ORIGINAL ARTICLE

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Blockade of TGF-β by Catheter-based Gene Transfer of a Soluble TGF-β Type II Receptor Inhibits Neointima Formed after Stenting

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ABSTRACT

Background: Enhanced extracellular matrix (ECM) accumulation is an important finding in coronary stent restenotic tissue, in which TGF-β, implicated in ECM formation, is expressed abundantly. We assessed the hypothesis that blockade of TGF-β by the local delivery of an adenovirus expressing a soluble form of the TGF-β type II receptor (AdTβ-ExR), inhibits stent-induced neointima in porcine coronary arteries. **Methods**: Two remote coronary arterial segments (n=20) per pig randomly received 1×10^9 pfu of either AdTβ-ExR or adenovirus expressing β-galactosidase (AdLacZ)/PBS, using an Infiltrator™. Stents (n=20) were deployed, after gene transfer, in each segment of 10 pigs. Localized transgene expression was confirmed by both reverse transcription-PCR and immunohistochemistry. Computer-based morphometric assessment was performed in the stented arteries 4 weeks after the gene transfer. **Results**: There was significantly less intimal area (1.57±0.49 vs. 2.13±0.34 mm^2), area ratio of intima/media (0.84±0.44 vs. 1.32±0.48) and higher neointimal cell density (3121±330 vs. 2812±183 cells/mm^2) in the arteries treated with AdTβ-ExR compared to the controls (all, $p<0.05$). Neither the cell proliferation rate, assessed by PCNA immunohistochemistry, nor the injury score were significantly different between the two groups. The distribution of hyaluronan in the intima was less in 4 of the 6 AdTβ-ExR treated arteries compared to the controls. **Conclusions**: Blockade of TGF-β, by a local in vivo gene transfer of a soluble TGF-β receptor, inhibits stent-induced neointima, probably by inhibiting the ECM accumulation in porcine coronary arteries, which may have therapeutic potential in the inhibition of restenosis after stenting. **(Korean Circulation J 2004;34(1):59-68)**

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

Introduction

According to previous studies, neointimal ingrowth, rather than tissue remodeling or stent recoil, is thought to play a key role in restenosis after stenting.[1-3] Pathological studies of human coronary stent neointima suggests that the ECM accumulation is an important factor for the development of an in-stent neointima.[4)5)] TGF-β1 (transforming growth factor-β1) is known as a multifunctional cytokine, which regulates cell proliferation and differentiation, angiogenesis, and the synthesis of a variety of ECM components, such as proteoglycans, hyaluronan, fibronectin and collagen.[6-13)] Most cells have three types of TGF-β receptors (types I, II and III) on their surface, and the types I and II receptor have been found necessary for all biological activities of TGF-β tested, whereas the type III is not known to mediate any.[7)10)] The type II receptor is a member of the transmembrane receptor serine-threonine kinase family, and the TGF-β

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signals through a heteromeric complex between the type I and II receptors.[7)10)14)] Binding of the TGF-β to the type I receptor requires the presence of the type II receptor, whereas the type II receptor binds to the TGF-β independently.[10)14)]

As a consequence of developments in the field of molecular biology, strategies targeting TGF-β have recently been developed. For example, ribozyme oligonucleotides cleave the mRNA of the TGF-β, leading to its decreased expression.[15)] An adenoviral vector, expressing the ectodomain of the type II TGF-β receptor (AdTβ-ExR) inhibits the action of TGF-β both in vitro and in vivo.[16)17)] This soluble receptor is also known to reach remote areas by means of systemic circulation.

The Infiltrator™ is a balloon catheter, with 21 injector ports in three lines, designed for direct intramural injection of a desired agent. Previous studies using an Infiltrator™, in general, agree that it is an effective tool for intravascular drug or gene delivery, and we confirmed the efficacy of gene expression using the Infiltrator™.[18)]

In this study, we tested the hypothesis that inhibition of TGF-β, by a catheter-based in vivo local gene delivery of a recombinant adenovirus expressing an ectodomain of the TGF-β type II receptor, inhibits in-stent neointimal formation in porcine coronary arteries. A pathological analysis revealed the possible role of TGF-β in the ECM accumulation in the formation of in-stent neointima.

Methods

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 a β-galactosidase (AdCALacZ) under a CA promoter, composed of a cytomegalovirus enhancer and a chicken β actin promoter, was constructed as described previously.[19)20)] The TGF-β type II soluble receptor is known to be secreted from AdTβ-ExR-infected cells, bind to TGF-β and inhibit its signaling.[17)] Both AdTβ-ExR and AdCALacZ were used as adenoviral vectors for the delivery of the gene into the porcine coronary arteries. The titer of virus was quantified by a plaque formation assay, using 293 cells, and expressed in plaque formation units (pfu).

Local gene delivery and stenting

Thirteen domestic female pigs (2−3 month old, weighing 25−30 kg) underwent coronary artery intravascular gene delivery using the Infiltrator™ (Interventional Technologies, San Diego, CA), with subsequent intracoronary stent (n=10) deployment. All animal care and handling were performed in accordance with the guideline specified by the National Institute of Health Guide for the Care and Use of Laboratory animals, and were approved by the Animal Care and Use Committee of Yonsei University. Animals took 100 mg/d Aspirin and 75 mg/d Clopidogrel 24 hours prior to the procedure, and continued to take these medications until sacrificed. Before anesthesia, the pigs were subjected to an intramuscular injection of Atropine (0.04 mg/kg) and Rompun (2 mg/kg). After endotracheal intubation, anesthesia was induced by inhalation of 2.5% Enflurane. Heparin (8,000 IU) was injected intravenously before the coronary arterial intervention. An 8 F Judkins right coronary artery guide catheter was inserted through the left carotid artery. The coronary arterial segments in either the left anterior descending (LAD), left circumflex (LCX) or right coronary artery (RCA), feasible for the intravascular gene delivery using the Infiltrator (3.0−3.5 mm), were selected. An infiltrator™ was used for the injection of 400 μL of either the adenoviral vector or phosphate buffered saline (PBS), following the inflation of the Infiltrator™ to 2 atmosphere. Coronary angiograms were performed before, immediately after and 4 weeks after the gene delivery. An intracoronary injection of isosorbide dinitrate (200 μg) was administered prior to the angiogram to relieve any possible vasospasm. To identify the optimal titer of adenoviral vector for an effective gene transfer, three different titers for the AdTβ-ExR (1×10^8, 5×10^8, and 1×10^9 pfu in 400 μL PBS) and two differ-

ent titers for the AdCALacZ (2.5×10^7 and 2.5×10^8 pfu in 400 μL PBS) were randomly injected into each coronary arterial segment of three pigs. The rest of pigs (n=10) underwent intravascular injections of AdTβ-ExR (1×10^9 pfu) and AdCALacZ (1×10^9 pfu)/PBS in two different segments per pig. After the gene delivery, a Palmaz-Schatz coronary stent was deployed in each treated arterial segment (9−11 atm, balloon/artery= 1.1). After the stenting catheters were removed, the left carotid artery was ligated.

To identify the efficiency of the gene transfer, three pigs were sacrificed 1 week after the delivery. Three out of the 10 pigs in which stents were deployed died due to complications during the procedure (intravascular thrombosis and spasm), therefore seven pigs were sacrificed 4 weeks after the delivery. Coronary arterial segments from each adenovirus injected site were retrieved. Thoracic aortae and remote coronary arterial segments were used as negative control tissues. The coronary arteries dissected 1 week after the gene delivery were snapfrozen in liquid nitrogen, and divided into two pieces: one embedded in OCT for cryostat sectioning and the other kept at −70℃ until the 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 stented arterial segment, identified by an angiography, to have a higher degree of stenosis, underwent tissue processing with Kulzer Histotechnik 8100 (Heraeus Kulzer, Germany) for morphometric analysis. The other segment was embedded in paraffin for various pathological staining.

Histochemical analysis for β-galactosidase

The pigs were sacrificed 1 week after the gene delivery with a lethal dose of sodium pentobarbital. The coronary arterial segments were excised, fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 2 h at 4℃, embedded in OCT compound, and subjected to cryostat sectioning at 20 μm thicknesses. The 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) at 37℃. The tissue sections were subsequently counterstained with hematoxylin-eosin.

Reverse transcription polymerase chain reaction (RT-PCR)

The total cellular RNA from each arterial segment was isolated using an RNeasy mini kit (Qiagen, Germany). For the reverse transcription and PCR reactions, a Perkin-Elmer/Cetus DNA thermal cycler (Foster city, CA) was used. Two microgram of the total RNA was reverse transcribed in a 25 μL reaction containing 5 μL reverse transcriptase buffer, 25 mM dNTPs, random hexamers (0.5 μg/μL), 20 units RNase inhibitor and 200 units MMLV reverse transcriptase at 37℃ for 1 h followed by 5 min at 70℃. The PCR amplification was performed in a 50 μL volume using 2 μL of the reverse transcription reaction plus 1 unit of Taq polymerase, 10×buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$ and 0.2 μM of each primer, under following conditions: 94℃ for 3 min; followed by 35 cycles of 30 sec at 94℃, 30 sec at 54℃, and 1 min at 72℃; followed by 7 min at 72℃. The primer sequences and the expected product sizes were as follows: TGFβ type II receptor: sense primer 5´-ACATCGTCCTGTGGACGCGTA-3´, antisense primer 5-CTAGCAACAAGTCAGGATTGC-3´, with an expected size of 450 bp; and 28S RNA: sense primer 5´-TTAAGGTAGCCAAATGCCTCG-3´, antisense primer 5´-CCTTGGCTGTGGTTTCGCT-3´, with an expected size of 102 bp. The PCR products were separated on a 1.2 % agarose gel, stained with ethidium bromide, and analyzed using an image analyzer (Bioprofil, Viber Lourmat, France).

Immunostaining

The soluble TGF-β type II receptor was identified by direct immunofluorescent staining to detect the human IgG fused to the soluble receptor, using fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako, Carpinteria, CA). Briefly, the frozen sections

were dried, placed in PBS for 10 min, incubated with FITC-conjugated rabbit anti-human IgG at room temperature for 1 h, rinsed with PBS for 10 min, and mounted with glass cover slip. The immunostained slides, with the FITC conjugated anti-human IgG complex, were assessed using an immunofluorescent microscope, and photographed immediately. T cells were identified in specimens taken at both 1 and 4 weeks after the gene delivery by immunohistochemical staining with rabbit polyclonal anti-human CD3 antibodies (Dako). The TGF-β1 positive cells were identified by immunohistochemical staining of the tissue specimens, taken 1 week after the delivery of the PBS, using the rabbit anti-porcine TGF-β1 polyclonal antibody (Cell Sciences Inc., Norwood, MA). Biotinylated link antimouse and antirabbit antibodies (Dako) were used as secondary antibodies for both T cell and TGF-β1 staining, with further staining performed using the avidin-biotin-peroxidase technique (Vectastain, Vector Lab., Burlingame, CA) and DAB (3, 3´-diaminobenzidine tetrahydrochloride, Sigma). Hyaluronan labeling was performed with biotinylated hyaluronan binding protein (gift from Dr. Underhill), as previously described.[5)]

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 (IEL area), the external elastic lamina (EEL area) and the stent (stent area), were measured in the minimal luminal area (MLA) site, and neointima (IEL area-lumen area) and media areas (EEL area-IEL area), the ratio of neointima/media areas, the percentage of the stenosis area ([neointima area/IEL area] $\times$100) were calculated. The extent of vessel wall injury induced by the stent was calculated as the mean injury score by the method of Schwartz et al: mean injury score = sum of weights for each wire/number of coil wires present.[21)] The modified Movat pentachrome[22)] and hematoxylin-eosin stains were used to identify the general histology and ECM components.

Statistical analysis

All statistics were calculated using SPSS ver. 10.0 for Windows (SPSS Science), and all data are expressed as the mean$\pm$SD. The morphometric parameters, cell density, cell proliferation rate and mean injury score were compared between the groups by a paired t test. A value of $p<0.05$ was used to indicate statistical significance.

Results

Identification of human TGF-β type II receptor gene

Three pigs were sacrificed 1 week after the gene delivery to analyze the efficiency of the gene transfection. As shown in Figure 1, the RT-PCR products from the porcine coronary artery (d-LAD) injected with AdTβ-ExR at 1×10^9 pfu showed a definite band of human TGF-β type II receptor gene product, while the artery (p-LAD) injected with 5×10^8 pfu showed only a faint band of the gene product. Neither arterial segments adjacent to the AdTβ-ExR injected site (m-LAD), as shown in Figure 1, nor that injected with the AdTβ-ExR at 1×10^8 pfu (data not shown) revealed any detec-

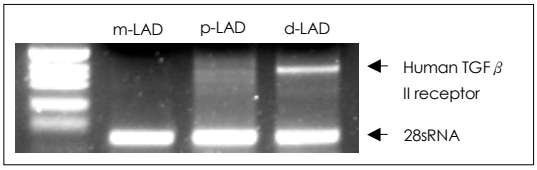

Figure 1. RT-PCR analysis for the detection of a transferred soluble TGF β type II receptor mRNA in each porcine coronary artery. The RT-PCR analysis showed the amplified gene product from the distal LAD injected with 1×10^9 pfu AdTβ-ExR. The proximal LAD injected with 5×10^8 pfu AdTβ-ExR showed a faint band of the soluble receptor gene product, whereas, the mid LAD in between the two injection sites showed no transgene product. Note the bands of 28S RNA product from each arterial segment, which verified the quantitative analysis of the human TGF-β type II receptor gene product. RT-PCR: reverse transcription polymerase chain reaction, LAD: left arterior descending artery.

table bands. These results suggest that the local gene delivery of the soluble human TGF-β type II receptor was successfully carried out using the Infiltrator, with no significant dissemination of the adenovirus vector. The RT-PCR products of the 28S RNA from each artery showed bands of equal density, which verified the quantitative analysis of the human TGF-β type II receptor gene product.

Identification of soluble receptor and β-galactosidase

Direct immunofluorescent staining, using FITC-conjugated rabbit anti-human IgG, identified the soluble TGF-β type II receptor. As shown in Figure 2, the coronary artery injected with 5×10^8 pfu AdTβ-ExR had multiple dispersed, immunofluorescent positive, soluble receptor-antibody complex particles around the media and adventitia areas. The soluble TGF-β receptor was more widely dispersed compared with the distribution of the β-galactosidase. Conversely, the remote arteries including the remote coronary artery and thoracic aorta had no discernable immunofluorescent positive particles. Thus, it is conceivable that the soluble receptor, delivered by a certain adenoviral vector, could be expressed locally, at the site of gene delivery, with no significant spread to remote sites. The gene transfection was also studied by an immunohistochemical assay of β-galactosidase in the arteries injected with the two different titers of AdCALacZ (2.5×10^7 pfu and 2.5×10^8 pfu). As shown in Figure 3, the expression of β-galactosidase was noted locally around the outer media and adventitia areas, at the arterial segment injected with the AdCALacZ at 2.5×10^8 pfu, and there was no significant spread of the β-galactosidase to the remote arterial segments. The coronary arterial segments injected with 2.5×10^7 pfu AdCALacZ showed no β-galactosi-dase expression (data not shown).

Morphometric changes after AdTβ-ExR treatment

Seven arteries from each of the AdTβ-ExR treated and control (either AdCALacZ treated or PBS) groups were analyzed for morphometric parameters. Since we found that either AdCALacZ or PBS has no significant effect on the artery, both AdCALacZ and PBS treated arteries were analyzed together as a control group. A pair of arteries was excluded from the statistical analysis, since the injury score was significantly different from the others. The results of the morphometric analysis are summarized in Table 1. The arteries treated with AdTβ-ExR had a significantly less neointimal area (1.57 ± 0.49 mm^2 vs. 2.13 ± 0.34 mm^2, $p<0.01$) and smaller area ratio of neointima/media (0.84 ± 0.44 vs. 1.32 ± 0.48, $p<0.01$) compared with the controls. There was no sig-

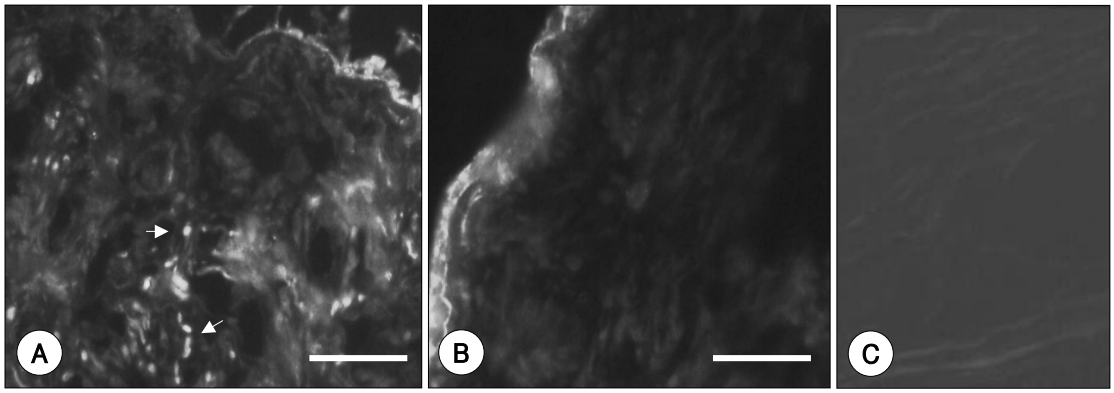

Figure 2. Immunohistochemical staining of the porcine coronary arteries for detection of the soluble TGF β type II receptor, with FITC-conjugated rabbit anti-human IgG. A) There are multiple dispersed tiny immunofluorescent positive particles (marked as arrows) of the soluble TGF β type II receptor located in the media and adventitia of the distal LAD injected with 5×10^8 pfu AdTβ-ExR. B and C) The right coronary artery of the same pig had no discernable soluble receptor: with (B) and without (C) FITC conjugated antibody, bar=50mm. TGF: transforming growth factor, LAD: left arterior descending.

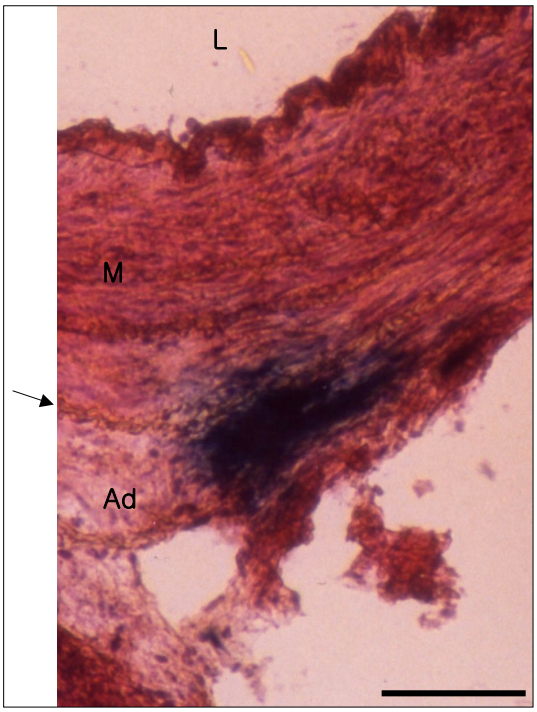

Figure 3. A porcine coronary artery injected with 2.5×108 pfu AdLacZ shows β-galactosidase activity, as identified by histochemical staining with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D galactopyranoside). Note the blue stained area showing the β-galactosidase expression in the outer media and adventitia. The arrow shows the external elastic lamina. Counterstaining with hematoxylin-eosin. Ad: adventitia, L: lumen, M: media.

nificant difference between the two groups in relation to the IEL, EEL, media areas, and their injury scores.

Pathological characteristics of in-stent restenotic tissue

Smooth muscle cells (SMC) were identified as the most common cell comprising the neointima, and there were variable numbers of macrophage like cells, eosinophils and multinucleated giant cells, especially around the stent struts (Figure not shown). The neointima cell density (cells/mm^2) in the AdTβ-ExR treated group was significantly greater than that in the control group (3,121±330 vs. 2,812±183, $p<0.05$), whereas the cell density of the media was not different between the two groups (2,123±309 vs. 2,205±245, p=NS). The calculated number of total cells per section in the neointima were

Table 1. Comparison of the morphometric parameters between the AdTβ-ExR and control groups

	AdTβ-ExR	Control
Lumen (mm^2)	3.81±1.45	3.95±1.00
intima (mm^2)	1.57±0.49 *	2.13±0.34
Media (mm^2)	2.06±0.70	1.75±0.57
Intima / media	0.84±0.44 *	1.32±0.48
Stent (mm^2)	5.72±0.70	5.99±1.04
EELA (mm^2)	7.44±1.34	7.81±1.02
% area stenosis	31±11	36 ±8
Injury score	0.93±0.31	0.80±0.33

Data are expressed as the mean±SD. EELA: external elastic lamina area, *: $p<0.01$ vs. control

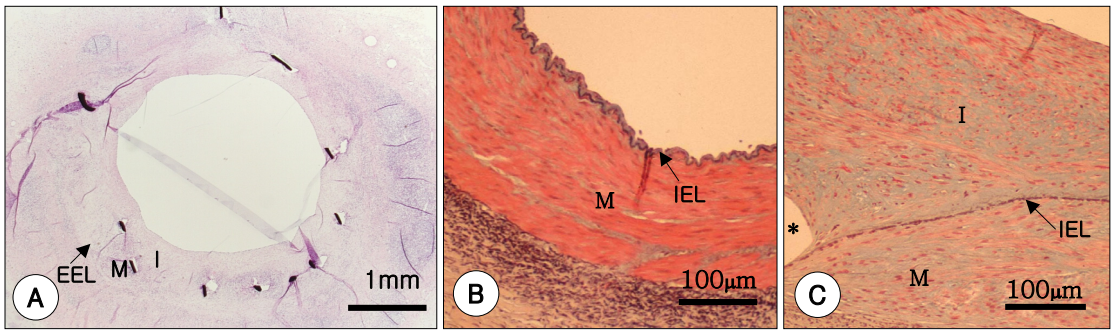

Figure 4. A: an example of a stented porcine coronary artery showing an in-stent neointima formed inside the stent struts. B: a normal porcine coronary artery showing a thin endothelial layer inside the internal elastic lamina. C: a stented porcine coronary artery showing intimal and medial ECM enriched with proteoglycans (blue color). I: intima, M: media, IEL: internal elastic lamina, *: stent strut (A: hematoxylin and eosin stain, B and C: modified Movat pentachrome stain). ECM: extracellular matrix.

significantly less in the AdTβ-ExR treated group compared with the control group (4,929±1762 vs. 5,998 ±1201 respectively, p<0.05). The cell proliferation rate of the AdTβ-ExR treated neointima at 4 weeks after stenting, as measured by the PCNA index ([PC-NA positive cells/total cells] ×100 (%)), was significantly greater than that of the normal intima (3.22±2.55% vs. 0.48±0.47%, p<0.05), but was not significantly different from that of the neointima of the control group (1.36±1.46%). The Movat pentachrome staining showed proteoglycans to be abundant in the in-stent neointima of both groups, as shown in Figure 4, and the occasional interruption of either the IEL, or EEL, was noted. Hyaluronan was found in the in-stent neointima, frequently around the stent struts with a high injury score, and to a lesser extent, in the media. Four of the six AdTβ-ExR treated arteries had less hyaluronan stained area compared with the control group (Figure 5). The CD3 positive T cells infiltrated around the adventitia and, to a lesser extent, the media in arteries 1 week and 4 weeks after injection of either AdCALacZ or AdTβ-ExR (Figure 6). Generally more T cells had infiltrated the segments injected with the AdTβ-ExR compared with the AdCALacZ. The remote coronary artery had no CD3 positive T cells (Figure 6).

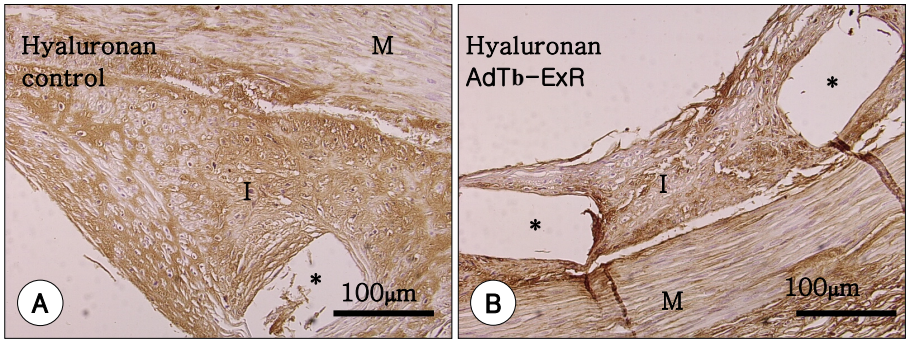

Figure. 5. The distribution of hyaluronan in a stented porcine coronary artery. The distribution of hyaluronan (brown color) in the neointima is less in the AdTβ-ExR treated artery (B) compared with a control (A). Counterstaining with hematoxylin. I: intima, M: media, *: stent strut.

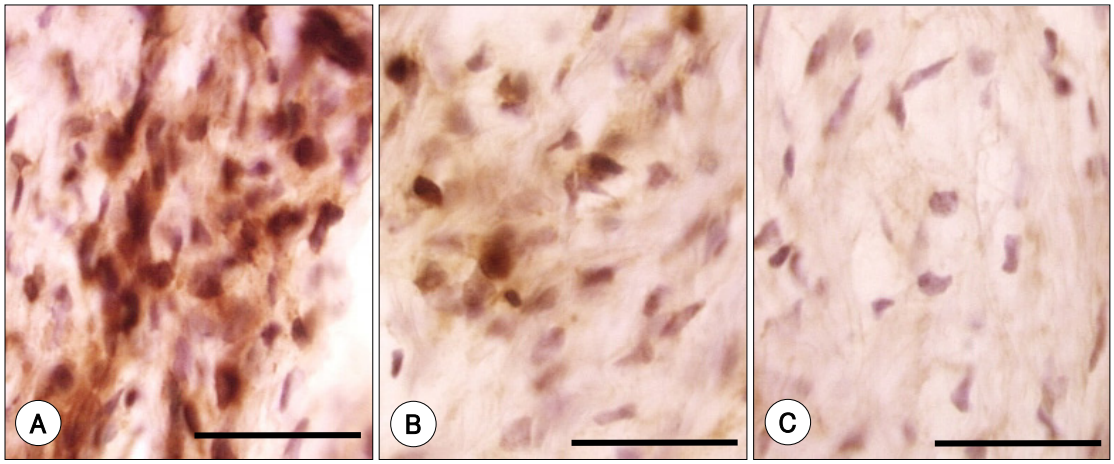

Figure 6. Infiltrated CD3 positive T cells in the porcine coronary arteries injected with each of the adenovirus vectors. Arteries 1 week after injection with either AdLacZ (2.5×10^8 pfu) (A) or AdTβ ExR (5×10^8 pfu) (B) shows CD3 positive T cells (brown color) infiltrated into the adventitia, whereas the remote control artery (C) has no CD3 positive cells. Counterstaining with hematoxylin. bar=50 μm.

Discussion

The main result of our study is that the inhibition of TGF-β signaling, using an adenoviral vector expressing a soluble TGF-β type II receptor, inhibits in-stent neointima in porcine coronary arteries. To address the possible mechanisms of this inhibition, we carried out pathological analyses on stented arteries. An in-stent neointima, treated with AdTβ-ExR, showed significantly less area, less total cell numbers and a greater cell density compared with the control group, with no significant difference in the cell proliferation rates between the two groups. The TGF-β1 positive cells were abundant in the neointima and media of our stented porcine coronary arteries in both groups, which verified the rationale of this as a targeting molecule. These data observed in AdTβ-ExR treated group are most likely the result of the reduced ECM accumulation. An increased cell death and reduced cell migration are other possible factors to explain our data. The former concept is supported by a previous in vivo study that addressed the association of the withdrawal of the TGF-β1 and increased apoptosis.[12] Similarly to our data, a balloon injured rat artery, treated with a soluble rabbit TGF-β type II receptor, showed significantly less neointima area, less total number of intima cells, and a tendency for a greater intimal cell density.[16] This study also showed that the soluble TGF-β type II receptor inhibits negative vascular remodeling by reducing adventitial collagen deposition, and subsequently induced a larger lumen in the injured rat artery. In another study, the truncated human exoplasmic domain of the TGF-β type II receptor also reduced the luminal loss at the MLA, in part, by inhibiting the negative vascular remodeling in balloon injured porcine coronary arteries, but this mutant receptor increased the adventitial collagen content.[23] The difference in the collagen content between these two studies may be related to the different experimental designs and animal models, but the exact cause remains uncertain.

ECM accumulation may play a key role in the formation, over time, of a human coronary in-stent neointima following stenting.[4][5] TGF-β1 has been implicated in the regulation of the coding of genes for ECM components.[8][24][25] Its expression was also significantly higher in restenotic lesions compared with primary lesions.[26] TGF-β also enhances the ECM accumulation by inhibiting the extracellular proteolysis and ECM degradation,[27] as well as the synthesis of various ECM components. Arteries overexpressing TGF-β1 are known to develop significant cellular and matrix rich neointimas.[12] There were increases in proteoglycan, procollagen and collagen synthesis in the TGF-β1 transfected artery, and the withdrawal of the TGF-β1 contributes to the neointimal regression, coupled with increased apoptosis.[8][12] Blocking of the TGF-β1, by either neutralizing antibodies[25] or by soluble TGF β type II receptor,[16] significantly reduced the size of the neointima in the balloon injured rat artery.

Although we were not able to identify any significant differences in the cell proliferation rates between both groups, TGF-β1 is known to affect cell proliferation. TGF-β1 inhibits the cell proliferation, by either extending the G2 phase[28] or arresting the G1 phase.[29] but other in vitro data indicated that the cell proliferation was affected according to the dose of TGF-β1 administered.[30][31] The data from in vivo studies are also confusing. TGF-β1 transfected rat arteries showed significantly greater intimal and medial cell proliferation rates compared with the controls 4 weeks after transfection,[12] whereas, inhibition of the TGF β signaling, using the soluble TGF β type II receptor, did not significantly change the cell proliferation rate of the rat artery 2 weeks following the balloon injury.[16] The lack of cell proliferation rate data for an earlier time period following the stenting in our study is a limitation that clearly needs to be addressed with this proliferation issue.

We identified proteoglycans/hyaluronan and collagen as the dominant ECM components making up the neointima and adventitia, respectively, in both groups. However, the distribution of hyaluronan in the in-stent neointima was less in 4 of the 6 AdTβ-ExR treated arteries compared with the controls. Proteoglycans and hyaluronan were frequently observed in human in-stent reste-

notic tissue at an early period following the stenting.[5] Hyaluronan has been implicated in the migration of SMCs, and the expression level in the cell surface receptors for hyaluronan mediated motility (RHAMM) is correlated with the locomotion of the SMCs.[32] Hyaluronan and proteoglycan versican are both regulated by TGF-β and have the ability to bind water (hygros-copic property) and therefore, by interacting with each other, may play an important role in the development of intimal thickening, by expanding the tissue space into which the cells can migrate easily. In our study, the stented arteries occasionally showed SMCs led across the interrupted IEL or EEL (figure not shown), where the ECM, enriched with hyaluronan and proteoglycans, were frequently found. This observation suggests a possible role for cell migration in the formation of an in-stent neointima. Although cell migration in the vessel wall has no reliable marker, our human aortic clonality study, addressing the high prevalence of homologous clonality relation in between intima and underlying media, suggests that the intima probably originates from the underlying media.[33] With regard to this, a smaller number of total intimal cells and a lesser distribution of hyaluronan in the neointima of the AdTβ-ExR treated arteries, compared with the controls, raises the possibility that the inhibition of the neointimal formation in the AdTβ-ExR treated arteries may be a result of the inhibition of cell migration.

Our experiment had certain limitations. Three pigs died during the Infiltrator^{TM} procedure. Drug delivery with an Infiltrator^{TM} occasionally ruptured vascular tissue, and caused intramural thrombosis and spasms.[18] Additionally, the host immune response, dose-dependent inflammatory and cytopathic effects, inhibit adenoviral gene expression, and impose limitations on the gene delivery.[34-36] Therefore, identification of the vector effective for gene expression, with a minimal inflammatory reaction, seems to be a crucial issue. Our experimental design unavoidably induced vascular injury, caused by the Infiltrator^{TM} and immunological reactions against the viral vector, all of which probably affected the vascular response to some extent. In conclusions, our study suggests that inhibition of the TGF-β signaling, caused by the catheter-based in vivo gene transfer of a soluble TGF-β type II receptor, inhibits stent neointimas in porcine coronary arteries, probably by inhibiting the ECM accumulation. A strategy for the inhibition of the TGF-β signaling may have therapeutic potential for the prevention of restenosis following stenting.

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