Inhibition of anastomotic intimal hyperplasia using a chimeric decoy strategy against NFκB and E2F in a rabbit model

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Aims Neointimal formation remains a major limitation after arterial reconstruction. To overcome this problem, we focused on two important transcription factors, nuclear factor-kappaB (NFκB) and E2F. The purpose of this study was to determine the effects of simultaneous inhibition of these transcription factors on the formation of neointimal hyperplasia.

Methods and results We employed chimeric decoy oligodeoxynucleotides (ODN) to inhibit both NFκB and E2F simultaneously, and examined the effects of chimeric decoy ODN on the proliferation and migration of cultured vascular cells and on the formation of neointimal hyperplasia using prosthetic graft placement in a rabbit hypercholesterolemia model. Our in vitro study demonstrated that transfection of chimeric decoy ODN inhibited platelet-derived growth factor (PDGF)-induced proliferation and migration of vascular smooth muscle cells, whereas endothelial cell proliferation was not inhibited. In an in vivo study, treatment with chimeric decoy ODN significantly inhibited proximal and distal anastomotic intimal hyperplasia, and accelerated re-endothelialization. α-Smooth muscle actin (α-SMA)-positive cell proliferation was inhibited at the anastomotic sites. Expression of PDGF-BB and PDGF receptor-β was also suppressed by chimeric decoy ODN, resulting in a reduction of α-SMA-positive cell accumulation. In addition, chimeric decoy ODN treatment inhibited macrophage accumulation, which was accompanied by a reduction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 gene expression.

Conclusion The present study demonstrates the feasibility of chimeric decoy ODN treatment for preventing neointimal formation. This strategy might be useful to improve the clinical outcome after arterial reconstruction.

1. Introduction

Prosthetic grafts are commonly used for infrainguinal bypass grafting to treat arterial occlusive disease, but long-term patency remains a critical problem. Prosthetic graft failure is mainly due to the progression of anastomotic intimal hyperplasia.¹ In addition, one major limitation of prosthetic bypass grafting is its usage in small diameter vessels, such as tibial or peroneal artery bypass. Despite having minimal impact in large vessels, a small increase in anastomotic intimal hyperplasia causes significant lumen narrowing in anastomosis with a small orifice, leading to graft failure. The luminal lining of the prosthetic graft is composed of pseudointima and lacks endothelial cells. The lack of an endothelial monolayer disrupts the homeostatic regulation of thrombosis, platelet activation, and leucocyte adhesion, resulting in vascular smooth muscle cell (VSMC) proliferation and migration.¹,² Inhibition of VSMC proliferation is thought to prevent progression of intimal hyperplasia, and E2F has attracted attention in this process, because it is a pivotal cell-cycle transcription factor. Indeed, inhibition of E2F activity using decoy oligodeoxynucleotides (ODN) suppressed VSMC proliferation, leading to the prevention of neointimal formation in animal models.³,⁴ Based on these data, clinical trials using E2F decoy ODN for graft failure have been performed, but treatment with E2F decoy ODN was no more effective in preventing restenosis after coronary and peripheral bypass grafting.⁵,⁶ These results suggest that simple inhibition of VSMC proliferation is not sufficient to prevent graft failure in humans, because multiple processes participate in the formation of intimal hyperplasia.

As neointimal formation can be viewed as an inflammatory response, we focused on another transcription factor, nuclear factor-kappaB (NFκB), which plays an important

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role in the transcription of pro-inflammatory genes. Furthermore, NFκB activity appears to be essential for the proliferation and migration of VSMCs. Activation of NFκB directly stimulates transcription of cyclin D1, which accelerates VSMC proliferation to promote G1- to S-phase transition. Indeed, we have previously reported a preventive effect of NFκB decoy ODN on the formation of intimal hyperplasia using in vitro strategies. However, the cyclin D1 promoter is regulated by multiple transcription factors such as retinoblastoma protein family and, other D-cyclins, cyclin D2 and D3, also promote G1- to S-phase transition. In addition, recent analysis of mice lacking D-cyclins revealed the presence of alternative mechanisms that induce cell cycle progression in cyclin D-independent fashion. Activation of cyclins E and A sufficient to allow proliferation of cyclin D-deficient fibroblasts. As E2F initiates transcription of the gene including cyclins D, E and A, we hypothesized that the effect of E2F blockade on VSMC proliferation would be more potent than that of NFκB.

From these viewpoints, we hypothesized that the simultaneous inhibition of both NFκB and E2F binding activity might be an effective approach to prevent neointimal formation and improve the clinical outcome after arterial reconstruction. Therefore, we developed a chimeric decoy strategy to inhibit both NFκB and E2F simultaneously. Chimeric decoy ODN could reduce the inflammatory response as well as VSMC proliferation and migration in the process of neointimal formation. The purpose of this study was to determine the inhibitory effects of chimeric decoy ODN on in vitro proliferation and migration of vascular cells and on the formation of anastomotic intimal hyperplasia using prosthetic graft placement in a rabbit hypercholesterolemia model.

2. Methods

2.1 Synthesis of oligodeoxynucleotides and selection of target sequences

The following sequences of phosphorothioate ODN were utilized (consensus sequences are underlined): chimeric decoy ODN: 5'-GAA GGGATTTCCCTCAATTTCCGGGAGAAGTAAAG CGGCCTTGG-3'; 5'-CTTCCCTAAAGGGAGGTAAAG CTTCCCGCG-3'; NFκB decoy ODN: 5'-GGCTTGAAGGGATTTCCCTC-3'; 3'-GGAACTTAAAGGGAGGTAAAG CGGCCTTGG-5'; E2F decoy ODN: 5'-TGACATGGACTGAATCG CCGG-3'; 3'-TAAGGGGCCTCTAG-5'; scrambled chimeric decoy ODN: 5'-CGTACCTGACTTAGGCTTGGGAGAAGTAAAG CGGCCTTGG-3'; 3'-GCACTGACTAATCGTAAAGCTTGG-5'.

Synthetic ODN were washed with 70% ethanol, dried, and dissolved in sterile Tris-EDTA buffer (10 μmol/L Tris, 1 μmol/L EDTA). The supernatant was purified through a NAP 10 column (Pharmacia, Piscataway, NJ, USA) and quantified by spectrophotometry. Fluorescent isothiocyanate-labelled chimeric decoy ODN (FITC-ODN) were used to evaluate the distribution of ODN after transfection.

2.2 Cell culture

Human aortic endothelial cells (HAEC) and VSMCs were obtained from Clonetics Corp. (San Diego, CA, USA). HAEC were cultured in endothelial cell basal medium (Clonetics) supplemented with 2% foetal calf serum (FCS), 4 ng/mL fibroblast growth factor, 10 pg/mL epidermal growth factor, and 1 μg/mL hydrocortisone. VSMCs were cultured in smooth muscle cell basal medium (Clonetics) with 5% FCS, 2 ng/mL fibroblast growth factor, 500 pg/mL epidermal growth factor, and 5 μg/mL insulin in the standard fashion. All cells were used within passages 5–8.

2.3 Cell proliferation assay

Cells were seeded onto 96-well tissue culture plates at a density of 1 × 10^3 cells per well for HAEC and 5 × 10^3 cells per well for VSMCs. After 24 h, cells were rendered quiescent by incubation for 48 h in medium with 0.5% FCS for HAEC or defined serum-free medium for VSMCs, and then transfected with decoy ODN (20 or 600 nmol/L) combined with Oligofectamin® reagent (Invitrogen, CA, USA). At 24 h after transfection, HAEC were stimulated with medium containing 5% FCS, and VSMCs were incubated in medium containing human platelet-derived growth factor-BB (PDGF-BB, 10 ng/mL, PeproTech, London, UK) for 24 h. An index of cell proliferation was determined using a WST-1 cell counting kit (Dojindo, Japan). All experiments were performed in triplicate.

2.4 Cell migration assay

VSMC migration was assayed by modified Boyden chamber method, which contained an 8 μm pore size polycarbonate membrane. VSMCs were grown on 60 mm tissue culture dishes. At 50% confluence, VSMCs were transfected with decoy ODN (200 nmol/L) combined with Oligofectamine reagent. At 24 h after transfection, VSMCs were trypsinized and resuspended in serum-free medium. Culture medium containing 2.5 × 10^4 cells was added to the upper compartment of the chamber, and the bottom compartment was filled with serum-free medium containing PDGF-BB (10 ng/mL). At 4 h after incubation at 37°C, cells that migrated to the lower side of the filter were fixed and stained with a Diff-Quick staining kit (International Reagents, Kobe, Japan). Migration was determined as the mean number of migrated cells per high-power field. All experiments were performed in triplicate.

2.5 Experimental animal models and intra-operative transfection of decoy ODN

Male Japanese white rabbits weighing 2.0–2.5 kg were used in this experiment for a period of 1 week before the operation until the time of prophylactic graft harvest to induce hypercholesterolemia in rabbits, as previously described. Animals were divided into five groups: control (saline perfusion only), treatment with scrambled decoy ODN, treatment with NFκB decoy ODN, treatment with E2F decoy ODN, and treatment with chimeric decoy ODN. A blood sample was taken from each rabbit to check serum cholesterol level before and 5 weeks after beginning a high cholesterol diet.

Animals were anesthetized by intravenous injection of pentobarbital, and a midline neck incision was made to expose the left common carotid artery. A 3–4 cm segment of common carotid artery was isolated just proximal to the carotid bifurcation. After systemic heparinization, the common carotid artery was clamped and divided. Transfection of decoy ODN into the anastomotic site of the carotid artery was performed by intra-arterial pressure-mediated transfection, without the use of viral vectors or other delivery techniques. The distal anastomotic site of the carotid artery was anastomosed with running 7-0 nylon sutures. At the end of the replacement, the operative area was rinsed with normal saline solution and the skin was closed.

To evaluate the transfection efficiency of intra-arterial pressure-mediated transfection, tissue sections of the carotid artery were subjected to histochemical measurement after transfection of FITC-ODN at pressures ranging from 50 to 200 mmHg for 10 min. Five equally spaced cryosections (5 μm thick) of carotid artery were checked.
transfected with FITC-ODN were examined by fluorescence microscopy, after staining in eriochrome black T solution. The sections were also stained with DAPI, and successfully transfected cells were defined as those with localized fluorescence in the nucleus. The numbers of FITC-labelled nuclei and total DAPI-labelled nuclei were calculated in high-power fields, and transfection efficiency was defined as the mean ratio of FITC-labelled nuclei to DAPI-labelled nuclei.

This study was performed under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law (No. 105), and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.6 Electrophoretic mobility shift assay

One week after operation, nuclear extracts were prepared from anastomotic sites of the carotid artery and the contralateral artery as an untreated sample. Electrophoretic mobility shift assay (EMSA) was performed to analyse the expression of NF-κB and E2F in nuclear extracts (10 μg) with a gel shift assay system (Promega Corp., WI, USA), according to the manufacturer’s specifications. Double-stranded ODNs containing the NFκB binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′, only sense strands are shown) or E2F binding site (5′-ATTAAAGTTTCGGCCCTTCTCAA-3′) were used as primers.

2.7 Evaluation of endothelialization

Four weeks after implantation, endothelialization of the graft surface was identified by staining with 1% Evans-blue solution injected intravenously 30 min prior to killing. The graft surface without endothelialization was stained with Evans-blue solution, but the area of endothelialization was not stained. The positive area (unstained area) was calculated by quantitative morphometric analysis with a computerized sketching program. Results were expressed as a percentage of the total area of the graft surface.

2.8 Histological study

Animals were sacrificed at 4 weeks after operation. The carotid arteries and prosthetic grafts were carefully dissected to remove adherent tissue, and processed for routine paraffin embedding. Three sections were divided longitudinally from the anastomotic site at equal distances, and the sections (6 μm) were stained with both haematoxylin and eosin and elastic van Gieson’s (EVG) stain in a standard manner. In spite of destruction by surgical procedure, both haematoxylin and eosin and elastic van Gieson’s (EVG) stain (Promega and conformed with the Government Animal Protection and Management Law (No. 105), and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.9 Immunohistochemical study

Immunohistochemical staining was performed using the immunoperoxidase avidin–biotin complex method, according to the manufacturer’s specifications (Vectorstain Elite ABC kit, Vector Laboratories). Immune complexes were localized using 0.05% 3,3′-diaminobenzidine, and slides were counterstained with haematoxylin. Paraffin sections were stained with mouse monoclonal antibody for rabbit macrophage (RAM11, 1:50, Dako, CA, USA) or proliferating cell nuclear antigen (PCNA, 1:100, Dako) at 1 week after operation. Positively stained cells and total cells were counted manually, and statistical analysis was performed. For negative control experiments, non-immune IgG was applied in place of primary antibody. Rabbit monoclonal antibody for α-smooth muscle actin (α-SMA, 1:300, NeoMarkers Inc., CA, USA) was used in the specimens at 4 weeks after operation. The positive area was measured, and the results expressed as a percentage of the intimal area of the cross section.

Double immunofluorescent staining was also performed on sections of artery with scrambled decoy ODN transfer, using antibodies for PCNA and α-SMA, to identify the expression of PCNA in α-SMA-positive cells. The images were visualized using fluorescence microscopy.

2.10 Western blot analysis

One week after transfaction, total protein was extracted from the distal anastomotic sites of the carotid artery with ISOGEN (Nippon Gene, Japan). Real-time RT–PCR was performed using a Superscript III two-step qRT–PCR kit with SYBR green (Invitrogen). Primers based on the published sequences for rabbit vascular cell adhesion molecule-1 (VCAM-1, sense: 5′-GAAGACTCTTACCTGTGCACAGC-3′, antisense: 5′-CCATCCTCTAGAATTAAGGTGAG-3′; 567 bp), monocyte chemoattractant protein-1 (MCP-1, sense: 5′-GTCTCTGCAACGTCTCCTGCC-3′, antisense: 5′-AGTCGTGTCTTGATGGTTGTG-3′; 327 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense: 5′-GGCCCTTGTGACCCAGGCTCTT-3′, antisense: 5′-TGCCGAA GTGGTCGTTGAGATAC-3′; 465 bp) were used. Equal amounts (0.5 μg) of RNA from each sample were reverse transcribed into cDNA for 50 min at 42 °C. Polymerase chain reaction amplification was performed under the following conditions: denaturation for 15 s at 94 °C, annealing for 40 cycles. Expression of VCAM-1 and MCP-1 was determined relative to the expression of GAPDH.

2.12 Statistical analysis

All values are expressed as mean ± SEM. For statistical analysis, a paired t-test was used for comparison between two groups. One-way ANOVA and Tukey–Kramer multiple range test were used for comparisons among multiple groups. P < 0.05 was considered significant.

3. Results

3.1 Effects of chimeric decoy oligodeoxynucleotides in vitro study

First, we assessed the effects of chimeric decoy ODN on the proliferation and migration of cultured vascular cells. Proliferation assay demonstrated that transfection of chimeric decoy ODN inhibited VSMC proliferation in a dose-dependent manner when compared with transfection of scrambled decoy ODN or control. Although single transfection of NFκB decoy ODN or E2F decoy ODN at high dose (600 nM) also inhibited VSMC proliferation, the effect of chimeric decoy ODN was significantly greater than that of NFκB decoy ODN alone (Figure 1A). In contrast, PDGF-induced VSMC
migration was inhibited by chimeric decoy ODN and NFκB decoy ODN, but not E2F decoy ODN (Figure 1B). Interestingly, endothelial cell proliferation was not inhibited by chimeric decoy ODN, as well as NFκB and E2F decoy ODN alone (Figure 1C). These results established that chimeric decoy ODN inhibited both VSMC proliferation and migration without the inhibition of endothelial cell growth.

3.2 Distribution of fluorescent isothiocyanate-labelled chimeric decoy oligodeoxynucleotides in anastomotic site

To confirm the successful transfer of naked decoy ODN into the carotid artery using pressure-mediated transfection, we evaluated the distribution of FITC-labelled decoy ODN. Histologically, fluorescence was detected in the intima and inner half of the media (Figure 2A). Untreated vessels and vessels transfected with non-FITC-ODN revealed no specific fluorescence (data not shown). Successfully transfected cells were defined as those with localized fluorescence in the nucleus (Figure 2A). With an infusion pressure of 150 mmHg for 10 min, the average transfection efficiency of decoy ODN was 60.0 ± 4.7%. This efficiency was significantly higher than that with an infusion pressure of 50 or 100 mmHg for 10 min, and was not further increased at 200 mmHg (Figure 2B). These findings established the appropriateness of pressure-mediated transfection with 150 mmHg pressure for 10 min to introduce decoy ODN into the anastomotic site of the carotid artery.

3.3 Inhibitory effects of chimeric decoy oligodeoxynucleotides in in vivo study

To assess the in vivo effect of chimeric decoy ODN, prosthetic 2 mm ePTFE grafts were placed in an end-to-end fashion in the carotid arteries in rabbits fed a high cholesterol diet. An increase in serum total cholesterol level was confirmed at the time of prosthetic graft harvest (pre: 37.5 ± 1.7 mg/dL, after 5 weeks: 1300.8 ± 154.1 mg/dL, P < 0.05). The effect of chimeric decoy ODN on transcriptional activation of NFκB and E2F was confirmed by EMSA. Activity of both transcription factors was increased at 1 week after placement of prosthetic grafts and scrambled decoy ODN transfer in control. In contrast, activity of NFκB and E2F was simultaneously inhibited by transfection of chimeric decoy ODN (Figure 2C).

Four weeks after operation, treatment with chimeric decoy ODN significantly accelerated re-endothelialization of the anastomotic site and a part of the graft surface as assessed by Evans-blue staining (Figure 2D). Importantly, EVG staining demonstrated that treatment with chimeric decoy ODN significantly inhibited proximal anastomotic intimal hyperplasia and reduced intima-to-media ratio when compared with treatment with control or scrambled decoy ODN (Figure 3A). Distal anastomotic intimal hyperplasia and intima-to-media ratio were also reduced by chimeric decoy ODN treatment (Figure 3B). In contrast, the single transfection of NFκB decoy ODN or E2F decoy ODN was not enough to prevent neoimal formation. Additionally, the inhibitory effect of chimeric decoy ODN was significantly greater than that of single transfection of NFκB or E2F decoy ODN (Figure 3A and B).
3.4 Mechanisms of inhibition of neointimal formation

To examine the molecular mechanisms of treatment with chimeric decoy ODN, we assessed proliferative activity of α-SMA-positive cells at the anastomotic site. Immunohistochemical study demonstrated that accumulation of α-SMA-positive cells within the neointima was inhibited by chimeric decoy ODN treatment (Figure 4A). In addition, PCNA-positive cells were markedly found within the neointima and media in specimens of control and scrambled decoy ODN transfer, but treatment with chimeric decoy ODN resulted in a significant decrease in the ratio of PCNA-positive cells (Figure 4B). Furthermore, double immuno-fluorescent staining revealed that PCNA was stained in α-SMA-positive cells in sections of scrambled decoy ODN transfer (Figure 4C).

The PDGF signal pathway is a key regulator in the development of intimal hyperplasia, and is implicated as a possible VSMC mitogen. Thus, we also investigated the expression of PDGF in the anastomotic sites by western blotting. Treatment with chimeric decoy ODN significantly inhibited the expression of PDGF-BB when compared with control or scrambled decoy ODN transfer. Furthermore, chimeric decoy ODN treatment also decreased the expression of PDGFR-β (Figure 5).

Finally, we investigated the anti-inflammatory effect of chimeric decoy ODN. Many macrophages infiltrated the anastomotic site in specimens transfected with scrambled decoy ODN, while macrophage recruitment was significantly inhibited by treatment with chimeric decoy ODN (Figure 6A). In addition, the expression of adhesion molecules and chemotactic factors is considered to induce migration of macrophages into the injured vessel wall. Real-time RT–PCR analysis demonstrated that the expression levels of VCAM-1 and MCP-1 were significantly increased at 1 week after operation. In contrast, treatment with chimeric decoy ODN significantly decreased VCAM-1 and MCP-1 gene expression, followed by inhibition of the recruitment of macrophages (Figure 6B and C).

Discussion

As a basis for treating vascular disease using a chimeric decoy strategy, we assessed the effects of chimeric decoy ODN on the proliferation and migration of cultured vascular cells. Transfection of chimeric decoy ODN inhibited VSMC proliferation in a dose-dependent manner. Importantly, the effect of chimeric decoy ODN was more potent than that of NFκB decoy ODN alone. Therefore, the inhibitory effect of chimeric decoy ODN may be mainly related to cell-cycle
arrest through the suppression of E2F activity, while NFκB also appears to be essential for cell-cycle regulation and promotes VSMC proliferation.9,10 In contrast, chimeric decoy ODN as well as NFκB decoy ODN inhibited VSMC migration. Several studies have demonstrated that VSMC migration was stimulated by NFκB through activation of multiple intracellular signalling pathways, such as mitogen-activated protein kinases (MAPK).16 In addition, it has been reported that activation of NFκB mediated VSMC migration rather than proliferation.17 Then, the inhibitory effect of chimeric decoy ODN on VSMC migration may be associated with suppression of NFκB activity. These results indicated that the therapeutic effects of chimeric decoy ODN on neointimal formation may be very potent when compared with single decoy ODN, because chimeric decoy ODN inhibited both proliferation and migration of VSMC, as a result of the concomitant blockade of both transcription factors. Interestingly, transfection of chimeric decoy ODN did not inhibit endothelial cell proliferation. Similar observations were reported in several studies. TNF-α-induced endothelial cell proliferation was associated with STAT activation, whereas this response was independent of several growth-signalling molecules including MAPK and NFκB.18

Blockade of cell-cycle protein expression by E2F decoy ODN or antisense against PCNA and cell division cycle 2 led to an improvement in endothelial dysfunction.14,19 Regulation of endothelial cell proliferation is thought as a result of very complex network of intracellular signalling systems, and there are no specific signal transduction pathways and transcription factors controlling the entire process of proliferation.20 Therefore, inhibition of NFκB and/or E2F resulted in a small effect on the proliferative activity of endothelial cells. This phenomenon might be favourable in clinical situations.

Based on our in vitro findings, we examined the effects of chimeric decoy ODN on the formation of anastomotic intimal hyperplasia in a rabbit hypercholesterolemia model. A prosthesis graft placement model causes significant intimal hyperplasia without remodelling process. Activation of both NFκB and E2F was markedly increased at the sites of anastomosis in this model. Pressure-mediated application was an easy approach to introduce naked decoy ODN into the arterial wall intra-operatively, and achieved high transfection efficiency, resulting in simultaneous inhibition of the binding activities of both transcription factors. However, the transfection efficiency to disease artery would worsen in

Figure 3  Effects of chimeric decoy ODN on neointimal formation at 4 weeks after implantation. (A) Representative histological sections stained with EVG stain, and thickness of intima and media in sections and ratio of intima to media at proximal anastomotic site. (B) Representative histological sections stained with EVG stain, and thickness of intima and media in sections and ratio of intima to media at distal anastomotic site. Arrows indicate the anastomotic intimal hyperplasia. L, lumen; G, prosthetic graft; M, medial layer; IH, intimal hyperplasia; Cont, prosthetic graft with saline perfusion only; Scr, prosthetic graft transfected with scrambled decoy ODN; NFκB, prosthetic graft transfected with NFκB decoy ODN; E2F, prosthetic graft transfected with E2F decoy ODN; Chi, prosthetic graft transfected with chimeric decoy ODN. *P < 0.05 vs. Control, Scrambled, NFκB, and E2F, n = 6 per group. Scale bar = 500 μm.
Figure 4  Inhibition of α-SMA-positive cell accumulation and proliferative activity. (A) Typical example of immunohistochemical staining of α-SMA and percentage of positive area at 4 weeks after operation. (B) Typical example of immunohistochemical staining of PCNA and percentage of positively stained cells at 1 week after operation. (C) Double immunofluorescent staining for PCNA and α-SMA in section of artery with scrambled decoy ODN transfer at 1 week after operation. *P < 0.05, n = 4 per group. Scale bar in (A) and (B) = 50 μm, in (C) = 10 μm.

Figure 5  Inhibition of PDGF-BB and PDGFR-β expression. Western blotting of PDGF-BB and PDGFR-β, and densitometric evaluation at 1 week after transfection. *P < 0.05, **P < 0.05 vs. Untreated, n = 4 per group.
clinical setting, because human arteries as targets of arterial reconstruction usually associated with atherosclerotic or non-atherosclerotic intimal thickening.

The present study clearly demonstrated that chimeric decoy ODN treatment significantly inhibited the formation of anastomotic intimal hyperplasia, despite being applied in hypercholesterolemic rabbits. In addition, chimeric decoy ODN was very potent when compared with single transfection of NFκB or E2F decoy ODN, consistent with in vitro observation. Although the inhibitory effects of NFκB or E2F decoy ODN on neointimal formation has been reported in vein graft and injured artery models,3,4,11 the present study demonstrated that single transfection of decoy ODN did not suppress the anastomotic intimal hyperplasia. Transfection of decoy ODN was performed native artery alone, but not prosthetic graft, and foreign body reaction of prosthetic graft lasted at the site of anastomosis of carotid artery. Therefore, the concentration of single decoy ODN may not be sufficient to prevent neointimal formation in anastomotic site.

Treatment with chimeric decoy ODN decreased DNA synthesis in α-SMA-positive cells at anastomotic sites, leading to inhibition of proliferation and accumulation of α-SMA-positive cells. More interestingly, it is noteworthy that in this study, the expression of both PDGF-BB and its receptor, PDGFR-β, was also decreased by chimeric decoy ODN treatment. The PDGF signalling cascade plays an important role in the initiation and progression of intimal hyperplasia. PDGF is a potent growth factor and is implicated as a possible VSMC mitogen.21 Especially, PDGF-BB induces a predominantly migratory response in VSMC when compared with other PDGF isoforms.22 In human studies, PDGF-B and PDGFR-β were detected in VSMC and macrophages at all stages of atherosclerotic lesion development, and were associated with induction of a mitogenic response.22,23 The expression of PDGF-B and PDGFR-β is regulated by activation of NFκB. NFκB can bind to the promoter region of PDGF-B and mediates PDGF-B gene expression.24 Similarly, inhibition of NFκB activation resulted in a marked reduction in promoter activity of the PGDFR-β gene and led to suppression of PGDFR-β gene expression.25 In addition, chimeric decoy ODN treatment suppressed PDGF expression indirectly through a significant decrease of inflammatory cells, because migrating macrophages, as well as platelets, are a major source of PDGF-BB secretion.23

Importantly, treatment with chimeric decoy ODN accelerated re-endothelialization of the anastomotic site and a part of the graft surface. Endothelial dysfunction or loss is also considered to have a key role in the initiation of intimal hyperplasia.26 Especially, prosthetic grafts lack an endothelial layer on the luminal surface, thus leading to migration of inflammatory cells, adhesion and activation of platelets at the anastomotic site, which trigger lesion formation.1,2 Recent clinical reports demonstrated that pharmacological agents for preventing restenosis, such as rapamycin, induced endothelial damage, and delayed re-endothelialization, despite the inhibition of VSMC proliferation.27 In contrast, chimeric decoy ODN did not inhibit proliferation of endothelial cells in vitro. Additionally, our previous in vitro data demonstrated that transfection of NFκB decoy ODN inhibited endothelial cell death through an increase in an anti-apoptotic molecule, bcl-2, under hypoxic conditions or oxidative stress.28 Furthermore, it has been reported that activation of NFκB induced by high glucose inhibited endothelial cell migration, which was restored by an NFκB inhibitor.29 These findings suggest...
that chimeric decoy ODN treatment may protect surviving endothelial cells from damage at the time of surgery, leading to re-endothelialization.

Inflammation of the vascular wall is another major factor in the development of lesion formation, and NF-kB plays a significant role in this phenomenon.  

Activation of NF-kB increases the expression of various pro-inflammatory genes, such as adhesion molecules and chemokines, leading to migration of inflammatory cells within the injured vessel wall.  

Inflammatory cells secrete cytokines and growth factors, further stimulating medial VSMC proliferation and migration. The present study also demonstrated that transfection of chimeric decoy ODN significantly inhibited the recruitment of macrophages in the anastomotic site, accompanied by a reduction of VCAM-1 and MCP-1 gene expression. This suppression of macrophage accumulation explains in part the mechanism of the inhibitory effect of chimeric decoy ODN on neointimal formation.

Here, we demonstrated the feasibility of treatment with chimeric decoy ODN against NF-kB and E2F for preventing neointimal formation in a rabbit hypercholesterolemia model. A chimeric decoy strategy might be useful to improve the clinical outcome after arterial reconstruction and, furthermore, it could be possible to develop a new clinical indication of prosthetic arterial grafting in small diameter vessels.

Conflict of Interest. Dr Morishita has stocks for AnGes MG and serves as Board Member of AnGes MG which developed the decoy ODN.

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