Antithrombotic Strategies in Gene Therapy

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Current Cardiology Reports 2000, **2**:34–38
Current Science Inc. ISSN 1523–3782

Advances in the field of molecular medicine are making gene therapy a viable treatment strategy for the next millennium. Indeed, over the past 10 years, a number of improvements have occurred that have resulted in an increased interest in gene therapy for the treatment of diseases in cardiovascular medicine. Because antithrombotic and anticoagulation therapy generally involves the systemic administration of agents that target a small region of the vasculature, localized and controlled delivery of specific genes could offer enormous potential to treat a number of life-threatening diseases. In addition, gene therapy may allow sustained antithrombotic or anticoagulant treatment when prolonged systemic administration is undesirable. Gene therapy for antithrombotic strategies can involve a number of different approaches. This could include inhibition of coagulation factors, over-expression of anticoagulant factors, o modulation of endothelial biology to make thrombus formation or propagation unfavorable. Preclinical data regarding these different strategies are reviewed and their potential limitations discussed.

Introduction

Overview of gene therapy in cardiovascular medicine

Cardiovascular disease is the leading cause of morbidity and mortality in the Western world. Gene therapy involves the introduction of genetic material into the cells of patients in order to correct an inherited or acquired disorder. Gene therapy works via the synthesis of missing or deficient protein products, or by directing expression of a recombinant protein to achieve its biological effect. The first successful human gene transfer occurred in 1989 in patients with cancer [1]. The therapeutic efficacy of gene therapy was shown in patients with adenosine deaminases deficiency [2]. Over the next decade, a number of improvements have occurred that have brought gene therapy closer to a clinical reality. This has led to a marked increase in the interest in gene therapy in cardiovascular medicine. This review outlines approaches that ultimately will permit us to utilize gene therapy for antithrombotic therapies.

Rationale for local gene therapy

Antithrombotic and anticoagulation therapies are employed millions of times each year for a vast array of syndromes. All antithrombotic and anticoagulation therapies involve the systemic administration of therapeutic agents, which in reality, are targeting a small region of the vasculature. In a number of life-threatening situations, such as an acute myocardial infarction or pulmonary embolus, the risks associated with systemic administration are clearly outweighed by the potential benefits [3]. Moreover, gene therapy for these emergency conditions is necessarily limited by the need for immediate, rather than delayed, production of an anticoagulant state. Despite this, localized gene therapy offers the potential to reduce the bleeding risks associated with systemic administration and this point is underscored by the fact that each year, a large number of patients are excluded from antithrombotic and anticoagulation therapy because of the reality, or perception, that there is an unacceptably high risk associated with systemic therapies.

There are numerous conditions in which a localized, sustained antithrombotic therapy might be beneficial, and where the time necessary to allow recombinant gene expression would not be a drawback. These potentially include recurrent venous thromboembolism, atrial fibrillation, prosthetic cardiac valves, vascular shunts and grafts, coronary interventions, and even acute coronary syndromes without infarction. Finally, even if the one-time systemic administration of an agent is deemed to be safe, there are a number of clinical situations where prolonged antithrombotic and anticoagulant therapy would be needed to prevent adverse long-term problems. An example would be in arterial bypass grafts where the increased risk of thrombotic occlusion lasts up to a year and repetitive thrombosis may predispose to long-term graft failure [4,5]. Clearly, the systemic administration of thrombolytic therapy for prolonged periods of time, targeting recurrence of subacute episodes is both unsafe and impracticable. It is in these situations that the transient

expression of gene products that can affect the coagulation or fibrinolytic system offers enormous clinical advantages.

Direct versus indirect antithrombotic strategies

Gene therapy for antithrombotic strategies can broadly be divided into approaches, corresponding to two components of Virchow's triad: modifying the blood, or modifying the vessel wall. First, therapeutic strategies can be used to modulate blood coagulability, by direct inhibition of proteins that initiate or accelerate the intrinsic and extrinsic coagulation cascade. Targets for these would include the over-expression of thrombin inhibitors (*ie*, hirudin) and soluble receptors for coagulation proteins (*ie*, soluble fibrinogen receptor). Second, the therapeutic approaches can increase the expression of the endogenous proteins that inhibit coagulation (*ie*, recombinant tissue factor pathway inhibitor). Finally, therapeutic strategies could be used to modify the biology of the vessel wall, in particular the endothelium, in order to make conditions for intravascular thrombosis unfavorable [6]. Examples might include increased expression of nitric oxide or prostacyclin, or expression of recombinant plasminogen activators. Alternatively, a more fundamental approach would aim to maintain endothelial integrity or increase endothelial repair by growth factor gene delivery.

Direct Modulation of Coagulation Factors

Inhibitors of intravascular coagulation

Hirudin is one of the most potent and specific inhibitors of thrombin. Rade *et al*. [7], used an adenovirus containing cDNA for the thrombin inhibitor hirudin in a model of arterial injury. The authors demonstrated that they were able to over-express the thrombin inhibitor and the over-expression was associated with a marked reduction in intimal hyperplasia. This reduction of intimal hyperplasia was achieved without any systemic change in the activated prothrombin time (APTT). This study provides clear "proof-in-concept" that therapeutic local delivery can be achieved in arteries without adverse systemic effects.

Gene therapy can be used as a prophylactic measure to inhibit intravascular thrombosis. In a rabbit carotid artery balloon injury model, cyclic flow variations are directly related to intravascular thrombus formation [8]. Using an adenovirus expressing the human cycloxygenase-1 gene, Zoldhelyi *et al*. [9] were able to effectively block cyclic flow variations. The mechanism appeared to be the alteration of prostacyclin levels, the inhibition of platelet aggregation and the maintenance of vascular tone through smooth muscle cell relaxation.

Thrombomodulin is a naturally occurring inhibitor of intravascular thrombosis [10]. In vitro the over-expression of thrombomodulin in endothelial cells via gene transfer enhances the endogenous activity of protein C and leads to the inhibition of thrombus formation on intact endothelial surfaces [11]. In this situation, a more favorable homeostatic balance in the blood vessel can be achieved.

The over-expression of thrombolytic proteins is an attractive and potentially useful approach to limit intravascular thrombosis [12]. This straightforward technique, however, may be associated with unexpected results. Dunn *et al*. [13] attempted to seed vascular grafts with endothelial cells that were modified with tissue plasminogen activator (TPA), with the intention that over-expression of TPA could serve to limit the episodes of recurrent intravascular thrombosis in small arterial bypass grafts. Somewhat unexpectedly, the seeding of these grafts with these modified endothelial cells markedly reduced the adherence of the endothelial cells to the graft surface. In this clinical situation, the homogenous seeding the graft is critical to maintain vascular integrity. Similarly, Ekhterae *et al*. [14] utilized gene transfer to increase TPA expression in smooth muscle cells. Although the authors again were able to demonstrate enhanced TPA expression, overall coagulation was unchanged and this lack of therapeutic efficacy was in part due to the resultant increase in the inhibitor of TPA. Therefore, approaches to over-express thrombolytic proteins to date have been met with only minimal success. The potential to locally over-express thrombolytic agents without systemic toxicity remains a goal of gene therapy [15].

Modulation of the Vessel Wall

Gene therapy strategies aimed at modifying the biologic properties of the vessel wall, while not intervening directly in the intrinsic or extrinsic coagulation cascade, may be equally important in modifying a prothrombotic tendency. These strategies may be particularly important when thrombosis is related to local abnormalities in the vessel wall, for example, in vascular bypass grafts and fistulas, or after local vascular injury due to a surgical or percutaneous intervention.

Nitric oxide synthase gene therap

Nitric oxide (NO), produced in the normal endothelium by endothelial nitric oxide synthase (eNOS; NOS 3) has pleiotropic actions in maintaining normal vascular homeostasis, including regulation of vascular tone, inhibition of endothelial adhesion molecule expression and inhibition of medial smooth muscle migration and proliferation. In particular, NO is a potent inhibitor of platelet aggregation and activation [16]. Loss of normal NO bioactivity is a characteristic feature of vascular disease states and pre-atherosclerotic conditions such as smoking, hypertension, hypercholesterolemia and diabetes, and seems to accelerate the tendency to thrombosis and progression to a more advanced disease.

Nitric oxide synthases have proven to be attractive and effective targets for experimental vascular gene therapy. In several in vitro and in vivo model systems, vascular gene transfer of NOS directs synthesis of active

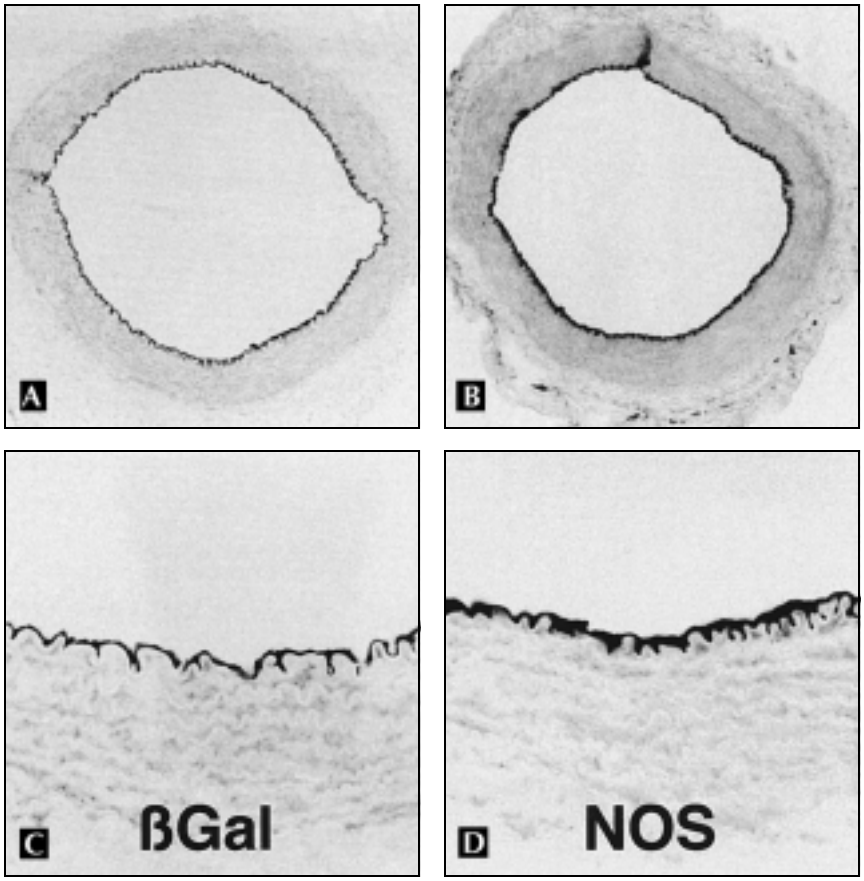

Figure 1. Increasing endothelial NOS activity by NOS gene transfer. Rabbit carotid arteries were infected with a replication deficient adenovirus encoding the neuronal isofor of nitric oxide synthase (nNOS), using luminal dwell of virus solution for 20 minutes [20]. Control arteries were exposed to an adenovirus encoding the marker gene *LacZ* (ßGal). Arteries were harvested 3 days after gene transfer and were stained for NADPH-diaphorase activity, a histochemical stain which produces a blue-purple coloration specific for nitric oxide synthase activity [21••]. Following nNOS gene transfer, endothelial NADPH-diaphorase staining was increased approximately three fold, in association with increased NO production and augmented NO-dependent vascular relaxation [20]. (Original magnifications: Upper panels x10, lower panels x100) NADPH—nicotinamide adenine dinucleotide phosphate, reduced form; NOS—nitric oxide synthase.

recombinant NOS enzyme, resulting in augmented NO production (Fig. 1). Importantly, increased NO production has functional effects on vessel biology, including restored or enhanced endothelial-dependent vasomotor function, and reduced vascular inflammation and endothelial adhesion molecule expression in atherosclerosis (Table 1). However, studies specifically examining the effects of NOS gene therapy on platelet deposition and thrombosis in these models have been only preliminary. Nevertheless, induction of endogenous inducible NOS in the vessel wall reduces local platelet adhesion after balloon injury [17] and NOS gene transfer increases cyclic guanosine monophosphate (cGMP) formation in neighboring smooth muscle cells [18–20], so by analogy, gene transfer of NOS isoforms would be expected to exert a local antiplatelet effect by increasing platelet cGMP levels. Further studies are required to examine the direct antithrombotic effect of NOS gene transfer in in vivo models (Table 1).

Maintenance of endothelial integrity and repair

Gene therapy strategies aimed at maintaining or restoring endothelial integrity are a potentially powerful approach to antithrombotic therapy in the setting of vascular injury. Not only does physical loss of endothelium expose blood constituents to a thrombogenic surface, but lost or dysfunctional endothelium deprives the vessel wall of the cells that normally elaborate potent antithrombotic and thrombolytic factors, as described above.

Vascular endothelial growth factor (VEGF), an endothelial-cell specific mitogen, has received considerable attention as a candidate for angiogenic gene therapy [28–29]. Topical VEGF protein accelerates endothelial regrowth after balloon denudation [30], so local VEGF gene therapy may be an indirect antithrombotic approach in injured vessels after surgery or percutaneous intervention [31••]. In addition to its direct effect on endothelial regrowth, VEGF may have important roles in maintaining normal endothelial cell function in the intact endothelium [32] and in inhibiting endothelial cell loss by apoptosis under adverse conditions [33].

Limitations of Gene Therapy

The gene therapy strategies discussed here are subject to several limitations and problems that continue to shadow their potential clinical utility. Principally, these limitation relate to the vector used to transfer and express the therapeutic gene. Adenovirus has received much attention as a vector for vascular gene therapy, due to its ease of preparation and use, and the high efficiency of gene transfer attained with high titer infection. However, within hours, high titers of adenovirus induce endothelial adhesion molecule expression, inflammation and impaired NO-medi-

Table 1 Studies of in vivo experimental gene therapy of nitric oxide synthase

Vessel	Species	NOS isoforms	Vector	End points	Study
Basilar artery	Canine	eNOS	Ad	Recombinant NOS, increased cGMP, increased vasomotor relaxation	Chen *et al.* [20]
Carotid artery, (±cholesterol)	Rabbit	nNOS, eNOS	Ad	Recombinant NOS, increased NO, increased vasomotor relaxation, reduced endothelial activation and inflammation	Channon *et al.* [21••], Qian *et al.* [22], Kullo *et al.* [23], and Kullo *et al.* [24]
Carotid artery, (+balloon injury)	Rat	eNOS	HVJ	Recombinant NOS, increased NO, increased vasomotor relaxation, reduced intimal hyperplasia	Von der Leyden *et al.* [25]
		eNOS	Ad		Janssens *et al.* [26]
Coronary artery (+balloon injury)	Pig	eNOS	Ad	Increased cGMP, reduced intimal hyperplasia	Varenne *et al.* [18]
Pulmonary artery	Rat	eNOS	Ad	Increased NO, increased cGMP, reduced hypoxic constriction	Janssens *et al.* [19]
Vein grafts	Canine	eNOS	HVJ	Recombinant NOS, reduced intimal hyperplasia	Matsumoto *et al.* [27]

Ad—adenovirus; cGMP—cyclic guanosine monophosphate; eNOS—endothelial nitirc oxide synthase; HVJ—hemagglutinating virus of Japan; NO—nitric oxide; NOS—nitric oxide synthase.

ated vasomotor function in infected arteries [34], likely to increase any prothrombotic tendency. These limitations can be substantially overcome by careful use of intermediate titers of adenovirus, avoiding inflammation while obtaining adequate transgene expression. However, the immunogenicity of first generation adenoviruses, relating to low-level viral gene expression by infected cells, also causes chronic vascular inflammation after 7 to 10 days [35] and complete loss of recombinant gene expression after approximately 14 days. A short duration of therap may not be a particular limitation for some antithrombotic gene therapy strategies, for example aimed at local vascular injury or endothelial regrowth. Chronic inflammation is a limitation to antithrombotic strategies, as endothelial adhesion molecule expression and tissue factorexpression would undoubtedly increase the local tendency to thrombosis. Newer adenoviral vector designs, incorporating more extensive deletions of the viral genome, or even total deletions, are now providing some potential solutions to these problems, resulting in reduced inflammation and prolonged expression. Future therapies may be based around combinations of liposomal, artificially engineered viral particles, or extensively modified recombinant viruses

Conclusions

Gene therapy offers the potential to safely and efficiently treat localized states of hypercoagulability in the human vasculature and thereby influence a number of potentially lifethreatening situations. Approaches with target antithrombotic strategies can include the transient expression of anti-coagulant proteins, the over-expression thrombolytic or fibrinolytic proteins, or can be used to modulate the biology of the vessel wall to make coagulation unfavorable. For antithrombotic gene therapy to become a clinical reality, considerable progress in preclinical evaluations is still required, to develop improved vectors, and to establish a more thorough understanding of the duration, magnitude, regulation and therapeutic benefit of recombinant transgene expression.

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