delivery after coronary stent implantation. Follow-up included the percent neointimal volume obstruction measured by IVUS, clinical outcome and quantitative coronary angiography. There was no reduction in either the neointimal volume obstruction or the angiographic restenosis rate after treatment with 10 mg of phosphorothioate-modified ODN directed against c-myc as demonstrated by the analysis of 77 patients.

IV. Conclusions

Excessive cell proliferation within the arterial wall is thought to contribute to neointimal thickening during the pathogenesis of atherosclerosis, in-stent restenosis, and vessel bypass graft failure. Animal models of atherosclerosis have demonstrated an inverse correlation between neointimal cell proliferation and atheroma size, suggesting that excessive cell growth prevails at the onset of atherogenesis. Cell proliferation may also predominate at the early stages of human atheroma development. Thus, given that patients frequently exhibit advanced atherosclerotic plaques when first diagnosed, the potential benefit of antiproliferative strategies for the treatment of human atherosclerosis is uncertain. The antiproliferative approaches used so far in the setting of vascular obstructive disease have focused on restenosis and graft atherosclerosis, during which neointimal hyperplasia is spatially localized and develops over a short period of time (typically 2-12 months). Gene therapy is emerging as an attractive strategy in the treatment of vascular proliferative disease due to minimally invasive and easily monitored gene delivery in vascular interventions. Antiproliferative gene therapy strategies that have proven efficient in inhibiting neointimal thickening in animal models of vascular obstructive disease include the use of antisense- and ribozyme-mediated inactivation of positive cell cycle regulators, overexpression of negative regulators of cell growth, and ‘decoy’ strategies to inactivate transcription factors that promote cell cycle progression. Although some of these strategies have shown encouraging results in humans, further studies are required to override the current practical barriers and limitations placed on most clinical trials before gene therapy strategies exhibit wide application in clinic. These should include the clarification of safety issues, development of better gene delivery vectors, and improvement of transgene expression. Aside from these technical improvements, significant effort in basic research is warranted to identify more effective and safer treatment genes.
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References


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by antisense cdk2 kinase oligonucleotides. *J Clin Invest* 93, 1458-1464.


Olson, NE, Kozlowski, J, and Reidy, MA (2000). Proliferation of intimal smooth muscle cells. Attenuation of basic fibroblast growth factor 2-stimulated proliferation is associated with intimal smooth muscle cell proliferation in vivo. *Proc Natl Acad Sci USA* 92, 5855-5859.


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muscle cells by c-myc antisense oligomers. *Circulation* 90, 1-147.


