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Inhibition of TGF-β by Gene Delivery does not Alter the Neointima Area, but Enhances Inflammation in In-Stent Stenosis of Pig Coronary Arteries

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ABSTRACT

Background : We have shown that extracellular matrix (ECM) rather than cell proliferation contributes to in-stent restenosis. Transforming growth factor-β (TGF-β), a positive regulator of ECM deposition by vascular cells, may be implicated in in-stent restenosis. We assessed if the blockade of TGF-β by catheter-based local delivery of an adenovirus expressing a soluble form of TGF-β type II receptor (AdTβ-ExR) can inhibit stent-induced neointima. **Methods** : AdTβ-ExR was applied onto a coronary arterial segment of a pig using an Infiltrator™, and either of adenovirus expressing β-galactosidase (AdLacZ) or PBS was applied onto other remote segment of the same pig (n=10). Then, stents (n=20) were deployed in the treated arterial segment. **Results** : Computer-based morphometric analysis 4 weeks after stenting showed no significant difference in neointima area between the AdTβ-ExR-infected and control groups (AdLacZ and PBS). However cell density of neointima was significantly increased in the AdTβ-ExR group compared with control group (3121±331 vs 2812±183 cells/mm^2, $p<0.05$). Notably, the AdTβ-ExR group had more extensive CD3 positive T cell infiltration. In addition matrix metalloproteinase (MMP)1 expression and accumulation of hyaluronan was greater in the AdTβ-ExR group. Cell proliferation rate was significantly increased in the media of the AdTβ-ExR group compared with control group (2.04±1.21% vs 1.18±1.06%, $p<0.05$). **Conclusion** : Blockade of TGF-β by use of catheter-based local *in vivo* gene delivery did not alter neointima formation significantly in our porcine coronary artery stent model, however it increased inflammation and pathological changes that could promote lesion formation. (**Korean Circulation J 2004;34(11):1022-1032**)

KEY WORDS : Extracellular matrix ; Hyaluronan ; TGF-β ; Coronary restenosis ; Stents.

Introduction

Neointimal ingrowth rather than tissue remodeling or stent recoil is thought to play a key role in restenosis after stenting.[1] Studies of human coronary in-stent neointima suggest that the enhanced ECM accumulation may be an important mechanism for the development of in-stent neointima.[2]

The TGF-β expression is thought to increase ECM accumulation. The level of TGF-β1 mRNA is increased in arteries as early as 6 hours after injury,[3] and its expression is significantly higher in restenotic lesions compared with primary lesions.[4] TGF-β1 up-regulates the synthesis of various ECM components, such as proteoglycans, hyaluronan, fibronectin, and collagen.[3][5-8] TGF-β1 also enhances ECM accumulation by down-regulating plasminogen activators as well as by increasing the plasminogen activator inhibitor.[4][5] Direct evidence showing that TGF-β1 is involved in the development of arterial lesion was reported.[6-8] Over-expres-

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sion of the TGF-β1 promotes the formation of a neointima enriched with ECM,[6)7)] and withdrawal of TGF-β1 contributes to neointimal regression with increased apoptosis.[7)] Treatment of balloon injured arteries with neutralizing anti-TGF-β1 antibodies reduced intimal hyperplasia.[8)] In contrast, other studies suggested the role of TGF-β in inhibition of inflammation and atherosclerosis.[9)]

Most cells express three types of TGF-β receptors (type I, II and III) on the surface, and type I and II receptors (TβRI and TβRII, respectively) are necessary for all biological activity of TGF-β.[10)11)] TGF-β exerts its effect by binding to heterodimeric complexes of TβRI and TβRII.[10)11)] TβRII is a transmembrane receptor of serine-threonine kinase family, and can itself bind free TGF-β, whereas TβRI can only recognize TGF-β that is already bound with TβRII.[11)] An adenoviral vector expressing the ectodomain of TβRII (AdTβ-ExR) specifically inhibits the function of the endogenous TβRII, and inhibits action of TGF-β, specifically TGF-β1, TGF-β3, and TGF-β5, but not TGF-β2.[12)13)] AdTβ-ExR can act as a specific dominant negative mutant of TβRII, thus preventing an interaction of the endogenous functional TβRII with TβRI.[10)]

In this study, we inhibited TGF-β using a catheter based local *in vivo* delivery of AdTβ-ExR in a porcine coronary stent model. Blockade of TGF-β did not reduce in-stent neointima formation, although it did reduce ECM formation to some extent. Furthermore, blockade of TGF-β induced increases in T cell infiltration, MMP 1 expression, deposition of hyaluronan-rich ECM, and medial cell proliferation rate, suggesting that inhibition of TGF-β activity promotes inflammation in injured arteries.

Methods

Materials

Antibodies against soluble human TβRII IgG (a fluorescein isothiocyanate (FITC)-conjugated, rabbit polyclonal), human CD3 (T cell marker, rabbit polyclonal), human α-actin (SMC marker, mouse monoclonal), and human proliferating cell nuclear antigen (PCNA) (cell proliferation rate marker, mouse monoclonal) were purchased from Dako, Carpinteria, CA. Mouse monoclonal anti-human MMP1 and rabbit polyclonal anti-porcine TGF-β1 were purchased from Oncogene, Cambridge, MA and Cell Sciences, Norwood, MA, respectively. Biotinylated anti-mouse IgG and anti-rabbit IgG were purchased from Dako. The Vectastain Eite ABC kit (Avidin DH and biotinylated horse-radish peroxidase H reagent) was purchased from Vector Lab., Burlingame, CA. DAB (3, 3'-diaminobenzidine tetrahydrochloride) and other chemicals were purchased from Sigma (St. Louis, MO).

Recombinant adenovirus vector

Replication-defective E1- and E3- recombinant adenovirus expressing either an entire ectodomain of the TGF-β type II receptor fused to the human immunoglobulin Fc portion (AdTβ-ExR) or β-galactosidase (AdLacZ) under a CA promoter (composed of cytomegalovirus enhancer and chicken β-actin promoter) was constructed as described previously.[14)] AdTβ-ExR-infected cells secrete soluble TGF-β type II receptor (TβRII), which inhibits TGF-β signaling by binding TGF-β.[13)] Adenovuruses were propagated and titered in HEK 293A cells, and were prepared by ultracentrifugation in cesium chloride gradient to yield concentrations of the order of 10^9-10^{10} plaque forming unit (pfu).

Local intravascular gene delivery and stent deployment

A total of thirteen large white female pigs (2−3 mo, 25−30 kg) were used for this study. Ten pigs underwent coronary artery intravascular gene delivery with subsequent intracoronary stent deployment. Briefly, animals took 100 mg/d Aspirin and 75 mg/d Clopidogrel 24 hours prior to the procedure and continued to take these until sacrifice. Before anesthesia, pigs subjected to intramuscular injection of Atropine (0.04 mg/Kg), Xylazine (2 mg/Kg), and Ketamine (10 mg/Kg). Anesthesia was induced by inhalation of 2.5% Enflurane. Heparin 6,000 IU was injected intravenously, and an 8 F Judkins right coronary artery (RCA) guide catheter was inserted through the left carotid artery. Two coronary arterial segments feasible for intravascular delivery

using a 3.0−3.5 mm Infiltrator^{TM} (Interventional Technologies, San Diego, CA) were selected for angioplasty by a blinded operator. One arterial segment was injected with AdTβ-ExR (1×10^9 pfu), and the other with either AdLacZ (1×10^9 pfu) or PBS. After gene delivery, a Palmaz-Schatz coronary stent was deployed in each treated arterial segment (9−11 atm, balloon/artery 1.1). After stent deployment, left carotid artery was ligated.

Three pigs were used for the analysis of efficiency of gene transfection at 1 week after gene delivery. To identify optimal titer of adenoviral vector for effective gene transfer, three different titers of AdTβ-ExR (1×10^8, 5×10^8, and 1×10^9 pfu in 400 (l PBS) and two different titers of AdLacZ (2.5×10^7 and 2.5×10^8 pfu in 400 μL PBS) were randomly injected into each arterial segments.

Three pigs died during angioplasity to vascular complications, thus seven pigs were sacrificed 4 weeks after stenting for the pathological analysis. Coronary arteries for the analysis of gene transfection were snap-frozen in liquid nitrogen, and divided into two pieces: one embedded in OCT for cryostat sectioning and the other kept −70℃ until RNA assay. The stented arterial specimens were pressure-fixed in situ with 4% formaldehyde, excised from the porcine heart, and divided into two segments by cutting the bridge portion of the Palmaz-Schatz stent. One bisected arterial specimen with higher degree of stenosis underwent tissue processing with Kulzer Histotechnik 8100 (Heraeus Kulzer, Germany) for morphometric analysis, and the other bisected specimen was embedded int paraffin for pathological staining.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from each arterial segment was isolated using RNeasy mini kit (Qiagen, Germany). Total cDNA synthesized from 2 μg of total RNA was amplified for 35 cycles at 94℃ for 30 s, 54℃ for 1 min, and 72℃ for 1 min. The primer sets for TβRII (5'-ACA TCG TCC TGT GGA CGC GTA-3' and 5'-CTA GCA ACA AGT CAG GAT TGC-3'), or 28S rRNA (5'-TTA AGG TAG CCA AAT GCC TCG-3', and 5'-CCT TGG CTG TGG TTT CGC T-3') were used for amplification of 450 bp and 102 bp fragments, respectively. Similarly, total RNA was isolated from the adenovirus-infected (6 and 60 moi) cultured rat arterial smooth muscle cells using Trizol (Gibco, Grand Island, NY) to study the effect of AdTβ-ExR on the expression of connective tissue growth factor (CTGF) mRNA. 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).

Immunohistochemical staining

Imunohistochemical staining was done on serial sections as described previously.[2] Briefly, after deparaffinization, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. Slides were incubated with primary antibodies as described. The frozen arterial segment sections were dried, placed in PBS for 10 min, and incubated with primary antibodies. Biotinylated anti-mouse IgG or anti-rabbit IgG was incubated as a secondary antibody, and further stained with avidin-conjugated peroxidase and DAB. Hyaluronan labeling was done with biotinylated hyaluronan binding protein. Transfected coronary arterial segments with AdLacZ subjected to cryostat section at 20 μm thickness. Expression of β-galactosidase was evaluated by incubation with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal : Sigma, St. Louis, MO). Tissue sections were subsequently counterstained with hematoxylin-eosin

Pathological analysis

The cross-sectional areas of the proximal and distal portions of the bisected stented arterial segments were measured with computerized digital morphometry software (Optimas 6.5). The areas bound by the luminal surface (lumen area), the internal elastic lamina (IELA),

the external elastic lamina (EELA) and stent (stent area) were measured at the minimal luminal area. Neointima area (IELA-lumen area), media area (EELA-IELA), and percent stenosis ([neointima area/IELA] × 100) were calculated. The extent of vessel wall injury induced by stent was calculated as mean injury score determined by Schwartz et al (mean injury score=sum of weights for each wire/number of coil wires present).[15] Modified Movat pentachrome stain[16] was used to identify ECM components. Collagen was identified in the picrosirius red-stained sections from paraffin-embedded tissue with polarized light on Olympus BX51 light microscope. The proportional area of either collagen or hyaluronan in each vessel layer was measured by analyzing at least 3 microscopic fields (×100) with Optimas 6.5 image software. To estimate semi-quantitatively the extent of distribution of CD3 positive T cells, we categorized the extent of T cells into three scores: one for CD3 positive cells <10% of total cells, two for ≥10% and <50%, three for >50%.

Statistical analysis

All data are expressed as mean±SD, and all statistics were calculated by use of SPSS 11.0 for Windows. Comparisons of the continuous variables between two groups were made by Wilcoxon signed ranks test. A value of $p<0.05$ indicates statistical significance.

Results

Identification of the infected human TβRII gene

Two coronary arterial segments, proximal and distal left anterior descending artery (LAD), from a pig were injected with two different titers of AdTβ-ExR. One week after gene delivery, the levels of mRNA or protein of Tβ RII were determined from each arterial segment. As shown in Figure 1 (A), RT-PCR products of Tβ RII mRNA from distal LAD injected with AdTβ-ExR of 1×10^9 pfu showed a definite single band, whereas proximal LAD injected with that of 5×10^8 pfu showed a faint band. Neither mid LAD nor other remote arteries

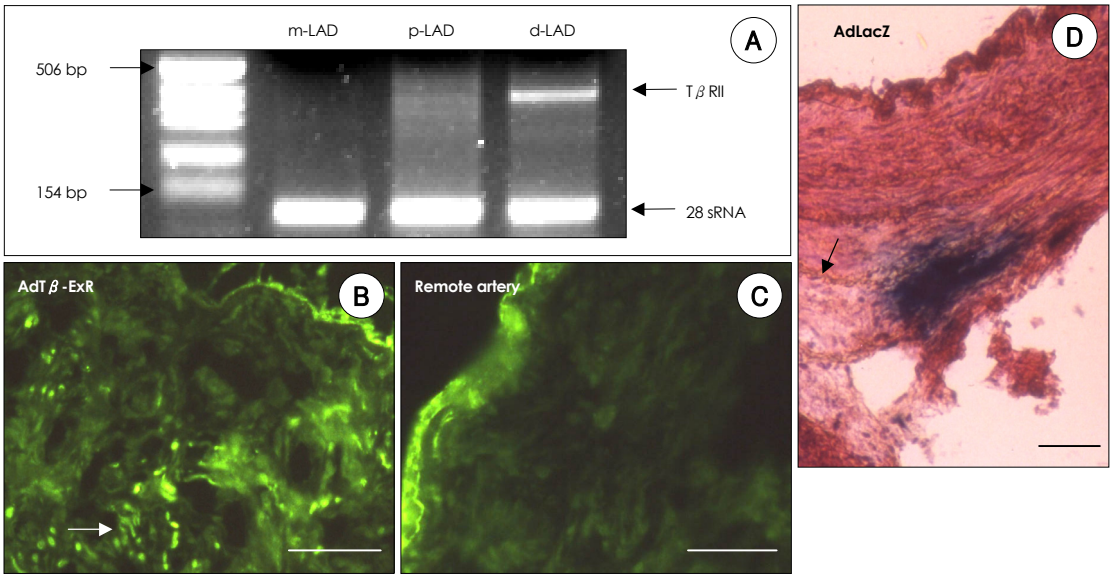

Figure 1. RT-PCR analysis for detection of a transferred Tβ RII mRNA in each porcine coronary arterial segments (A). The amplified RT-PCR gene product from the distal LAD injected with 1×10^9 pfu AdTβ-ExR was shown as a definite band of ~450 bp, whereas a faint band was shown by that from proximal LAD injected with 5×10^8 pfu AdTβ-ExR. Adjacent mid LAD did not have any this transgene product. Immunohistochemical staining with a FITC-conjugated rabbit anti-human IgG showed multiple dispersed immunofluorescent positive particles (marked as arrows) of Tβ RII in distal LAD injected with 5×10^8 pfu AdTβ-ExR (B), but not in remote coronary artery (C). Artery injected with 2.5×10^8 pfu AdLacZ showed β-galactosidase (dark blue color) around media and adventitia identified by histochemical staining with X-gal (D). Arrow indicates exteranl elastic lamina. Counter staining with hematoxylin-eosin. AdLacZ: adenovirus expressing β-galactosidase, AdTβ-ExR: adenoviral vector expressing the ectodomain of the type II TGF-β receptor, Tβ RII: TGF-β type II receptor. Bars=50μm. LAD: left anterior descending artery, FITC: fluorescein isothiocyanate.

revealed any detectable band. RT-PCR products of 28S RNA from each arterial segments had a band of equal density, verifying a quantitative nature of transfected gene delivery.

Immunofluorescent staining for soluble TβRII coronary artery injected with 5×10^8 pfu AdTβ-ExR showed multiple dispersed immunofluorescent positive TβRII-antibody complexes (Figure 1B). On the other hand, neither remote coronary arteries nor remote thoracic aorta showed any discernable immunofluorescent particles (Figure 1C). These results suggest that successful local catheter-based delivery of soluble TβRII was accomplished.

Efficiency of gene transfection was also confirmed by X-Gal staining of β-galactosidase in two coronary arterial segments injected with two different titers of AdLacZ (2.5×10^7 pfu and 2.5×10^8 pfu). The expression of β-galactosidase was shown in around outer media and adventitia in the arterial segment injected with AdLacZ of 2.5×10^8 pfu (Figure 1D), but not in the segment with 2.5×10^7 pfu (figure not shown).

The effect of AdTβ-ExR on CTGF mRNA in cultured rat arterial SMCs

There are CTGF-dependent and CTGF-independent signaling pathways activated by TGF-β, and CTGF functions as a downstream mediator of TGF-β in fibroblastic cells.[17] We analyzed to know if the expression of CTGF mRNA in cultured rat arterial SMCs can be affected by either AdTβ-ExR or AdLacZ. The expression of CTGF mRNA was inhibited by AdTβ-ExR at moi 60 (Figure 2). Therefore AdTβ-ExR can down-regulate CTGF expression, thereby inhibiting ECM synthesis.

The effect of blockade of TGF-β on morphometric parameters

Seven pairs (AdTβ-ExR and control group) of coronary arteries from seven pigs were analyzed for the comparison of morphometric parameters, as summarized in Table 1. Both AdLacZ and PBS treated arteries were pooled together as a control group. Cell density of neointima was significantly greater in the AdTβ-ExR group than in control group ($3,121\pm331$ vs $2,812\pm183$ cells/mm^2, $p<0.05$). However there was no significant difference in lumen area, intima area, percent area stenosis, area ratio of intima to media, or adventita area between the AdTβ-ExR and control group.

Table 1. Comparisons of morphometric parameters between two groups

	AdTβ-ExR (n=7)	Control (n=7)	p
Lumen (mm^2)	3.73±1.39	4.20±0.98	NS
Intima (mm^2)	2.02±0.92	2.06±0.37	NS
Media (mm^2)	1.83±0.41	1.90±0.60	NS
Adventitia (mm^2)	4.17±3.38	3.56±4.18	NS
Intima/media	1.13±0.52	1.19±0.51	NS
Area stenosis	36±15	35±8	NS
Stent (mm^2)	5.72±0.70	5.99±1.04	NS
EELA (mm^2)	7.61±1.22	8.15±1.26	NS
Intima CD	3121±331	2812±183	<0.05
Injury score	0.93±0.31	0.80±0.33	NS

CD: cell density (cells/mm^2), EELA: external elastic lamina area; % area stenosis=(neointima area/IEL area)×100. Data are expressed as mean±SD

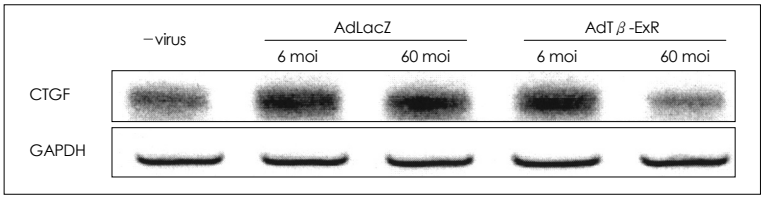

Figure 2. Inhibition of CTGF mRNA in cultured rat arterial SMCs transfected with AdTβ-ExR. Rat arterial SMCs were transfected with either AdTβ-ExR or AdLacZ at two different concentration (6 and 60 moi). After incubation for 48 hours, total RNA was extracted and subjected to RT-PCR as described in method. Signals of 420 bp for CTGF and 370 bp for GAPDH are shown. CTGF: connective tissue growth factor, AdTβ-ExR: adenoviral vector expressing the ectodomain of the type II TGF-β receptor, AdLacZ: adenovirus expressing β-galactosidase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, SMCs: smooth muscle cells.

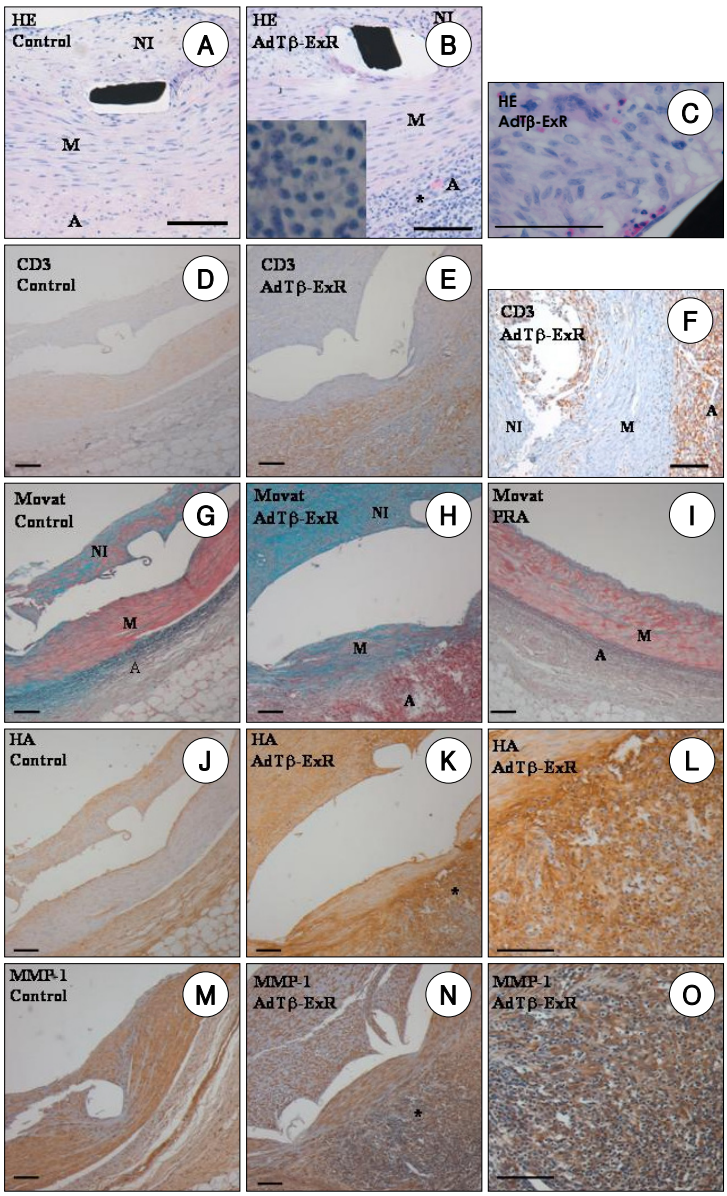

Figure 3. Effects of blockade of TGF-β on the pathological characteristics. A pair of porcine coronary arteries treated with either AdTβ-ExR or AdLacZ (control) are shown. Adjacent sections were stained. Hematoxylin-eosin (HE) staining shows an increase in inflammatory cells (mostly round nucleus cells and macrophages) at around stent struts and adventitial in the AdTβ-ExR artery (B, C) compared with control artery (A). Higher magnification of inflammatory cells are shown in an inset (B). Immunohistochemical staining identified CD3 positive T cells to be a main component of these inflammatory cells. CD3 positive cells were more abundant in the AdTβ-ExR artery (E, F) compared with control artery (D). Movat staining shows glycosaminoglycans (blue color) to be the main ECM component of neointima (G, H). Proximal reference artery (PRA) had thin intimal and adventitial layers (I). Hyaluronan detected with hyaluronan binding protein was expressed moderately in neointima of control artery (J), however its expression in AdTβ-ExR artery was more abundant in around neointima and adventitia enriched with inflammatory cells (K, L). Expression of MMP1 was localized in neointima and media of control artery (M), on the contrary its expression in AdTβ-ExR artery was also additionally found in inflammatory cell-rich adventitia (N, O). *: marks the site of higher magnification. NI: neointima, M: media, A: adventitia, PRA: proximal reference artery, HA: hyaluronan. counterstaining with hematoxylin. AdTβ-ExR: adenoviral vector expressing the ectodomain of the type II TGF-β receptor, AdLacZ: adenovirus expressing β-galactosidase, TGF-β: transforming growth factor-β, ECM: extracellular matrix, MMP1: matrix metalloproteinase 1. Bars=100 μm.

The effect of blockade of TGF-β on pathological characteristics

Infiltration of inflammatory cells was more marked in the AdTβ-ExR group compared with control group as shown in Figure 3 (A, B). The inflammatory cells were present throughout the tissue, but accumulated especially at around stent struts and adventitia (Figure 3C). Immunohistochemical staining identified CD3 positive T cells as the main component of inflammatory cells (Figure 3D-F), while the rest were composed of macrophages, eosinophils, and multinucleated giant cells (Figure 3C). T cell distribution in the AdTβ-ExR group was significantly larger than that of control group (Figure 4). In general, α-actin positive smooth muscle cell (SMC) was the predominant cell type in neointima and media of both groups, and most of these SMCs were TGF-β1 positive by immunohistochemical staining (figure not shown).

Movat staining identified that glycosaminoglycans were the predominant ECM component in the in-stent neointima in both AdTβ-ExR and control groups (Figure 3G, H). Hyaluronan, detected by hyaluronan binding protein, was abundantly expressed in stented arteries (Figure 3J, K), whereas its expression was scarce in proximal reference artery. The accumulation of hyaluronan was diffuse and variable, but tended to be increased in neointima especially near the stent strut and in adventitia containing many inflammatory cells

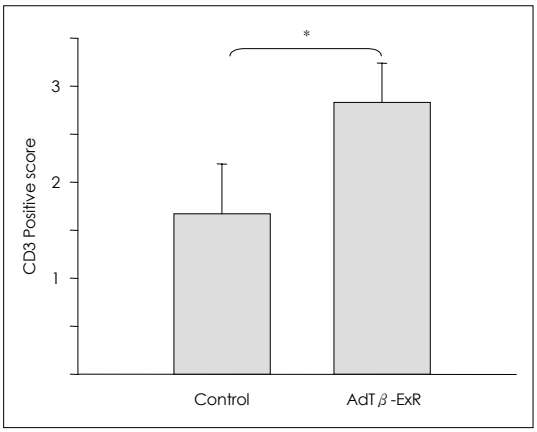

Figure 4. Comparison analysis of the distribution of T cells with use of a score to estimate the proportion of CD3 positive cells semi-quantitatively (1: positive cells <10% of total cells, 2: ≥10% and <50%, 3: ≥50%). The AdTβ-ExR group had significantly larger extent of CD3 positive cells compared with control. Data are mean±SD. *: $p<0.05$, AdTβ-ExR: adenoviral vector expressing the ectodomain of the type II TGF-β receptor.

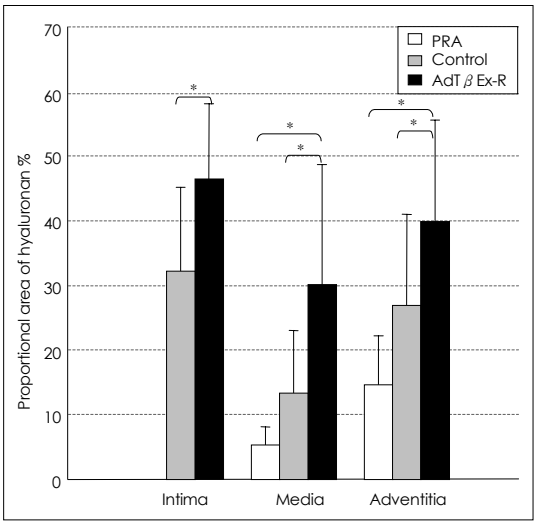

Figure 5. The effect of of TβRII on the proportional area of hyaluronan in each layer. The proportional areas of hyaluronan in all three arterial layers were significantly larger in the AdTβ-ExR group compared with control group. Data are mean±SD. *: $p<0.05$, AdTβ-ExR: adenoviral vector expressing the ecto-domain of the type II TGF-β receptor, TβRII: TGF-β receptor type II, PRA: proximal reference artery.

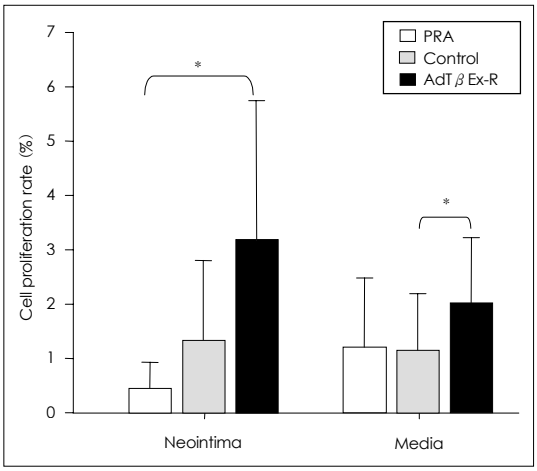

Figure 6. The effect of TβRII on cell proliferation rate in stented arteries. The cell proliferation rates of neointima and media in AdTβ-ExR group were significantly higher than those of PRA group and control group, respectively. Data are mean±SD. *: $p<0.05$, AdTβ-ExR: adenoviral vector expressing the ecto-domain of the type II TGF-β receptor, TβRII: TGF-β receptor type II, PRA: proximal reference artery.

(Figure 3L). Proportional areas of hyaluronan in all three layers of AdTβ-ExR arteries were significantly increased compared with those of control arteries (Figure 5). Polarizing microscopy on picrosirius-red stained slides was used to estimate collagens content. Areas of collagen in all three layers were not significantly different between the AdTβ-ExR and control group. (figure not shown). The expressions of MMP1 in neointima and media were similarly increased in both AdTβ-ExR and control groups, however its expression in adventitia tended to be higher in the AdTβ-ExR group containing many inflammatory cells (Figure 3M−O).

Cell proliferation rate, as measured by PCNA index (PCNA positive cells/total cells ($\times$100 (%)), was increased in the media of the AdTβ-ExR group compared with that of control group (2.04$\pm$1.21% vs 1.18$\pm$1.06%, $p<0.05$), however there was no significant difference in intimal cell proliferation rate between two groups (Figure 6).

Discussion

Blockade of TGF-β did not alter neointima area in a pig in stent model of restenosis. On the other hand, a key determinant of stent restenosis, ie vascular inflammation was affected. An increase in cell density without any area change shown in neointima of arteries treated with TGF-β blockade probably reflect modest decrease in ECM formation. If an inhibition of ECM accumulation was the sole mechanism by blockade of TGF-β, then less neointimal area with higher cell density, compared with control group, would be an expected outcome. Pathological analysis of stented arteries treated with TGF-β blockade showed increases in hyaluronan-rich ECM and in MMP1 expression in association with T cell infiltration, all of which could promote cell migration and, together with an increase of medial cell proliferation rate, lesion growth. These findings illustrate why blockade of TGF-β by gene transduction promotes or does not change rather than inhibits events associated with neoinitmal growth in stent restenosis.

An increase in T cell infiltration was observed especially at around adventitia and stent strut in the AdTβ-ExR group, in which MMP1 tended to be highly expressed. At least three factors in our experiment, such as adenoviral vector,[18] angioplasty induced vascular injury,[19] and inhibition of TGF-β signaling,[9)20)] could induce inflammation. Adenoviral gene delivery can induce host cellular and humoral immune response and dose-dependent inflammation, which in turn can adversely affect gene expression.[18] Inflammation also tends to be aggravated with high degree of vascular injury.[19] However the significance of TGF-β blockade probably outweighs other factors in our study, since the infiltrated T cells were much more in the AdTβ-ExR group compared with control group. Our data supports the notion that TGF-β has an anti-inflammatory property.[9)20)] Blockade of TGF-β with use of a neutralizing antibody in apo E-deficient mice induced an aggravation of inflammatory reaction, such as infiltration of macrophages and T cells, activation of NF-κB, and increased arterial lesion size compared with control group.[9] An anti-inflammatory property of TGF-β results from a competitive interaction between Smad proteins, downstream effectors of TGF-β1, and NF-κB known as the central transcriptional control mediator of vascular inflammation. Smad proteins inhibit transcriptional NF-κB activity by blocking the association of cAMP response element-binding protein-binding protein, present in endothelial cells, with p65/NF-κB.[20]

Hyaluronan is a high molecular weight polysaccharide, and is implicated in various biological processes including cell migration and proliferation, tumor invasion, angiogenesis, and wound healing.[21] Hyaluronan, found in human in-stent neointima especially at early period (<6 mo) after stenting,[2] can serve as a sustrate for leukocytes and promote leukocyte adhesion and therefore can be considered a proinflammatory component of ECM. Hyaluronan may significantly contribute to the formation of neointima[21] and vascular remodeling[22] after angioplasty. Hyaluronan can stimulate the locomotion of SMCs mediated by specific receptor. Migrating and proliferating SMCs organized pericellular matrices enriched with hyaluronan and versican, and interference with the binding of hyaluronan to the cell surface by use of hyaluronan oligosaccharides can inhibit SMC proliferation and migration.[21] Hygroscopic property of hyaluronan may promote intimal thickening

by expanding the tissue space into which cells can migrate easily. Since TGF-β1 can stimulate the synthesis of hyaluronan, a decrease in hyaluronan would be an simple expectation by the AdTβ-ExR treatment. However hyaluronan was unexpectedly more abundant in all three vascular layers of the AdTβ-ExR group compared with control group. This finding is consistent with previous studies that hyaluronan expression can be enhanced by proinflammatory cytokines. Expression of hayluronan on microvascular endothelial cells can be inducible by the proinflammatory cytokines, such as TNF-β and IL-1β, which in turn can enhance CD44-dependent adhesive interaction between circulating T cells and endothelial cells thus promoting extravasation of inflammatory cells.[23]

No significant change in collagen content by AdTβ-ExR shown in our study might reflect that collagen content does not change significantly at early period after angiplasty, and this observation is consistent with previous report that collagen becomes the dominant ECM component by replacing early hyaluronan and proteoglycan rich loose ECM at later period (>6mo) after angioplasty.[2]

Inflammatory cells are known to be important sources of MMPs in a wide variety of inflammatory conditions, and inflammatory mediators can stimulate the expression and activation of MMPs and other proteases, which in turn can facilitate the proteolytic degradation of matrix, promote cell migration, thus contributing to the development of neointima and plaque instability. Cellular interaction between T cells and VCAM-1 positive endothelial cells is known to be able to induce 72-kD gelatinase in T cells.[24] TGF-β1 typically inhibits expression of MMPs including MMP1.[25] The abundant expression of MMP1 in the area of T cell accumulation in the AdTβ-ExR group supports the aforementioned interaction between inflammation and MMP production.

Only a few studies have been published for the effect of blockade of TGF-β on a balloon injured artery[8][12][28] and on a Apo E deficient artery.[9] But its effect on stent restenosis is not known well yet. Interference of TGF-β inhibited loss of lumen area in balloon injured arteries mainly by decreasing negative vascular remodeling.[12][26] However the effect of inhibition of TGF-β on the newly developed lesion area was diverse: a decrease in,[8][12] no significant change in,[26] or an increase in lesion size.[9] In addition, the effects of blockade of TGF-β on adventitial collagen area are also contradictory. An increase in collagen content was noted in porcine coronary arteries,[26] whereas a decrease was observed in rat carotid arteries.[12]

The exact causes for the discrepancies in neointima area, collagen content, inflammation profiles, and proliferation rates in ours and other studies are not clear. As a possible factor to explain these discrepancies, a context dependent property of TGF-β should be in consideration. TGF-β exerts bifunctional effects that are dependent upon the context, in which the particular cell encounters the TGF-β signaling.[27] In our data, cell proliferation rate of the AdTβ-ExR group was significantly increased, compared with control group, in media. TGF-β can affect cell proliferation differently at different settings. TGF-β1 inhibits cell proliferation either by extending G2 phase or by arresting in G1 phase.[28] But other *in vitro* data showed that cell proliferation was affected in a different way by different doses of TGF-β1.[29] Data of other in vivo studies are also confusing. Either exogenous infusion of TGF-β1[3] or adenovirus mediated TGF-β1 transfection[7] increased cell proliferation rate in rat neointima. However, similar to our study, inhibition of TGF-β signaling tends to increase, though not significant, cell proliferation rate in intima of balloon injured artery.[12] SMC proliferation can also be affected by degradation of collagen. Human newborn arterial SMCs plated on monomeric type I collagen proliferate in responding to platelet-derived growth factor, whereas SMCs grown on polymerized type I collagen are arrested in the G1 phase of the cell cycle owing to upregulation of cyclin dependent kinase 2 inhibitors.[30] Therefore enhanced MMP1 expression in the AdTβ-ExR group might promote degradation of collagen, which in turn could increase cell proliferation rate.

Our experiment has other several limitation factors. Drug delivery using an Infiltrator™ may induce a vascular injury causing dissection, intramural thrombosis, and vasospasm. In addition, host immune response and

dose-dependent inflammatory and cytopathic effects can inhibit adenoviral gene delivery and target gene expression.[18] Therefore these methodological problems might affect vascular tissue response, thus may complicate the interpretation of the effects of inhibition of TGF-β.

In conclusions, blockade of TGF β signaling using a catheter-based gene transfer of a soluble TβRII could not inhibit in-stent neointima formation, although it reduced ECM formation modestly. Pathological property characterized by increases in T cell infiltration, MMP1 expression, medial cell proliferation, and ECM enriched with hyaluronan, induced by blockade of TGF-β, might contribute to lesion progression and offset modest benefit provided by inhibition of ECM formation.

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