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Blockade of TGF- 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 (AdT β -ExR) was applied onto a coronary arterial segment of a pig (n=10) using an InfiltratorTM, 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.

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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 AdT β -ExR group and control (n=7 for each). However the AdT β -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 AdT β -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.

Keywords

hyaluronan; inflammation; restenosis; stents; transforming growth factor- β

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- β 1, owing to its role as a potent up-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- β 1 is involved in the development of arterial lesion has been reported [5-7]. Over-expression of TGF- β 1 promotes the formation of a neointima enriched with ECM [5,6], and withdrawal of TGF- β 1 contributes to neointimal regression with increased apoptosis [6]. Treatment of balloon-injured arteries with neutralizing anti-TGF- β 1 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- β 1 initiates cell signaling by binding to the ectodomain of the TGF- β 1 receptor type II (T β RII) first, then recruiting and dimerizing with TGF- β receptor type I (T β RI) [4]. By forming heterotrimeric complex (TGF- β 1, T β RII and T β RI), TGF- β 1 exerts its biological effects via the Smad-dependent and -independent signaling pathways [4]. An adenoviral

vector expressing the ectodomain of T β RII (AdT β -ExR) acts as a dominant negative mutant of T β RII by adsorbing TGF- β , thus preventing an interaction of the endogenous functional T β RII with T β RI [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 AdT β -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 T β RII 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 T β RII fused to the human immunoglobulin Fc portion (AdT β -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 titered in HEK 293A cells, and were prepared by ultracentrifugation in cesium chloride gradient to yield concentrations on the order of 10⁹-10¹⁰ 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 AdT β -ExR. A small piece of aorta from male Sprague-Dawley rat (200-250g) 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 μ g/ml streptomycin. SMCs of passage 5-6 were infected for 48 h with either AdT β -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 with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Female pigs (2-3 mo, 25-30kg, n=13) were used in this study. To identify the optimal titer of adenoviral vector for effective gene transfer, different titers of AdT β -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 AdT β -ExR / PBS and 5 pairs of AdT β -ExR / AdLacZ) with subsequent stent deployment. Animals continued to take 100mg/d Aspirin and 75 mg/d Clopidogrel from the day before the procedure until sacrifice. Pigs were subjected to intramuscular injection of Atropine (0.04mg/Kg), Xylazine (2mg/Kg), and Ketamine (10mg/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 6,000 IU, one arterial segment was injected with AdT β -ExR (1×10^9 pfu in 400 μl , n=7), 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 $\approx$ 1.3), and the left carotid artery was ligated after procedure. Three pigs (3 pairs of AdT β -ExR / AdLacZ) died immediately after gene transfection. For the morphometric analysis, seven pigs were sacrificed with a lethal dose of sodium pentobarbital 28d 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 AdT β -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[.proportional]g 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 420bp and 370bp 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 ($[(\text{neointima area}/\text{IELA}) \times 100]$) 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 BX51 light microscope. The proportional area (%), occupied by collagen, hyaluronan, CD3 positive T cells, or MMP1, and cell density (cells/mm²) in arterial layers were measured by analyzing entire cross-section ($\times 100$).

2.7. Immunohistochemical staining

Immunohistochemical staining was done on serial paraffin-embedded sections as previously described [3]. Hyaluronan labeling was done with biotinylated hyaluronan binding protein. IgG fused to soluble human T β RII was identified by direct immunofluorescent staining with FITC-conjugated rabbit anti-human IgG. Briefly, frozen sections were incubated with FITC-conjugated rabbit anti-human IgG at room temperature for 1 h, rinsed with PBS, and assessed by using immunofluorescent microscope. Tissue sections were subsequently counterstained with hematoxylin.

2.8. Statistical Analysis

Values are presented as mean $\pm$ SEM, and all statistics were calculated by use of SPSS 14.0 for Windows. Comparisons of the continuous variables between two groups were made by Wilcoxon signed ranks test. Significance was assigned as $p < 0.05$.

3. Results

3.1. AdT β -ExR decreases CTGF mRNA expression in SMCs

Expression of transgene in the arteries was verified and described previously [23]. Briefly, immunofluorescent staining for soluble T β RII identified multiple T β RII-antibody complexes dispersed in an artery injected with 5×10^8 pfu AdT β -ExR, whereas few, if any, immunofluorescent particles were shown in remote arteries (Fig. not shown). RT-PCR product of T β RII mRNA from the arterial segments injected with 1×10^9 pfu AdT β -ExR was also verified (Fig. not shown). The expression of CTGF mRNA in cultured rat arterial SMCs was slightly increased by AdLacZ (moi 6 and 60) and AdT β -ExR at moi 6, but it was inhibited by the AdT β -ExR at moi 60 (Fig. 1). This result, confirmed by repetitive experiment, indicates that the AdT β -ExR can down-regulate expression of CTGF mRNA in SMCs, thereby possibly inhibiting ECM synthesis.

3.2. The effect of blockade of TGF- β on morphometric parameters

Coronary arterial specimens from AdT β -ExR / PBS (5 pairs) and AdT β -ExR / AdLacZ (2 pairs) injected segments in 7 pigs were analyzed for the morphometric parameters. The two

control groups (AdLacZ group and PBS group) were assessed by pooling together as a control group or separately. Expectedly, both control group (PBS and AdLacZ) and the AdT β -ExR group had in-stent neointima (Fig. 2A,B), whereas proximal reference artery segment had only thin endothelial layer (Fig. not shown). Comparisons of morphometric parameters between the AdT β -ExR group and control group are shown as in Table 1. Cell density of in stent neointima was significantly increased in the AdT β -ExR group than control ($p<0.05$), suggesting that AdT β -ExR induced less ECM accumulation per cell than control. However there were no significant differences in other morphometric parameters such as intima area and % area stenosis between the AdT β -ExR group and control. When two control groups, ie the AdLacZ control and PBS control, were analyzed separately, no significant difference in intima area or % area stenosis was also observed between the AdT β -ExR group and each control (Fig. not shown).

3.3. Blockade of TGF- β increases CD3⁺T cell accumulation

Notably, infiltration of a variety of inflammatory cells was enhanced in the AdT β -ExR group (Fig. 2B,C) compared with control (Fig. 2A). Inflammatory cells were present throughout the tissue, but accumulated more around stent struts and adventitia. Immunohistochemical staining identified CD3⁺ T cells as the main inflammatory cell component (Fig. 2D-F), while the rest were macrophages, eosinophils, and multinucleated giant cells (Fig. 2C). The area of CD3⁺ T cells was significantly greater in the AdT β -ExR group (Fig. 2E,F), especially in the adventitia ($p<0.05$, Fig. 2G) and stent struts, compared with control (Fig. 2D). Similar findings of CD3⁺ T cell distribution were also observed, when two control groups were analyzed separately (Fig. not shown).

3.4. Blockade of TGF- β increases hyaluronan deposition

Movat staining identified glycosaminoglycans (GAG) as the predominant ECM of the in-stent neointima in both control (Fig. 3A) and the AdT β -ExR group (Fig. 3B). Occasionally, there were foci of SMCs with GAG-rich ECM across the interrupted internal elastic lamina in stented arteries in both groups (Fig. 3C), suggesting that substantial portion of SMCs comprising in-stent neointima probably migrated from outside of intima. Then we analyzed GAG hyaluronan that is implicated in restenosis pathophysiology including vascular cell migration and inflammation [3,24-26]. The accumulation of hyaluronan was diffuse and variable, but tended to be increased in the portion enriched with inflammatory cells (Fig. 3D-F). Area of hyaluronan immunoreactivity was significantly increased in all three layers of arteries in the AdT β -ExR group compared with control (all, $p<0.05$, Fig. 3L). Similar findings of hyaluronan were also observed, when two control groups were analyzed separately (Fig. not shown).

Collagen area, quantified in the picrosirius red-stained sections using a polarized light microscope, tends to be decreased in neointima compared with adventitia in both groups (Fig. 3G,H). Collagen area was not significantly different between control and the AdT β -ExR group (Fig. 3M).

3.5. Effect of blockade of TGF- β on MMP1 expression and cell proliferation

Inflammatory cells and mediators can stimulate the expression and activation of MMPs [14]. The expression of MMP-1, assessed by immunohistochemical staining, was generally increased in all three layers of stented arteries in both control (Fig. 3I) and the AdT β -ExR group (Fig. 3J) compared with proximal reference arteries (Fig. not shown). The area of MMP-1 immunoreactivity was increased, especially in adventitia, in the AdT β -ExR group compared with control ($p < 0.05$, Fig. 3N). MMP-1 immunoreactivity tends to be more abundant around inflammatory cell-rich area (Fig. 3J,K).

Cell proliferation rate, measured by PCNA index (PCNA positive cells / total cells $\times$ 100 %), was increased in the AdT β -ExR group compared with control (Fig. 4). Similar findings of MMP-1 and PCNA index were also observed, when two control groups were analyzed separately (Fig. not shown).

Discussion

The main finding of the present study is that blockade of TGF- β by local intravascular gene delivery of an adenoviral vector expressing the ectodomain of T β RII enhanced vascular inflammation without inhibition of in-stent neointima formation in the pig coronary stent model of restenosis. Inhibition of TGF- β enhanced infiltration of inflammatory cells, mostly consisting of CD3⁺ T cell, and a constellation of pathological changes related with vascular inflammation, ie increases in hyaluronan accumulation, cell proliferation, and expression of MMP-1. An increase in cell density of neointima in arteries treated with AdT β -ExR possibly reflects, to some extent, a decrease in ECM accumulation, thus potentially inhibiting neointima formation. However these findings illustrate why blockade of TGF- β by gene transduction promotes or does not change rather than inhibits events associated with neointimal growth in restenosis after stenting.

At least three factors in our experiment potentially induce vascular inflammation: 1) an adenoviral vector [27], 2) mechanical vascular injury [28], and 3) inhibition of TGF- β signaling [12-17]. Adenoviral gene delivery can induce innate immune response followed by adaptive immune response to both capsid proteins and the transgene product [27]. Inflammation also tends to be aggravated by a high degree of mechanical injury [28]. Nevertheless the significance of TGF- β blockade as a contributing factor for vascular inflammation probably outweighs other factors, since the infiltrated CD3⁺ T cells were greater in the AdT β -ExR group than AdLacZ/PBS control. The present data is consistent with other studies in support of anti-inflammatory and anti-atherosclerotic properties of TGF- β [12,17,18]. Mallat *et al* demonstrated that blockade of TGF- β with a neutralizing antibody in apo E-deficient mice induced an activation of NF- κ B and increased T cell infiltration and lesion size in arteries [12]. Robertson *et al* showed that ApoE-knock out mice with abrogated TGF- β signaling in T cells exhibited a dramatic increase in atherosclerotic lesion size along with marked increases in IFN- γ mRNA expression, CD3⁺ T cell infiltration, and increased T cell activation [17]. Similarly, Lutgens *et al* showed that inhibition of TGF- β with use of recombinant soluble T β RII in apo E-deficient mice increased CD3⁺ T lymphocytes and macrophages, CD40 and CD40L immunoreactivity, lipid core area, MMP-2 and MMP-9 immunoreactivity, and intraplaque hemorrhage [18].

Intriguingly, in our AdT β -ExR group, CD3⁺ T cells were accumulated predominantly around the adventitia and stent struts where expressions of hyaluronan and MMP-1 were enhanced. The present data suggests that activation of adventitial T cells may play a significant role in the inflammation induced by blockade of TGF- β , and is consistent with Galkina's study [29] that most T and B lymphocytes accumulate in the adventitia of normal and atherosclerotic mouse aorta. Galkina *et al.* through adoptive transfer experiments suggest that lymphocytes accumulate in the adventitia through the migration from the adventitial vasa vasorum rather than from the intimal lumen. TGF- β regulates T cell development and homeostasis, and T cell specific deletion of T β RII developed lethal inflammation along with T cell activation and differentiation [30]. TGF- β also has an inhibitory effect on T cell proliferation by inhibiting interleukin-2 production, and inhibits the acquisition of most effector functions by naive T cells and expression of Gata-3, thereby inhibiting T_H2 development [16]. In addition, Smad proteins, downstream effectors of TGF- β 1, competitively interact with NF- κ B, a central transcriptional control mediator of inflammation, thereby endowing TGF- β with an anti-inflammatory property [15].

In the present study, ECM enriched with hyaluronan was observed in stented arteries in both AdT β -ExR group and control, which is similar to our previous study of human coronary stent restenosis [3]. Interestingly hyaluronan was more abundant, especially around inflammatory cells, in the AdT β -ExR group than control. Hyaluronan has been known to be implicated in cell migration, proliferation, inflammation, and wound healing, thereby contributing significantly to neointima formation [24,25]. Expression of hyaluronan can be inducible on endothelial cells by proinflammatory cytokines, which in turn can enhance CD44-dependent adhesive interaction between circulating T cells and endothelial cells thus promoting extravasation of inflammatory cells [26].

The present data showed that expression of MMP-1 immunoreactivity was enhanced, especially in the inflammatory cell rich area, in the AdT β -ExR group, suggesting the possible interaction between inflammation and MMP-1 production [14,31]. TGF- β 1 typically inhibits expression of most MMPs [32]. Inflammatory cells and mediators can stimulate the expression and activation of MMPs, along with other proteases, thus potentially contributing to plaque instability and lesion development [14,31,33]. For instance, cellular interaction between T cells and VCAM-1 positive endothelial cells can induce 72-kD gelatinase in T cells [33]. We studied MMP-1 due to following reasons. Firstly, substrates of MMP-1, including collagen type I, III, gelatin, and proteoglycan, comprise most of in-stent restenotic ECM [3,32,34]. Proteoglycans comprise most of early stage ECM, whereas collagen tends to become the main ECM component over time after stenting [3]. Secondly, expression of MMP-1 tends to correlate with plaque progression [34].

We observed no significant difference in collagen area between the AdT β -ExR group and control, although there was a tendency of decrease in collagen content in the adventitia of the AdT β -ExR group compared with AdLacZ control. Since TGF- β 1 is the up-regulator of collagen synthesis in vascular SMCs [4,34], the present collagen data is somewhat confusing to interpret. Although the answer is not clear, one possible explanation is that local increases

in MMPs may act to augment the bioavailability of TGF- β 1 thus creating a feedback loop promoting intimal thickening [4].

Previous studies reported heterogeneous effects of blockade of TGF- β , using various strategies such as ribozyme oligonucleotides [35], truncated T β RII [18,36,37], transgenic CD4-dnT β RII Tg⁺¹⁴ [12], and antibodies [7], on a balloon injured artery model or a Apo E deficient mice. According to these studies, blockade of TGF- β inhibits loss of lumen area mainly by decreasing negative vascular remodeling [36,37]. On the other hand, the effects of TGF- β blockade on the neointima formation are variable: a decrease [7,18,35,36], no significant change [37], and an increase [12,17]. Furthermore, the effects of TGF- β blockade on adventitial collagen content are also contradictory [17,35-37]. Although it is unclear to explain these discrepancies, one possible explanation is a context dependent property of TGF- β . TGF- β exerts bifunctional effects that are dependent upon the context, in which the particular cell encounters the TGF- β signaling [38]. Given the differences in mechanism for restenosis between the balloon injury model and the stent model [39], the biological effects of TGF- β could be different depending on experimental model.

In the present study, the cell proliferation rate was increased in the AdT β -ExR group compared with control. TGF- β can affect cell proliferation differently in different settings. TGF- β 1 inhibits cell proliferation either by extending G2 phase [40] or by arresting in G1 phase [41]. However cell proliferation could be affected heterogeneously by TGF- β 1 depending on different doses of TGF- β 1 *in vitro* study [42] and on different *in vivo* study conditions [6,35,36]. SMC proliferation can also be affected by the type of collagen components. Monomeric type I collagen can allow SMC proliferation in response to growth factor, whereas polymerized type I collagen has inhibitory effect on SMC proliferation owing to upregulation of cyclin dependent kinase-2 inhibitors [43]. Thus it is a reasonable deduction that enhanced cell proliferation in the AdT β -ExR group might be at least partially induced by monomeric collagen produced by enhanced MMP-1.

The study limitations of the present study are as follows. Firstly, we deployed stent in normal coronary arteries instead of pre-existing plaque. The biological response to injury of the normal artery could be different from that of the pre-existing lesion in terms of cell proliferation, death, and inflammation [44]. Therefore the biological effects of TGF- β blockade on the stented pre-existing lesion may be different from the present data. Secondly, we found only negligible level of soluble T β RII detected in remote coronary arteries. However this soluble T β RII can be circulated, thus potentially affecting in the remote segments to some extent [19]. Thirdly, pathological analysis was performed only at 4 weeks after stenting, thus limiting the sequential biological assessment. Finally, gender has an important influence on the development of cardiovascular diseases, with men and postmenopausal women having a greater incidence than reproductive women. Estrogen, a key sex steroid hormone in female, can regulate inflammation by modulating nitric oxide synthesis, rennin-angiotensin system, and oxidative stress, thus influencing development of cardiovascular disease [45]. Therefore the biology of male may not be simply extrapolated from the present female pig data.

In conclusions, a modest inhibition of ECM accumulation by blockade of TGF- β is probably offset by vascular inflammation characterized by infiltration of inflammatory cells, especially CD3⁺ T lymphocytes, hyaluronan accumulation, and enhanced MMP-1 expression and cell proliferation. Therefore blockade of TGF- β may not inhibit in-stent neointima, but rather potentially can promote lesion progression owing to enhanced inflammation in a stented coronary artery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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
TGF-β	transforming growth factor- β

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Fig. 1.

The effect of the AdT β -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 AdT β -ExR or AdCALacZ at two different concentrations (6 and 60 m.o.i). After incubation for 48 hours, 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; AdT β -ExR: an adenovirus expressing the ectodomain of the type II TGF- β receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; moi: multiplicity of infection.

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Fig. 2. Histological staining of stented pig coronary arteries in the AdTβ-ExR group and control. Hematoxylin and eosin staining shows that in-stent neointima was formed around stent strut in both control (A) and AdTβ-ExR group (B). An increase in inflammatory cells, such as

round nucleus cells (higher magnification in an inset, B), macrophages, multinucleated giant cells (black arrow, C), and eosinophils (white arrow, C) around stent struts and adventitia was noted in the AdT β -ExR treated artery compared with control (A). CD3⁺ T cells (brown), identified by immunohistochemical staining, were more abundant in the AdT β -ExR treated artery (E, F) compared with control (D). Bar graph showing the comparison analysis of CD3⁺ T cell positive areas, identified by immunohistochemical staining and measured with computerized digital morphometry software, between the AdT β Ex-R group and control (G). Data are mean $\pm$ SEM. The asterisk in panel B marks the site of high-power micrograph of inset. Counterstaining with hematoxylin. A: adventitia; HE: hematoxylin and eosin stain; M: media; NI: neointima; St: stent strut. Bars=100 μ m. Other abbreviations are as in Fig. 1.

* p <0.05.

Fig. 3. Histological characteristics of the extracellular matrix in stented pig coronary arteries in the AdTβ-ExR group and control. Glycosaminoglycans (GAG, blue), identified by Movat staining, are abundant in in-stent neointima of both control (A) and AdTβ-ExR treated artery (B). Panel C shows the foci of SMCs in the GAG-rich ECM across the interrupted internal elastic lamina (IEL) in a stented artery in control group. Hyaluronan immunoreactivity was more abundant, especially around neointima and adventitia enriched with inflammatory cells, in the AdTβ-ExR treated artery (E, F) compared with control (D). Panel F is a high-power micrograph of the area in Panel E indicated by the asterisk and shows that hyaluronan

is accumulated in T cell-rich area. Hyaluronan area was significantly increased in the three vascular layers of the AdT β -ExR group than control (L). Picrosirius red staining viewed under polarized light microscopy detected birefringence of collagen fiber in control (G) and the AdT β Ex-R group (H). Collagen areas were not significantly different between control and the AdT β Ex-R group (M). Immunoreactivity of matrix metalloproteinase-1 (MMP-1) was detected diffusely in in-stent neointima and media in both control (I) and the AdT β Ex-R group (J, K). Panel K is a high-power micrograph of the area in Panel J indicated by the asterisk and shows that expression of MMP-1 is enhanced in T cell-rich foci in adventitia. Panel N shows that MMP-1 positive area is increased in adventitia of the AdT β Ex-R group than control group. Counterstaining with hematoxylin. HA: hyaluronan; Data are mean $\pm$ SEM. Other abbreviations are as in Fig. 1 and 2. * p <0.05. Bars=100 μ m.

Fig. 4.

Comparison analysis of cell proliferation rate measured by PCNA (proliferating cell nuclear antigen) index (PCNA positive cells / total cells $\times$ 100), between the AdTβEx-R group and control. Data are mean $\pm$ SEM. Abbreviations as in Fig. 1. * p <0.05.

Table 1

Morphometric assessment of stented coronary arteries at site of minimum luminal area 28 days after stenting: comparisons between the AdT β -ExR group and control group

	AdT β -ExR (n=7)	Control (n=7)
lumen (mm ²)	3.73 $\pm$ 0.53	4.20 $\pm$ 0.37
intima (mm ²)	2.02 $\pm$ 0.35	2.06 $\pm$ 0.14
media (mm ²)	1.83 $\pm$ 0.15	1.90 $\pm$ 0.23
adventitia (mm ²)	4.17 $\pm$ 1.28	3.56 $\pm$ 1.58
intima / media	1.13 $\pm$ 0.20	1.19 $\pm$ 0.19
% area stenosis	36 $\pm$ 5.67	35 $\pm$ 3.02
stent (mm ²)	5.72 $\pm$ 0.26	5.99 $\pm$ 0.39
EELA (mm ²)	7.61 $\pm$ 0.46	8.15 $\pm$ 0.48
intima CD	3121 $\pm$ 125 *	2812 $\pm$ 69
injury score	0.93 $\pm$ 0.12	0.80 $\pm$ 0.12

Data are expressed as mean $\pm$ SEM. area stenosis (%) = (intima area/internal elastic lamina area) $\times$ 100; CD: cell density (cells/mm²); EELA: external elastic lamina area.

*
p <0.05 vs control group.