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Blockade of TGF-β by catheter-based local intravascular gene delivery does not alter the in-stent neointimal response, but enhances inflammation in pig coronary arteries

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ABSTRACT

Background: Extracellular matrix (ECM) accumulation significantly contributes to in-stent restenosis. In this regard, transforming growth factor (TGF)-β, a positive regulator of ECM deposition, may be implicated in in-stent restenosis. The goal of this study was to assess the effect of blockade of TGF-β on stent-induced restenosis in porcine coronary arteries.

Methods: An adenovirus expressing the ectodomain of the TGF-β type II receptor (AdTβ-ExR) was applied onto a coronary arterial segment of a pig ($n=10$) using an Infiltrator™, followed by stent deployment. Controls consisted of adenoviruses expressing β-galactosidase (AdLacZ) or phosphate-buffered saline (PBS) applied onto the other segment ($n=10$) of the same pig.

Results: Computer-based pathological morphometric analysis of stented coronary arteries, performed 4 weeks after stenting, demonstrated no significant difference in morphometric parameters such as in-stent neointimal area and % area stenosis between the AdTβ-ExR group and control ($n=7$ for each). However the AdTβ-ExR group had increased neointimal cell density, infiltration of inflammatory cells mostly consisting of $CD3^+$ T cell, accumulation of hyaluronan, cell proliferation rate, and adventitial matrix metalloproteinase-1 (MMP-1) expression compared with control. The expression of connective tissue growth factor mRNA, measured by reverse transcription PCR, in cultured rat arterial smooth muscle cells was inhibited by AdTβ-ExR at moi 60.

Conclusions: Blockade of TGF-β by catheter-based local intravascular gene delivery does not reduce stent-induced neointima formation 4 weeks after stenting in spite of modest inhibition of ECM accumulation, but it induces vascular inflammation and associated pathological changes that may potentially aggravate lesion progression.

1. Introduction

Restenosis after stenting has been referred to as the Achilles' heel of percutaneous coronary intervention (PCI). The rates of restenosis for drug-eluting and bare-metal stents are about 9% and 29% respectively at 6 months after PCI [1]. Neointimal ingrowth, rather than tissue remodeling or stent recoil, is thought to play a key role in restenosis after stenting [2]. Although the exact mechanism for restenosis after stenting is not clear, our previous study suggested that enhanced extracellular matrix (ECM) accumulation may play a crucial role in the development of in-stent neointima in human coronary arteries [3]. In this regard, TGF-β1, owing to its role as a potent up-

Abbreviations: AdLacZ, adenoviruses expressing β-galactosidase; AdTβ-ExR, adenovirus expressing the ectodomain of the TGF-β type II receptor; CTGF, connective tissue growth factor; ECM, extracellular matrix; EELA, the external elastic lamina area; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IELA, internal elastic lamina area; MMP-1, matrix metalloproteinase-1; moi, multiplicity of infection; PBS, phosphate-buffered saline; PCI, percutaneous coronary intervention; SMC, smooth muscle cell; TβRII, TGF-β1 receptor type II; TGF-β, transforming growth factor-β.

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regulator of ECM accumulation such as proteoglycans, hyaluronan, fibronectin, and collagen, may play a significant role in the development of in-stent neointima [4–7]. In addition, TGF-β may contribute to ECM accumulation by down-regulating matrix metalloproteinases (MMPs) and upregulating protease inhibitors [8,9]. TGF-β also exerts other biological effects such as growth inhibition, cell migration and differentiation, and immunomodulation [8]. Expression of TGF-β is significantly higher in human restenotic lesions after stenting [3] or balloon angioplasty [10] compared with primary lesions. Direct evidence showing that TGF-β1 is involved in the development of arterial lesion has been reported [5–7]. Overexpression of TGF-β1 promotes the formation of a neointima enriched with ECM [5,6], and withdrawal of TGF-β1 contributes to neointimal regression with increased apoptosis [6]. Treatment of balloon-injured arteries with neutralizing anti-TGF-β1 antibodies reduces intimal hyperplasia [7]. In contrast to these proatherosclerotic effects of TGF-β, other studies suggested the protective role of TGF-β in atherosclerosis by regulation of inflammation, MMPs, and cell proliferation [9,11–18].

TGF-β1 initiates cell signaling by binding to the ectodomain of the TGF-β1 receptor type II (TβRII) first, then recruiting and dimerizing with TGF-β receptor type I (TβRI) [4]. By forming heterotrimeric complex (TGF-β1, TβRII and TβRI), TGF-β1 exerts its biological effects via the Smad-dependent and -independent signaling pathways [4]. An adenoviral vector expressing the ectodomain of TβRII (AdTβ-ExR) acts as a dominant negative mutant of TβRII by adsorbing TGF-β, thus preventing an interaction of the endogenous functional TβRII with TβRI [19].

To know if blockade of TGF-β inhibits in-stent neointima formation, we blockaded TGF-β using a catheter-based local delivery of the AdTβ-ExR in a porcine coronary artery stent model which bears a marked resemblance to humans [20]. In the present study, blockade of TGF-β reduced ECM formation to some extent, however it did not reduce in-stent neointima formation. Furthermore, blockade of TGF-β enhanced $CD3^+$ T cell infiltration, MMP-1 expression, deposition of a hyaluronan-rich ECM, and cell proliferation, suggesting that blockade of TGF-β enhances inflammation in stented arteries. The present study's strength, unlike from most prior studies, is that stent was used to induce neointima in porcine coronary arteries, thus achieving a situation that more closely resembles the clinical situation.

2. Materials and methods

2.1. Materials

Antibodies against soluble human TβRII IgG (a fluorescein isothiocyanate (FITC)-conjugated, rabbit polyclonal), human CD3 (rabbit polyclonal), and human proliferating cell nuclear antigen (PCNA, mouse monoclonal) were purchased from Dako (Carpinteria, CA). Mouse monoclonal anti-human MMP-1 was purchased from Oncogene (Cambridge, MA).

2.2. Recombinant adenovirus vector

Replication-defective E1- and E3-recombinant adenovirus expressing either an entire ectodomain of the TβRII fused to the human immunoglobulin Fc portion (AdTβ-ExR) or bacterial β-galactosidase (AdLacZ) under a CA promoter (composed of cytomegalovirus enhancer and chicken β-actin promoter) was constructed as previously described [19]. Adenoviruses were propagated and tittered in HEK 293A cells, and were prepared by ultracentrifugation in cesium chloride gradient to yield concentrations on the order of 10^9–10^{10} plaque forming unit (pfu).

2.3. Cell culture and infection

There are connective tissue growth factor (CTGF)-dependent and -independent signaling pathways activated by TGF-β, and CTGF functions as a downstream mediator of TGF-β action on connective tissue cells where it stimulates ECM synthesis [21]. We examined whether the expression of CTGF mRNA of cultured rat arterial smooth muscle cells (SMCs) can be affected by AdTβ-ExR. A small piece of aorta from male Sprague–Dawley rat (200–250 g) was incubated in 1 mg/ml collagenase for 3 h at room temperature, and fixed to a culture flask for explantation in minimal essential medium containing 10% fetal calf serum, 1% nonessential amino acids, 100 mU/ml penicillin, and 100 μg/ml streptomycin. SMCs of passage 5–6 were infected for 48 h with either AdTβ-ExR or AdLAcZ at a multiplicity of infection (moi) of either 6 or 60.

2.4. Gene delivery and stent deployment

The animal experiment conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Female pigs (2–3 mo, 25–30 kg, $n=13$) were used in this study. To identify the optimal titer of adenoviral vector for effective gene transfer, different titers of AdTβ-ExR (1×10^8–1×10^9 pfu) and AdLacZ (2.5×10^7–2.5×10^8 pfu) were randomly injected into each coronary arterial segments of pigs ($n=3$) using an Infiltrator™ (Interventional Technologies, San Diego, CA). Arteries, dissected 1 wk after gene delivery, were snap-frozen in liquid nitrogen and kept −70 °C until assay.

Ten pigs underwent intravascular gene delivery (5 pairs of AdTβ-ExR/PBS and 5 pairs of AdTβ-ExR/AdLacZ) with subsequent stent deployment. Animals continued to take 100 mg/d Aspirin and 75 mg/d Clopidogrel from the day before the procedure until sacrifice. Pigs were subjected to intramuscular injection of Atropine (0.04 mg/kg), Xylazine (2 mg/kg), and Ketamine (10 mg/kg), and anesthesia was induced by inhalation of 2.5% Enflurane. An 8 F Judkins coronary artery guide catheter was inserted through the left carotid artery. Two coronary arterial segments per each pig feasible for intravascular delivery using a 3.0–3.5 mm Infiltrator™ were selected. After intravenous injection of heparin 6000 IU, one arterial segment was injected with AdTβ-ExR (1×10^9 pfu in 400 μl, $n=10$), and the other with either phosphate-buffered saline (PBS, $n=5$) or AdLacZ (1×10^9 pfu, $n=5$). Then a Palmaz–Schatz stent was deployed in each injected arterial segment (9–11 atm, balloon/artery ≈ 1.3), and the left carotid artery was ligated after procedure. Three pigs (3 pairs of AdTβ-ExR/AdLacZ) died immediately after gene transfection. For the morphometric analysis, seven pigs were sacrificed with a lethal dose of sodium pentobarbital 28 d after stenting. The stented arterial specimens were pressure-fixed *in situ* with 4% formaldehyde, excised, and divided into two segments by cutting the bridge portion of the stent. One bisected arterial segment with a higher degree of stenosis shown in angiography underwent tissue processing with Kulzer Histotechnik 8100 (Heraeus Kulzer, Germany) and was sectioned with Jung RM 2065 (Leica, Germany) for morphometric analysis. The other bisected segment was embedded in paraffin after careful manual removal of stent filament for other pathological analyses.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

To study the effect of the AdTβ-ExR on the expression of CTGF mRNA, total RNA was isolated from the adenovirus-infected cultured rat arterial SMCs using Trizol (Gibco, Grand Island, NY). Total cDNA, synthesized by reverse transcription from 2 μg of total RNA, was amplified by PCR for 35 cycles at 94 °C for 30 s, 54 °C for 1 min, and 72 °C for 1 min. The PCR primer sets for CTGF (5′-CGC CTG TTC TAA GAC CTG T-3′ and 5′-GAA AGC TCA AAC TTG ACA GG-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-TCA TTG ACC TCA ACT ACA TGG T-3′ and 5′-CTA AGC AGT TGG TGG TGC AG-3′) were used for amplification of 420 bp and 370 bp fragments, respectively. PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and analyzed using an image analyzer (Bioprofil, Viber Lourmat, France).

2.6. Pathological analysis

The cross-sectional areas of the bisected stented coronary arterial segment were measured with computerized digital morphometry software (Optimas 6.5). The areas bound by the luminal surface, by the internal elastic lamina (IELA), by the external elastic lamina (EELA), and by stents, were measured using hematoxylin and eosin-stained stented arterial sections and averaged at the minimal luminal area from each vessel along with mean injury score [22]. Neointima area (IELA − lumen area), media area (EELA − IELA), and % area stenosis ([neointima area / IELA] × 100) were calculated. Modified Movat pentachrome stain was used to identify ECM components. Collagen was identified in the picrosirius red-stained sections with polarized light on Olympus

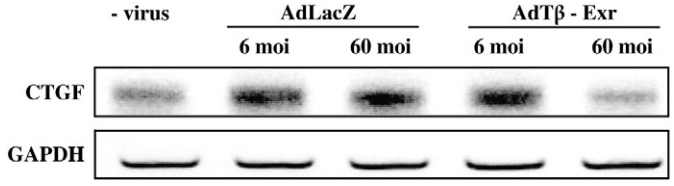

Fig. 1. The effect of the AdTβ-ExR on expression of connective tissue growth factor (CTGF) mRNA in cultured rat arterial smooth muscle cells (SMCs). Rat arterial SMCs were transfected with either AdTβ-ExR or AdCALacZ at two different concentrations (6 and 60 moi). After incubation for 48 h, total RNA was extracted and subjected to RT-PCR as described in method. Signals of 420 bp for CTGF and 370 bp for GAPDH are shown. AdLacZ: an adenovirus expressing β-galactosidase; AdTβ-ExR: an adenovirus expressing the ectodomain of the type II TGF-β receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; moi: multiplicity of infection.