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Stent-based delivery of decoy oligodeoxynucleotides against activator protein-1 binding site decreased restenosis in rabbits

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Abstract: The application of drug eluting stents (DES) significantly reduced the rate of restenosis but is associated with increased risk of late stage thrombosis. Thus, development of more effective and safer drugs for in stent delivery to inhibit restenosis is of great clinical interest. Decoy oligodeoxynucleotides against activator protein-1(AP-1) binding site (dec-ODN) delivered with one time infusion or transfer have been shown to effectively inhibit neointimal proliferation and thickening. In this study, we delivered dec-ODN against AP-1 in-stent and examined the inhibitory effects on restenosis. Synthetic dec-ODN targeting consensus AP-1 binding site was coated onto DESs. Thirty male rabbits were randomly divided into three groups: Control stent (CS), scrambled oligodeoxynucleotides (scr-ODN) stent and dec-ODN stent groups with 10 rabbits in each group. All stents were implanted in the abdominal aorta of rabbits. Eight weeks after stent implantation, the neointimal hyperplasia and re-endothelialization in the abdominal aortas of implanted segments were examined as well as the expression of TGF-β1 and TGF-β1 target gene connective tissue growth factor (CTGF). Dec-ODN delivered in-stent significantly inhibited the mRNA and protein expression of TGF-β1 and CTGF, as well as neointimal thickening and restenosis in abdominal aortas as compared to control (CS) and scr-ODN. Re-endothelialization was not evidently affected by the delivery of Scr-ODN and dec-ODN. Our data demonstrate that in-stent delivery of dec-ODN against AP-1 effectively inhibited neointimal hyperplasia and support further investigation of DES/dec-ODN against AP-1 as a potential long-term therapeutic agent against in-stent restenosis.

1. Introduction

Although the application of stents has dramatically improved the survival of patients with coronary artery diseases, the formation of in-stent restenosis with bare-metal stents(BMS) has a overall occurrence rate of about 20% (Dangas et al., 2010; Ip et al. 1991) Recently, a new generation of stents, drug-eluting stents (DESs) has been developed and applied in clinical practice. DES further reduced the rate of restenosis to below 10%, but early generation DES increase the risk of in-stent thrombosis (Laskey et al. 2007; Serruys et al. 2006; Shuchman, 2006). For example, DESs coated with sirolimus or paclitaxel have been demonstrated to be effective in preventing in-stent restenosis by inhibiting VSMCs proliferation. However, patients with these stents developed sub-acute or late thrombosis due to the inhibition of endothelial cell proliferation and delayed re-endothelialization (Farooq et al. 2011; Fishbein et al. 2008). An effective drug that inhibits restenosis with improved safety for in-stent delivery is of great clinical interest.
Various drugs and drug candidates including antisense oligonucleotide (Bennett and Schwartz 1995) have been investigated for in-stent delivery to prevent restenosis (Khan et al. 2012; Kipshidze et al. 2005). For example an antisense against PDGF-A was reported to decreased in-stent restenosis in a pig model (Li et al., 2006). Recently, decoy ODNs, which reduce the *trans*-activity of transcription factors have been applied as an innovative and attractive strategy for gene therapy. Decoy ODNs against various transcription factors such as EGR-1, E2F and NF-κB have been shown to be effective in reducing the proliferation of smooth muscle cells and restenosis in different animal models (Han and Liu 2010; Hashiya et al. 2004; Takeuchi et al. 2007). However, no study has been reported, to the best of our knowledge, on the prevention of in-stent restenosis using dec-ODN delivered in-stent.
Activator protein-1 (AP-1) is a dimerized complex of c-Jun and c-Fos which is activated by MAPK signaling (Karin 1995; Whitmarsh and Davis 1996). AP-1 binds specific DNA sequences present in a large number of genes associated with cell proliferation including SMC mitogen TGF-beta and Endothelin (Buchwald et al. 2002; Karin 1995; Kume et al. 2002; Whitmarsh and Davis, 1996). It has been reported that ERK and JNK are activated after balloon injury suggesting important role of AP-1 on restenosis (Hu et al. 1997; Pyles et al. 1997). Targeting AP-1 with decoy ODN has been shown to inhibit the expression of TGF-beta and ET-1 and neointimal proliferation in rabbit and pig model (Buchwald et al. 2002; Kume et al. 2002). Thus decoy ODN against AP-1 represent a good candidate for in-stent delivery to inhibit restenosis. In this study, we assessed the effects

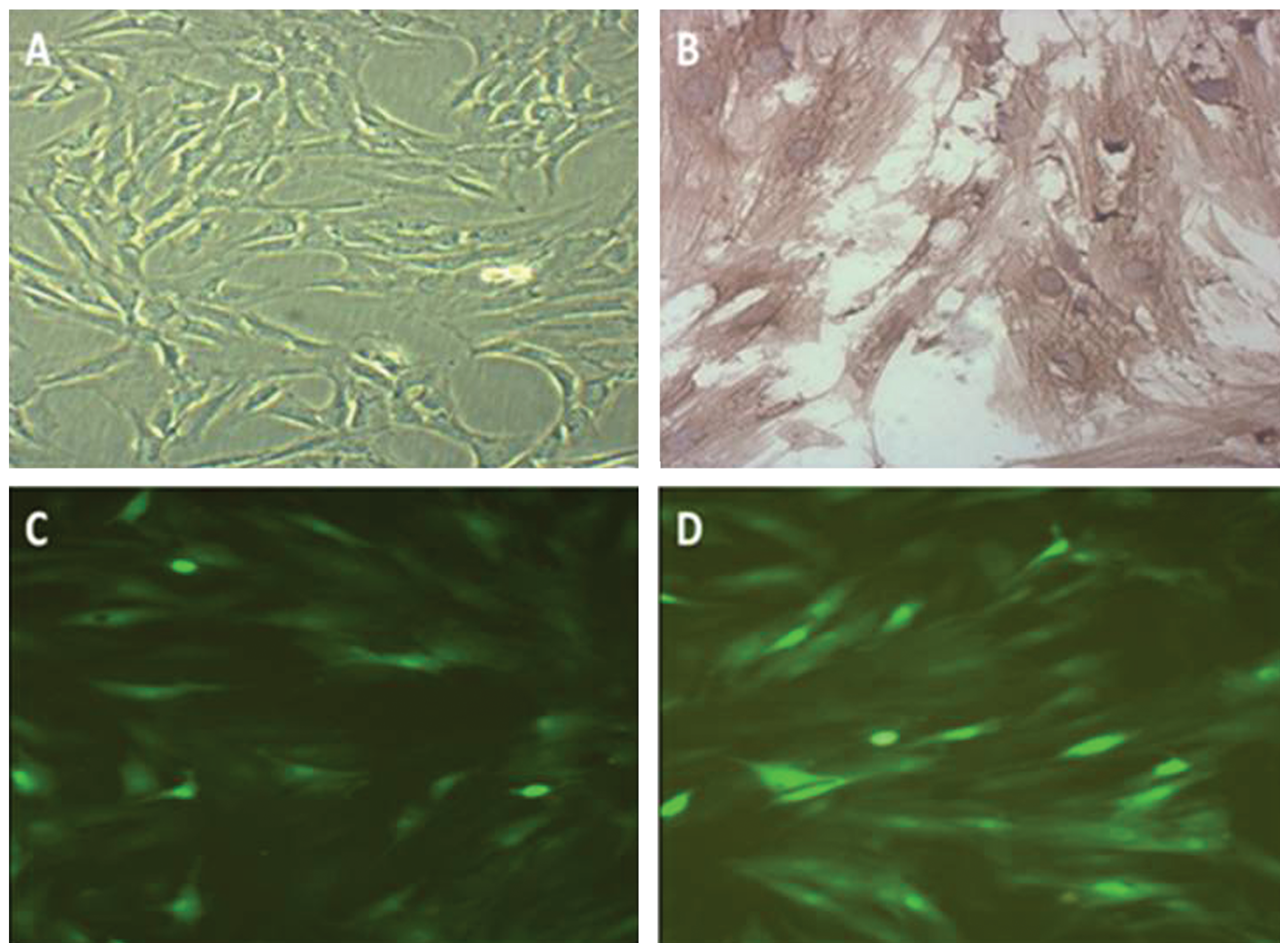

Fig. 1: Validation of decoy ODN uptake by primary rabbit vascular smooth muscle cells (VSMC). (A) Morphology of isolated primary rabbit VSMC. (B) Immunohistochemistry Showing the expression of SMC marker α-actin in primary rabbit VSMC. The distribution of FITC-labeled dec-ODNs examined with fluorescence microscopy (C, D). Incubation of primary rabbit VSMC with 10 μg/ml of FITC-labeled dec-ODNs for 48 hours (C) and 72 hours (D) displayed prominent presence of fluorescence inside the cells. (Magnification: 100x).

of decoy ODN targeting AP-1 consensus sequence delivered in-stent on neointimal formation in rabbit model. The expression of TGF-β1 and TGF- β1 target gene connective tissue growth factor (CTGF), and re-endothelialization were also examined.

2. Investigations and results

2.1. *Distribution of ODN in vascular smooth muscle cells*

Isolated primary rabbit vascular smooth muscle cells(VSMC) demonstrated the characteristic morphology of smooth muscle cells under the light microscope (Fig. 1A). The cells were positively stained with antibody against α-actin, marker of smooth muscle cells (Fig. 1B). To test whether ODN could be taken by primary VSMCs, FITC-labeled dec-ODN was synthesized and incubated with primary rabbit VSMCs at the concentration of 10 μg/ml. dec-ODN was prominently up-taken into primary VSMCs after 48 hours incubation (Fig. 1) and the uptake was further increased with 72 hour incubation (Fig. 2).

2.2. *dec-ODN against AP-1 delivered in DESs inhibited the neointimal formation and restenosis*

As dec-ODN could be effectively introduced into primary VSMCs *in vitro*, we then investigated whether the dec-ODN delivered in DESs could prevent the development of in-stent restenosis. Eight weeks after DES implantation, the abdominal aorta sections were stained with H &E (Fig. 2A-C) and elastic staining (Fig. 2 D-F) to evaluate the neointimal formation and in-stent restenosis. dec-ODN delivery significantly reduced neointimal hyperplasia (NIH) by 61.6% ($n = 10$, $p < 0.001$, Fig. 2G) as measured by neointimal area (mm^2) and reduced the area ratios of intima to media by 66.3% ($n = 10$, $p < 0.001$, Fig. 2H) as compared to the scr-ODN. Dec-ODN also significantly attenuated restenosis (control group = 42.2 ± 3.0%, scr-ODN = 42.0 ± 3.1%, dec-ODN = 23.0 ± 1.5%, $n = 10$, $p < 0.001$, Fig. 2I). Thus, in-stent delivery of dec-ODN against AP-1 significantly decreased in-stent restenosis.

2.3. *dec-ODN against AP-1 inhibited the expression of TGF-β1 and CTGF*

TGF-β1 is intimately involved in stenosis and restenosis. Its promoter has AP-1 binding sequence (Birchenall-Roberts et al, 1990) and is the target gene of PDGF which stimulate the proliferation of VSMC (Kume et al. 2002). To see if dec-ODN inhibited restenosis involved in changed TGF-β1 expression, we measured the expression of TGF-β1 mRNA and protein in the aortic segment of DES implantation. Dec-ODN decreased TGF-β1 mRNA (Fig. 3A) and protein expression (Fig. 3B) by 53.8% and 56.1% respectively as compared to scr-ODN. As confirmation of decreased TGF-β1 expression, the expression of CTGF, a target gene of TGF-β1, was also significantly reduced by dec-ODN (Fig. 3C,D). These data suggest that in-stent delivery of dec-ODN against AP-1 efficiently suppresses the expression of TGF-β1 at both transcriptional and translational levels.

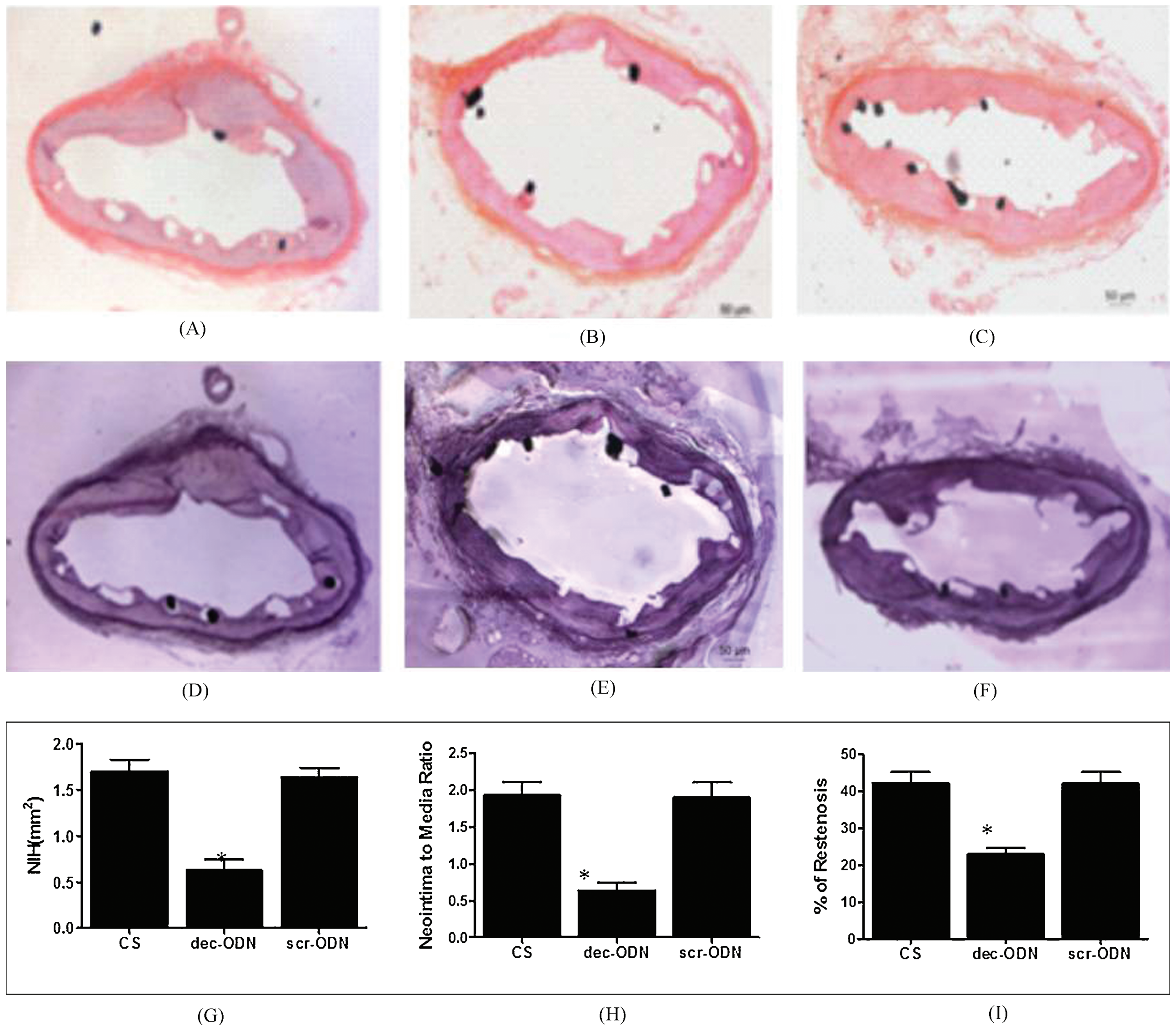

Fig. 2: Delivery of dec-ODN against AP-1 in DESs prevented the neointimal formation and in-stent restenosis in abdominal aorta. Eight weeks after DES implantation. Abdominal aortic sections were stained with hematoxylin–eosin staining (A-C) and Weigert's elastic staining (D-F). Cross-sectional area of intima and media and intima/media area ratios were measured and analyzed with a computerized apparatus and NIH ImageJ. Rabbit group implanted DES with dec-ODN against AP-1 (dec-ODN) showed significantly reduced neointimal hyperplasia (NIH) (G), intima/media ratios (H) and in-stent restenosis (I) as compared to the control group(CS) and scramble ODN group (scr-ODN). * $p < 0.001$ vs CS or scr-ODN, $n = 10$. (Magnification: 40x).

2.4. dec-ODN delivered in DESs did not affect re-endothelialization in the target artery

Delayed re-endothelialization has been reported after implantation of DESs in some studies, which may be related to the presence of increased stent thrombosis (Joner et al., 2006). We compared the impact of dec-ODN on the re-endothelialization after implantation of stents with or without dec-ODN under scanning electron microscope. No significant difference was observed among the three groups (Fig. 4), suggesting that the delivery of dec-ODN in DESs did not affect the re-endothelialization.

3. Discussion

In the current study, we demonstrated that in-stent delivery of dec-ODN against AP-1 suppressed neointimal formation and in-stent restenosis in rabbits. Dec-ODN against AP-1 inhibited the expression of TGF-β1 and TGF-β1 target gene CTGF but did not affect re-endothelialization after angioplasty.

Restenosis is characterized by neointimal formation due to vascular smooth muscle cell (VSMC) hyperplasia (Marx et al. 2011). Neointimal formation is a complex process in which a variety of factors and signaling pathways are involved (Curcio et al. 2011; Marx et al. 2011). Various studies have investigated the effectiveness of targeting different factors and pathways involved in stenosis (Curcio et al. 2011; Khan et al., 2007; Marx et al. 2011; Papafaklis et al. 2012). Activation of AP-1 has been demonstrated in injured arteries (Hu et al. 1997; Kim et al. 1998; Pyles et al. 1997). Growth factors and cytokines involved in stenosis such as PDGF and angiotensin have been shown to activate AP-1 (Hu et al. 1999; Kim and Iwao, 2003; Kim et al. 1998). Furthermore, mitogenic molecules of smooth muscle cells such as TGF- β1 and endothelin-1 (ET-1) are target genes of AP-1. Thus AP-1 represents an excellent target of stenosis therapy. Indeed, decoy ODN against AP-1 has been shown to effectively inhibited neointimal proliferation (Buchwald et al., 2002; Kume et al. 2002). In this study, we examined the strategy of delivering decoy ODN against AP-1 in stent on inhibition of restenosis. Our data demonstrated that decoy ODN against AP-1 was efficient in inhibiting restenosis in rabbits when delivered in-stent.

Transforming growth factor- β1 (TGF- β1) is a profibrotic cytokine playing an integral role in promoting intimal thick-

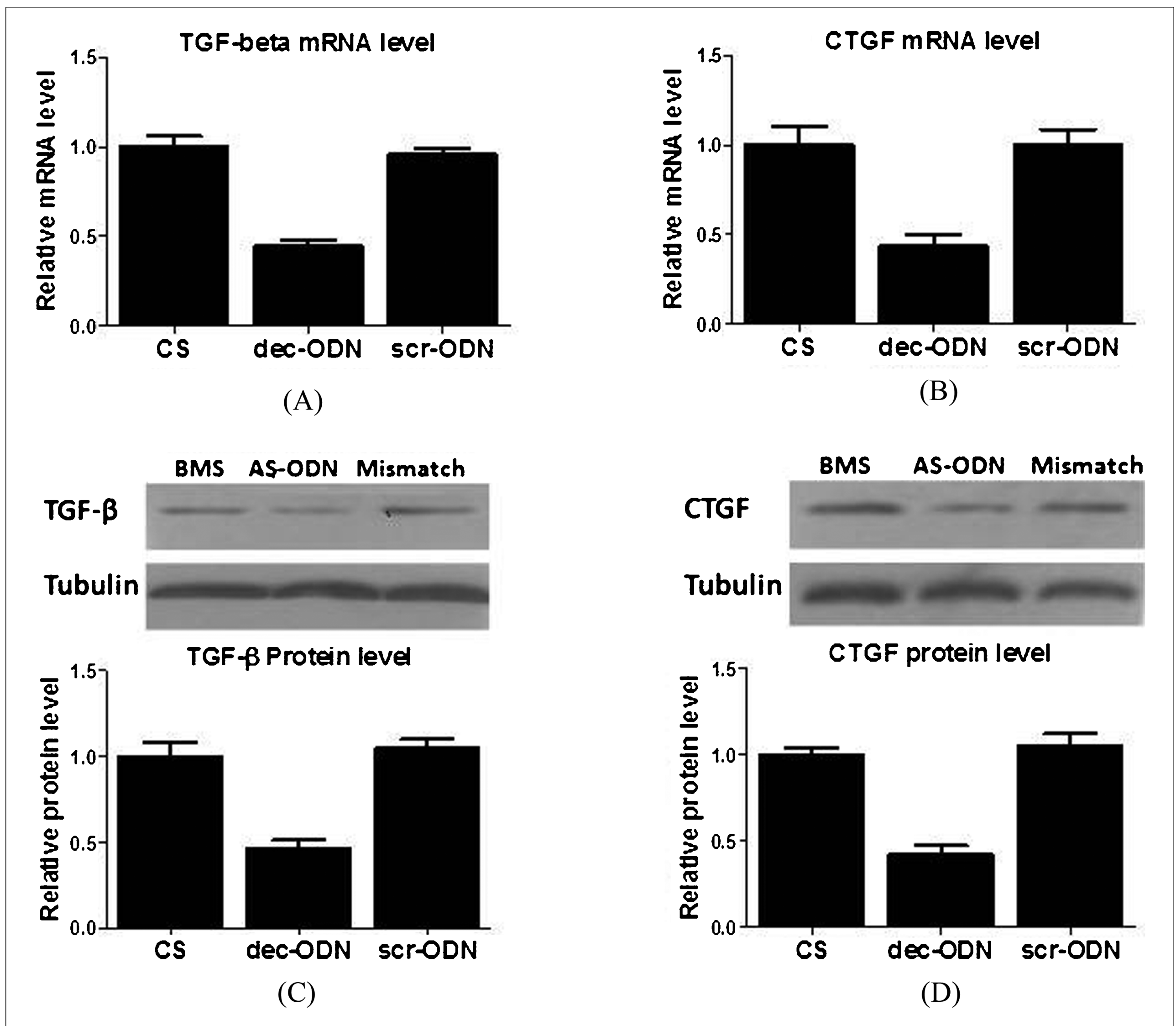

Fig. 3: Delivery of dec-ODN against AP-1 in DESs inhibited the expression of TGF-β1 and CTGF. mRNA and protein were isolated from abdominal aorta of rabbits 8 weeks after DES implantation. The expression of TGF-β1(A) and CTGF(C) mRNA was examined by RT-PCR and quantified with Agilent 2100 Bioanalyzer using GAPDH as internal control. The expression of TGF-β1(B) and CTGF (D) protein was measured by western blot using Tubulin as loading control. Dec-ODN significantly inhibited the expression of TGF-β1 and CTGF. * $p < 0.001$ vs CS or scr-ODN, $n = 10$.

ening (Khan et al. 2007). TGF-β1 expression has been shown to be associated with artery injury and luminal narrowing whereas inhibition of TGF- β1 attenuated intimal thickening and stenosis (Ando et al. 2004; Chamberlain, 2001; Khan et al. 2007; Matsuda et al., 2006). In this study, we observed that TGF-β1 expression was reduced in the aortic segments delivered with dec-ODN against AP-1, confirming the role of TGF- β1 in restenosis and suggesting the effect of dec-ODN against AP-1 on restenosis was mediated at least partially by modulating the expression of TGF- β1. We found that dec-ODN against AP-1 also significantly reduced the expression of TGF- β1 target gene CTGF (Gressner and Gressner, 2008), which is consistent with the inhibition of TGF-β1 by dec-ODN against AP-1. AP-1 signaling is activated by multiple stimuli and regulates the expression of multiple genes. The inhibitory effect of dec-ODN against AP-1 on in-stent restenosis is likely mediated by mechanisms more than modulating TGF- β1 expression. Modulation of other AP-1 target genes like ET-1 may also contribute the inhibitory effects of in-stent delivered dec-ODN on restenosis (Buchwald et al. 2002).

Though DES effectively reduce in-stent restenosis, early generation of DES has the potential risk of late stage stent thrombosis due to impaired re-endothelialization (Bennett 2007; Finn et al. 2005; Guagliumi et al. 2003) with persistent fibrin formation, inflammation and incomplete strut coverage, and delayed, if not incomplete, re–endothelialisation. Restoration of intact endothelium is critical for the prevention of in-stent restenosis and stent thrombosis. In our study, we observed that DES with dec-ODN against AP-1 reduced restenosis without inhibiting re-endothelialization. Thus in-stent dec-ODN against AP-1 may represent a safe and effective DES drug with effectiveness. Taken together, our study indicated that dec-ODN against AP-1 in DES significantly inhibited neointimal formation and in-stent restenosis associated with attenuated expression of TGF-β1 and CTGF and without altering re-endothelialization in rabbits. Our study paves the basis for further studies to evaluate the efficacy and safety of implanting DES with dec-ODN against AP-1 for clinical application.

4. Experimental

4.1. Isolation and culture of primary vascular smooth muscle cells(VSMC)

VSMCs were isolated from the aorta of New Zealand rabbits (purchased from SLAC Laboratory animal, Inc., Shanghai, China). Briefly, the abdom-

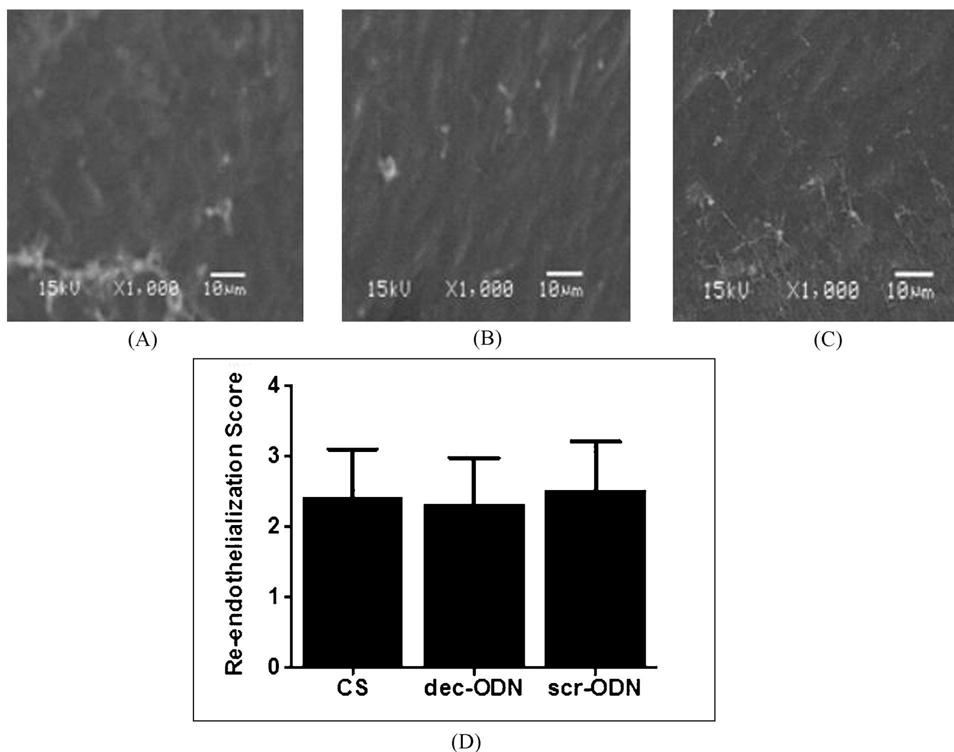

Fig. 4: Impact of ODN delivery in DES on re-endothelialization. Eight weeks after DES implantation, the extent of re-endothelialization of arteries was examined with scanning electron microscope (A-C). No significant difference was observed among the groups (D).

inal segments of aortas was longitudinally cut into pieces and digested with collagenase. The digested cells were cultured in 100 mm dishes overnight and the attached cells were collected as primary VSMC. The primary VSMCs were identified and morphologically characterized by microscopy and VSMC-specific α-actin immunohistochemistry. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS (RPMI/10%FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

4.2. Synthesis of dec-ODN against activator protein-1 binding site

Decoy oligodeoxynucleotides(dec-ODN) were designed to contain consensus AP-1 binding sequence and a scrambled oligodeoxynucleotides (scr-ODN) was synthesized as experimental control. The dec-ODN and scrambled ODN were prepared from complementary phosphorothioate-bonded ODN synthesized by Sangon Biotech Co., Ltd., Shanghai, China (Lot No. M427514). The sequences of dec-ODN (the consensus AP-1 sequence was bolded) and scrambled ODN were as follows:

dec-ODN: 5′-AGCTTGTGAGTCAGAAGCT-3′;
3′-TCGAACACTCAGTCTTCGA-5′
Scr-ODN: 5′-AATGGCATGGACTGTATCG-3′;
3′-TTACCGTACCTGACATAGC-5′

4.3. ODN Coating and stent implantation in rabbits

All animal protocols received prior approval from the Institutional Animal Care and Use Committee and all experiments were performed in accordance with relevant government guidelines and regulations.

Bare metal stents made of 316L medical stainless steel (diameter: original 0.9 mm, expanded to 2.5 mm after implantation, length: 12 mm, manufactured by Beijing Lepu medical instrument Inc.) were coated with polymer gel. The stents were then dip-coated with 20 ug/mL ODN solution containing 100 ug/mL polyethylenimine reagent for 10 min and were air-dried for 10 min. Control stents (CS) were coated with identical procedure but without ODN.

Thirty New Zealand rabbits (SLAC Laboratory animal, Inc., Shanghai, China) weighing between 2.0 and 2.5 kg were randomly divided into 3 groups: Control stent group (CS, n = 10), scrambled ODN stent group (scr-ODN, n = 10), and dec-ODN stent group (dec-ODN, n = 10). To place the stents, animals were anesthetized by intramuscular (i.m.) injection of ketamine (50 mg/kg) and xylazine (0.75 mg/kg). After stable anesthesia, the left femoral artery was exposed, and a 0.9 × 25 mm I.V. catheter (Becton Dickinson Medical Devices Co., Ltd., Suzhou, China) was inserted into the artery. After the intravenous infusion of heparin (50 IU/kg), angiography was performed. The stents were guided by the 0.35 mm catheter wire and implanted in the segment of infrarenal abdominal aorta at the pressure of 8 atmospheres through the wire. After the procedure, the femoral artery was ligated, and the wound was cleaned and sewed up. Aspirin (5 mg/mg/day) and clopidogrel (75 mg/rabbit/day) were administered for the following 8 weeks after the procedure to prevent thrombosis. The rabbits were euthanized and the abdominal aorta segments with the stent were collected after perfusion with saline. The abdominal aortas were divided into four segments for histology, scanning electron microscope, RNA isolation and protein extraction.

4.4. Histological examination and morphometric analysis

One segment of the abdominal aortas were soaked in 4% followed by being submerged in 4% paraformaldehyde for 48 hr and embedded in methyl acrylic acid methyl ester. For histological examination, the embedded abdominal aortas were cross-sectioned at a thickness of 5 μm and stained with hematoxylin-eosin and Weigert's elastic staining. The cross-sectional intima, media area and area ratios were measured and analyzed with a computerized apparatus and the Image software program from National Institutes of Health.

4.5. Scoring of re-endothelialization

Segments of the abdominal aortas were fixed in 4% glutaraldehyde for 2 hr, washed with PBS, placed in osmium tetroxide and washed again with PBS. The tissues were dehydrated using ethanol and dried. After covered with gold and palladium, a segment was cut longitudinally, opened along the longitudinal cut, and examined under a scanning electron microscope. Three sites were viewed and re-endothelialization was scored and averaged for each animal. The re-endothelialization was scored according to the following criteria: score 1, endothelial cells cover less than one quarter of the stent surface; score 2: endothelial cells cover between one quarter to one third of the stent surface; score 3: endothelial cells cover more than one third of the stent surface.

4.6. Western blot

The segments of the abdominal aortas for protein extraction were immediately frozen in liquid nitrogen after cut until the protein extraction. The frozen arteries were cut into small pieces and were homogenized completely in lysis buffer [50 mM Tris(pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 Mm EDTA, 0.1% SDS, 1% deoxycholate sodium] to extract protein. After the total proteins were extracted, a 10% SDS- polyacrylamide gel (SDS–PAGE) was used for electrophoresis, and then electro-transferred onto PVDF membranes (Amersham Biosciences, Uppsala, Sweden). After

transfer, membranes were blocked in 5% nonfat dry milk in Tris-saline buffer (150 mM Tris, 50 mM NaCl, 0.1% Tween-20) for 1 h and incubated overnight at 4 °C with a primary anti TGF-β1 or CTGF polyclonal antibody (AbFrontier Co., Ltd., Seoul, Korea), and then followed by incubation with HRP conjugated secondary antibody (DingGuo Biotech Co., FuZhou, China). Signals were developed with enhanced chemiluminescence (ECL Kit, RPN2106, Amersham) substrate and exposure to X-ray films. The bands were quantified by scanning densitometry with the NIH Image Software Program. Western blots using antibody against Tubulin were performed to normalize loading for data analysis.

4.7. Quantification of TGF-β1 and CTGF mRNA expression

mRNA expression of TGF-β1 and CTGF were analyzed with reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was isolated from the abdominal aorta segments frozen in liquid nitrogen after cut using Trizol reagent (Sigma, Missouri, USA). Aliquots of total RNA were reverse transcribed into complementary DNA (cDNA) with Oligo(dt) and Moloney murine leukemiavirus (M-MLV) reverse transcriptase (Promega Wisconsin, USA) at 37 °C for 1 h. The cDNA products were used as template for PCR amplification (one cycle of initial denaturation at 94 °C for 5 min and 32 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56.0 °Cand 45 s extension at 72 °C). The primers used to amplify TGF-β1 and CTGF were as the follows: sense: 5′-AAGGACCTGGGCTGGAAG-3′and antisense: 5′-CACGATGGGCAGTGGCTC-3′ for TGF-β1(Tm = 57.3°C, product size = 309 bp); sense: 5′-GGAAATGCTGCGAGGAGTG-3′ and antisense: 5′-GCATCATGGTTGGGTCTGG-3′ for CTGF (Tm = 60.4°C, product size = 114 bp). GAPDH was used as an internal control. PCR was performed in GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The quality and concentration of the amplified PCR products were analyzed with Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) and normalized to GAPDH.

4.8. Statistical analysis

Data are expressed as mean ± SD. Comparisons among groups were analyzed with one-way ANOVA for multiple groups and with Student's t-test for two groups. A p value < 0.05 was considered statistically significant.

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