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Inhibition of Experimental Abdominal Aortic Aneurysm in the Rat by Use of Decoy Oligodeoxynucleotides Suppressing Activity of Nuclear Factor κB and ets Transcription Factors

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Background—Two phenomena, inflammation and matrix degradation, contribute to the progression of abdominal aortic aneurysm (AAA). Importantly, the inflammation is regulated by the transcription factor nuclear factor (NF)–κB, whereas the destruction and degradation of elastin fibers by matrix metalloproteinases (MMP) are regulated by ets. Thus, we developed a novel strategy to treat AAA by simultaneous inhibition of both NF-κB and ets by using chimeric decoy oligodeoxynucleotides (ODN).

Methods and Results—AAA was induced in rats by transient aortic perfusion with elastase, whereas transfection of decoy ODN was performed by wrapping a delivery sheet containing decoy ODN around the aorta. Gel-mobility shift assay at 7 days after treatment demonstrated that both NF-κB and ets binding activity were simultaneously inhibited by chimeric decoy ODN. Transfection of chimeric decoy ODN resulted in significant inhibition of the progression of AAA such as aneurysmal dilation at 4 weeks after treatment as compared with control, accompanied by a reduction of MMP expression. Moreover, the destruction of elastin fibers was inhibited in the aorta transfected with chimeric decoy ODN. Importantly, transfection of chimeric decoy ODN demonstrated potent inhibition of aneurysmal dilatation compared with NF-κB decoy ODN alone, whereas scrambled decoy ODN had no effects. Interestingly, the migration of macrophages was significantly inhibited by chimeric decoy ODN.

Conclusions—We demonstrated that inhibition of the progression of AAA was achieved by a novel strategy with chimeric decoy ODN used against NF-κB and ets in rat model. NF-κB and ets are considered to play an important role in the pathogenesis of AAA. (***Circulation.*** **2004;109:132-138.)**

Key Words: aneurysm ■ aorta ■ inflammation ■ gene therapy ■ metalloproteinase

Destruction of elastin is considered to be one of the major pathogenetic mechanisms of abdominal aortic aneurysm (AAA). Although elastic fibers normally maintain the structure of the vascular wall against hemodynamic stress, proteolytic degradation induces remodeling of extracellular matrix, resulting in aneurysmal development and finally rupture. Matrix metalloproteinases (MMPs) play important roles in such mechanisms of AAA, and pathological vascular remodeling is considered to be mediated by MMPs secreted by invasive macrophages, migrating vascular smooth muscle cells, and endothelial cells.[1–3] The expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12 are significantly increased in harvested human aneurysms.[1–6] These findings suggest that MMP are strongly associated with the activity of the disease. From this viewpoint, pharmacological strategies to inhibit MMP might prevent the progression from asymptomatic to critical large AAA, resulting in a delay or avoidance of surgical repair. Some researchers have reported the inhibitory effects of MMP inhibitors on the progression of experimental AAA.[3,7–10]

To consider the pathophysiology of AAA, we focused on the transcription factors nuclear factor (NF)–κB and ets-1. In addition to mediating inflammatory changes, NF-κB regulates the transcription of MMP-1, MMP-2, MMP-3 and MMP-9.[11–14,15] In contrast, the ets family activates the transcription of genes encoding MMP-1, MMP-3, MMP-9 and

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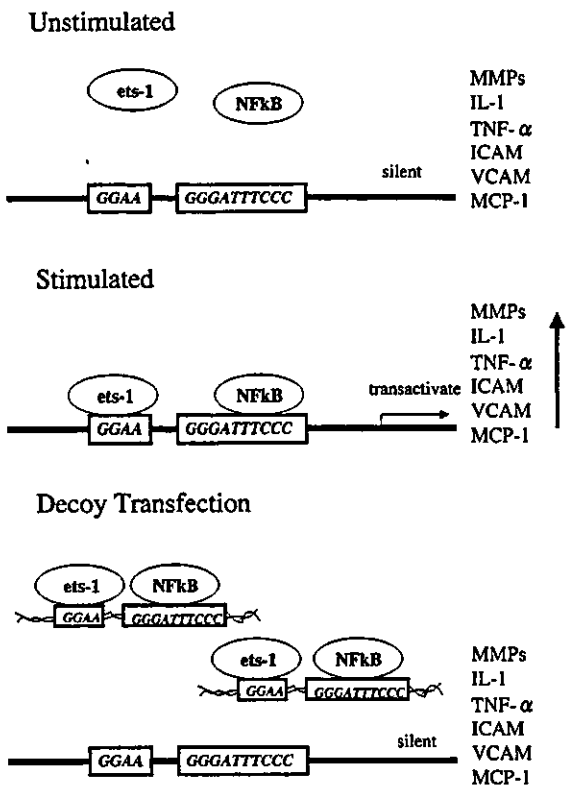

Figure 1. Chimeric decoy strategy. CCCATTTCCC and GGAA are consensus sequences for NF-κB and ets binding sites, respectively. Chimeric decoy *cis* element double-stranded ODN binds to free NF-κB and ets, preventing transactivation of various genes described in schema.

urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation.[16-19] Also, the AP-1 site in MMP promoters is known to be a dominant *cis* element responsible for regulating transcription of these genes. Among them, we focused on NF-κB and ets and hypothesized that inhibition of both NF-κB and ets activation would prevent aneurysmal development by a reduction of MMP expression. To test this hypothesis, we used a novel strategy to inhibit the activation of multiple transcription factors by using chimeric decoy ODN in this study (Figure 1). We demonstrated successful treatment of AAA by using chimeric decoy ODN against NF-κB and ets in a rat model.

Methods

Synthesis of ODN and Selection of Target Sequences

Sequences of the phosphorothioate ODN used were as follows: chimeric decoy ODN (consensus sequences are underlined), 5'-ACCGGAAGTATGAGGGATTTCCCTCC-3' and 3'-TGGCCTTCATACTCCCTAAAGGGAGG-5'; scrambled chimeric decoy ODN, 5'-TCGAGCATATACGTGACTGCGCTCAG-3' and 3'-AGCTCGTATATGCACTGACGCGAGTC-5' ; NF-κB decoy ODN (consensus sequences are underlined), 5'-CCTTGAAGGGATTTCCCTCC-3' and 3'-GGAACTTCCCTAAAGGGAGG-5'; and scrambled NF-κB decoy ODN, 5'-TTGCCGTACCTGACTTAGCC-3' and 3'-AACGGCATGGACTGAATCGG-5'.

NF-κB decoy ODN have been shown to bind the NF-κB transcription factor (Figure 1), consistent with previous reports.[20-23]

Immunohistochemical Studies

Anti-NF-κB p65 (F-6, Santa Cruz Biotechnology) antibody and anti-CD31 antibody (Serotec Ltd) were used for analysis. Immunohistochemical staining was performed with the use of the immunoperoxidase avidin-biotin complex system with nickel chloride (NiCl) color modification. Diluted primary antibodies (anti-p65 1:100) were then applied to the sections, and these sections were incubated for 30 minutes. With intervening washing in TBS, they were serially incubated with a 1:400 dilution of biotinylated rabbit, anti-mouse IgG (DAKO) in TBS for 30 minutes; streptavidin-biotinylated horseradish peroxidase complex (DAKO) diluted 1:100 in TBS for 30 minutes; and 0.05% 3,3'-diaminobenzidine (DAB, Sigma Chemicals) in 200 mL TBS, to which had been added 0.2 mL 30% hydrogen peroxide and 1.0 mL 8% NiCl solution for 5 minutes.

Procedure of AAA Model

Male Wistar rats (400 to 500 g; Charles River Breeding Laboratories) were anesthetized and underwent laparotomy.[3,7-10] Briefly, the abdominal aorta was isolated from the level of the left renal vein to the bifurcation. The right femoral artery was exposed, and a PE-10 polyethylene tube (Baxter Healthcare Corp) was introduced through the femoral artery to the distal aorta. The aorta was clamped above the level of the tip of the PE tube and ligated with a silk suture near the aortic bifurcation, followed by perfusion with 0.2 mL saline containing 50 U type I porcine pancreatic elastase (Sigma Chemicals). Aortic perfusion with 2 mL saline containing 25 U elastase was performed for 30 minutes at 100 mm Hg. Transfection of decoy ODN was performed by wrapping a delivery sheet around the abdominal aorta at the same time as surgery. To make the sheet, 73 mg hydroxypropyl cellulose and 7.3 mg polyethylene glycol 400 (PEG) were dissolved in 70% ethanol, and 400 nmol decoy ODN was mixed into this solution. Drying overnight resulted in a 4-cm^2 thin sheet containing 100 nmol decoy ODN/cm^2. When the sheet is wrapped around the aorta, the sheet immediately changes to a gel to allow incubation of ODN around the aorta for at least 1 week. 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).

To confirm the successful transfer of ODN into the aorta of rats, we used FITC-labeled ODN. The aorta was harvested 1 week after transfection and fixed with 4% paraformaldehyde, followed by processing for routine paraffin embedding. Sections were examined by fluorescence microscopy, after staining in eriochrome black T solution. Elastic fibers stained dark red and were readily distinguishable from the specific FITC-labeled ODN.[24,25]

Electrophoretic Mobility Shift Assay

Rats were killed 1 week after the operation, and nuclear extracts were prepared from transfected or untransfected aortic aneurysms.[22] In brief, rat aortas were homogenized with a Potte-Elvehjem homogenizer in 4 volumes of ice-cold homogenization buffer (10 mmol/L HEPES [pH 7.5], 0.5 mol/L sucrose, 0.5 mmol/L spermidine, 0.15 mmol/L spermin, 5 mmol/L EDTA, 0.25 mol/L EGTA, 7 mmol/L β-mercaptoethanol and 1 mmol/L phenylmethylsulfonyl fluoride). After centrifugation at 12 000*g* for 30 minutes at 4°C, each pellet was lysed in 1 volume of ice-cold homogenization buffer containing 0.1% NP-40 by homogenization in a Dounce homogenizer. It was then centrifuged at 12 000*g* for 30 minutes at 4°C, and the pellet nucleus was washed twice with ice-cold buffer containing 0.35 mol/L sucrose. After washing, the nucleus was preextracted with 1 volume of ice-cold homogenization buffer containing 0.05 mol/L NaCl and 10% glycerol for 15 minutes at 4°C. The nucleus was then extracted with homogenization buffer containing 0.3 mol/L NaCl and 10% glycerol for 1 hour at 4°C. After pelleting the extracted nucleus at 12 000*g* for 30 minutes at 4°C, 45% $(NH_4)_2SO_4$ was added to the supernatant. The precipitated protein was collected at 17 000*g* for 30 minutes, resuspended in homogenization buffer containing 0.35 mol/L sucrose, and stored in aliquots at −70°C.

ODN containing the NF-κB binding site (5′-CCTTGAA GGGATTTCCCTCC-3′; only sense strands are shown) and/or ets binding site 5′-GTGCCGGGGTAGGAAGTGGGCTGGG-3′; only sense strands are shown) were labeled as primers at the 3′ end, using a 3′ end-labeling kit (Clontech Inc). After end-labeling, ^{32}P-labeled ODN were purified by application to a Nick column (Pharmacia). Binding mixtures (10 μL) including ^{32}P-labeled primers (0.5 to 1 ng, 10 000 to 15 000 cpm) and 1 μg polydeoxyinosinic-deoxycytidic acid (Sigma Chemicals) were incubated with 10 μg nuclear extract for 30 minutes at room temperature and then loaded onto 5% polyacrylamide gel. As a control, samples were incubated with an excess (×100) of nonlabeled ODN, which completely abolished binding. Gels were analyzed by autoradiography.

In Situ Zymography

Gelatinolytic activity in rat aorta was analyzed with gelatin-coated film (Fuji Photo Film Co Ltd) by the methods previously reported.[26] Aneurysms of rats were excised 1 week after the operation, and frozen sections (5 μm) of tissue samples were placed on this film. Films with specimens were incubated in a humidified chamber at 37°C overnight. Then the film was stained with 1% Amido Black 10B (WAKO Inc) in 70% methanol and 10% acetic acid for 15 minutes. After destaining with a solution of distilled water, 70% methanol, and 10% acetic acid, lysis of the substrate was observed by light microscopy.

Ultrasonography

Ultrasonography was used to demonstrate dilation of the AAA. A cardiovascular ultrasound system (Power Vision 6000, Toshiba) and a linear transducer (15 MHz) were used to image the abdominal aorta noninvasively in anesthetized rats. Rats were scanned transversely to obtain images for measurement of the luminal diameter and the area of the lumen of the aneurysm at the segment with maximum diameter. The aortic size was measured before and after laparotomy once per week up to 4 weeks after the operation.

Histology

Rats were killed 4 weeks after the operation. The excised aorta was fixed in 10% neutral buffered formalin and processed for routine paraffin embedding. Aortic tissue cross sections (6 μm) were stained with both hematoxylin and eosin and Miller's elastin and van Gieson's stain in a standard manner.

Statistical Analysis

All values are expressed as mean±SEM. ANOVA was used to determine the significance of differences in multiple comparisons. A value of $P<0.05$ was considered significant.

Results

Prevention of Aneurysmal Dilation by Chimeric Decoy ODN in Rat AAA Model

To clarify the important role of NF-κB and ets in the pathogenesis of AAA, it is extremely important to understand the expression of NF-κB and ets in human aneurysms. Our immunohistochemical study showed that NF-κB was expressed mainly in the adventitia and intima in specimens from human AAA (Figure 2). Also, binding activity of NF-κB and ets was demonstrated by gel shift mobility assay. These findings suggest that activation of NF-κB or ets might be one of the major factors regulating the process of aortic dilatation. To confirm the successful transfer of decoy ODN into the aorta, we first transfected FITC-labeled decoy ODN by using a delivery sheet. Fluorescence could be detected mainly in the adventitia and part of the media (Figure 3A). Even at 7 days after wrapping in the sheet, as shown in Figure 3, fluorescence

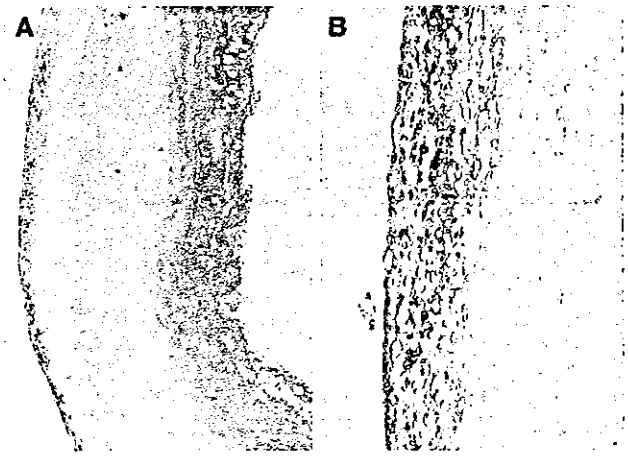

Figure 2. Immunohistochemical staining of p65 protein (a component of NF-κB) in human harvested AAA. A, Low magnification (×200). B, High magnification (×400).

was still readily detected mainly in the adventitia and part of the media, whereas no fluorescence was detected in untransfected artery. The fluorescence was localized primarily in cell nuclei. Untreated vessels and vessels transfected with non–FITC-labeled decoy ODN revealed no specific fluorescence. These findings established the feasibility of transfection of naked decoy ODN with the use of a delivery sheet to treat vascular disease, since the delivery sheet readily changed to a gel in wet conditions. Of importance, we observed NF-κB–positive macrophages mainly in the adventitia (Figure 2). Therefore, our approach to transfer decoy ODN in in vivo experiments appears to be ideal to test our scientific hypothesis.

Of importance, the binding affinity of NF-κB and ets was markedly increased in the aorta after perfusion with elastase as compared with sham-operated rats (Figure 3, B and C). As shown in Figure 3B, transfection of NF-κB decoy ODN by a delivery sheet suppressed elastase-induced activation of NF-κB. Also, treatment with chimeric decoy ODN suppressed not only activation of NF-κB but also activation of ets (Figure 3C). In contrast, the increased binding of NF-κB or ets was not inhibited by chimeric scrambled decoy ODN. These results demonstrated the simultaneous inhibition of NF-κB and ets binding activity by chimeric decoy ODN.

Indeed, in situ zymography showed that the expression of MMPs in the adventitia was markedly reduced in the aorta of an AAA model transfected with chimeric decoy ODN, whereas MMP expression was markedly increased in an AAA model transfected with scrambled decoy ODN (Figure 4A). There was no significant difference in MMP expression between untransfected rats ("control" elastase-infused aorta) and rats transfected with scrambled decoy ODN. Moreover, gel zymography demonstrated that the transfection of chimeric decoy ODN reduced the activity of MMP-9 (Figure 4B). Pro and active MMP-9 could be distinguished by the size. The 116-kDa band is the proform of MMP-9 and the 80-kDa band is active MMP-9.[27,28] Both the proform and the active form of MMP-9 were sup-

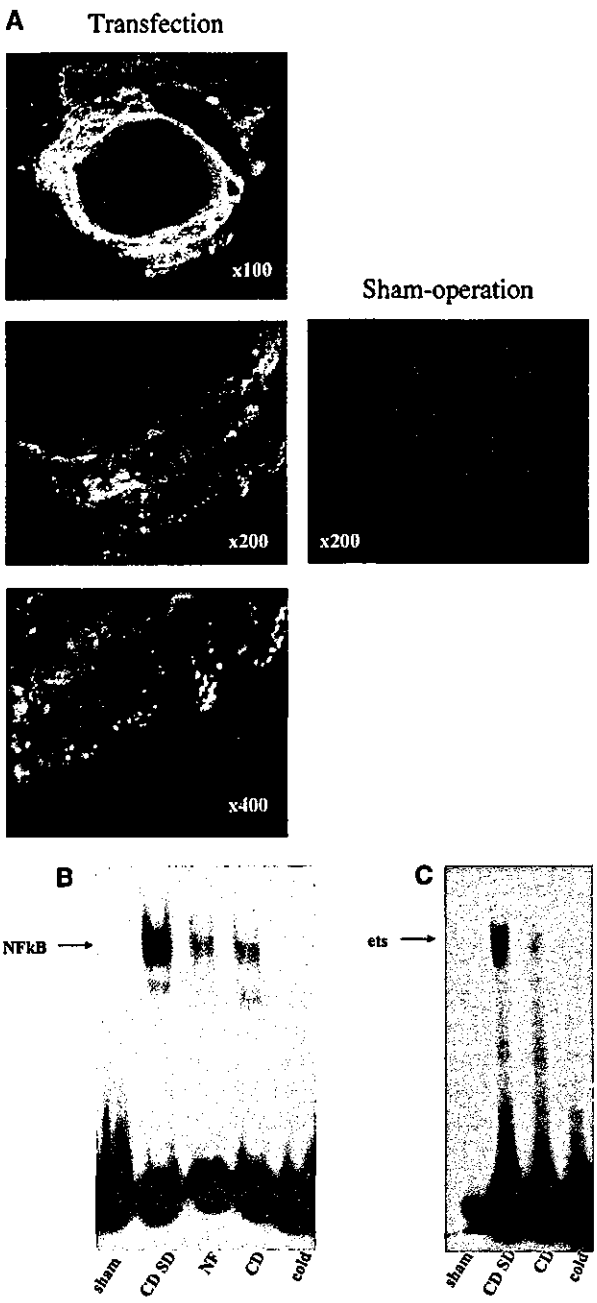

Figure 3. A, Typical photograph of fluorescence in rat aorta transfected with FITC-labeled ODN, using delivery sheet (B and C). B, Gel-mobility shift assay for NF-κB binding site. Sham indicates nuclear extract (30 μg) from aorta with saline perfusion; CD SD, nuclear extract (30 μg) from aorta transfected with chimeric scrambled decoy ODN; NF, transfected with NF-κB decoy ODN; CD, transfected with chimeric decoy ODN; Cold, nonlabeled NF-κB probe (×100 excess). Experiments were repeated 4 times. C, Gel-mobility shift assay for ets binding site. Sham indicates nuclear extract (30 μg) from aorta with saline perfusion; CD SD, nuclear extract (30 μg) from aorta transfected with scrambled chimeric decoy ODN; CD, transfected with chimeric decoy ODN; Cold, nonlabeled ets probe (×100 excess). Experiments were repeated 4 times.

pressed by the treatment with chimeric decoy ODN. Also, the suppression of MMP-9 expression in tissue transfected with chimeric decoy ODN was confirmed by Western blot (Figure 4C).

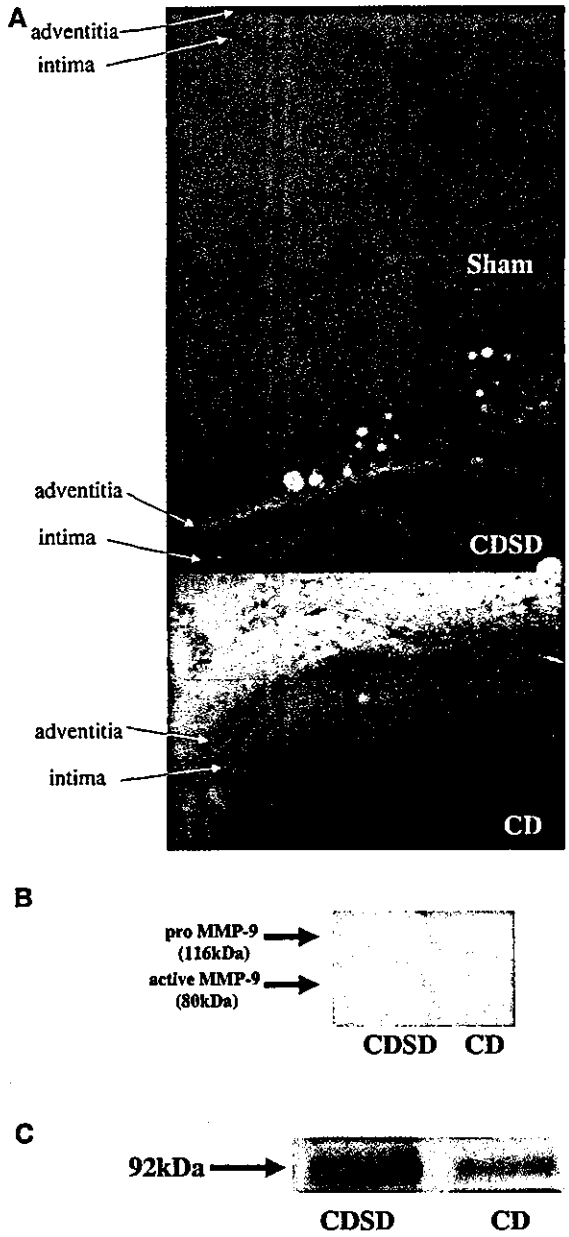

Figure 4. A, Representative cross sections of in situ gelatin zymography (×200). Scrambled decoy ODN (CDSD) did not suppress gelatinolytic activity. Gelatin beneath adventitia of vessels was degraded by induction of MMP in rat elastase infusion aneurysm model treated with CDSD. However, chimeric decoy ODN prevented gelatin degradation. B, Gel zymography. Experiments were repeated 4 times. C, Western blot for MMP-9. Sham-operated indicates aorta from sham-operated rats without elastase perfusion; CD SD, aorta transfected with chimeric scrambled decoy ODN; CD, aorta transfected with chimeric decoy ODN. Experiments were repeated 4 times.

Evaluation of Preventive Effects of Chimeric Decoy ODN in Rat AAA Model

We therefore examined the inhibitory effects of chimeric decoy ODN on aortic dilation by using a delivery sheet. As shown in Figure 5, A and B, ultrasound analysis demonstrated that treatment with chimeric decoy ODN prevented the progression of aortic dilation after elastase perfusion ($P<0.01$). Even 4 weeks after transfection, the progression of

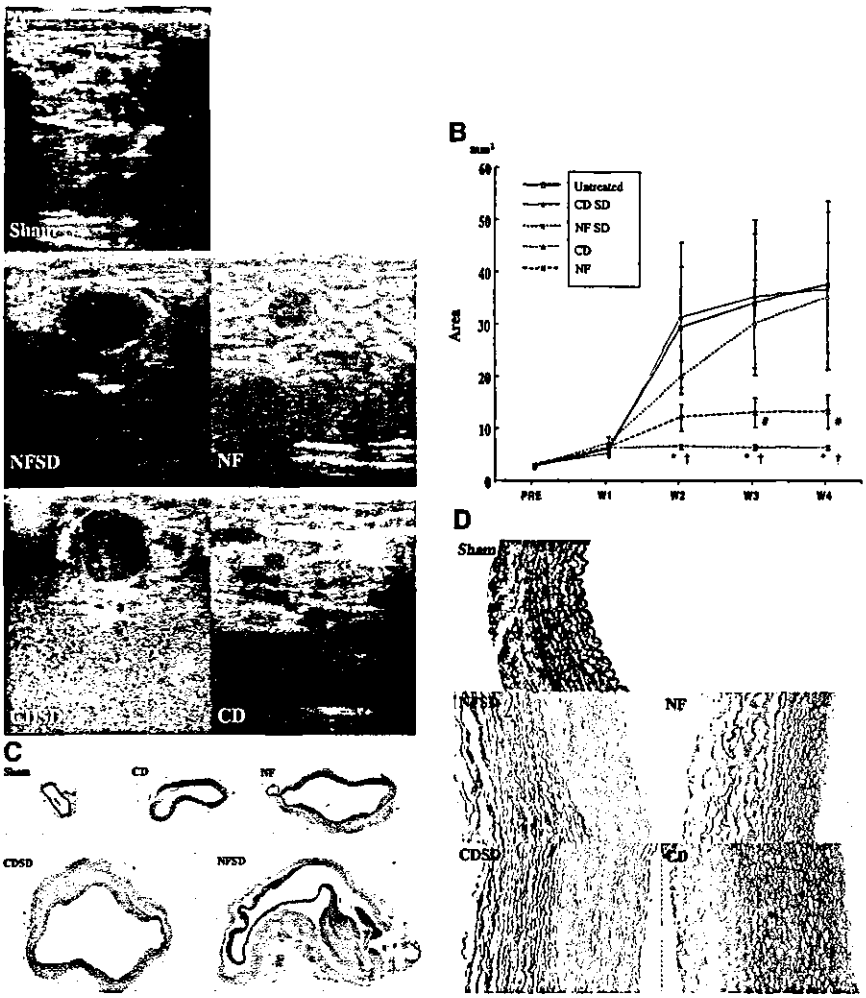

Figure 5. A, Representative ultrasound of aortic dilation. B, Time course of aortic size after elastase perfusion assessed by ultrasound. *$P<0.05$ vs untreated (with elastase perfusion), CDSD, NFSD, #$P<0.05$ vs CDSD, NFSD, †$P<0.05$ vs NF. n=6 per group. C, Representative cross sections with hematoxylin and eosin staining (×40). D, Histological sections of rat aorta stained with Miller's elastin and van Gieson's stain (×400). Sham-operated, Aorta from sham-operated rats without elastase perfusion; Untreated, aorta with elastase perfusion only; NF SD, AAA model transfected with NF-κB scrambled decoy ODN; NF, AAA model transfected with NF-κB decoy ODN; CD SD, AAA model transfected with chimeric scrambled decoy ODN; CD, AAA model transfected with chimeric decoy ODN.

AAA was still inhibited by chimeric decoy ODN. The inhibitory effect of chimeric decoy ODN on aortic dilation was also confirmed by histological studies (Figure 5C). Interestingly, the inhibitory effect of chimeric decoy ODN on aortic dilation was more potent than single transfection of NF-κB decoy ODN, whereas transfection of NF-κB decoy ODN also resulted in a significant decrease in aortic dilation as assessed by ultrasound and histological examination ($P<0.05$, Figure 5B). In contrast, both scrambled decoy ODN against NF-κB and chimeric decoy ODN failed to prevent aortic dilation (Figure 5B).

Finally, one of the major pathogenetic mechanisms of AAA is considered to be degradation of the extracellular matrix. As elastic fibers maintain the structure of the vascular wall against hemodynamic stress, resulting in prevention of aortic dilation, we therefore evaluated the effect of chimeric decoy ODN on the destruction of elastic fibers. As shown in Figure 5D, treatment with chimeric decoy ODN markedly inhibited the proteolysis of elastin as compared with scrambled decoy ODN as a control. Similarly, VEG staining demonstrated that transfection of NF-κB decoy ODN also inhibited the destruction of elastic fibers, though it was not as potent as chimeric decoy ODN. There was no significant difference between aorta transfected with both scrambled decoy ODN and sham-operated aorta. Infiltration of the aortic wall by monocytic leukocytes is considered to be a key mechanism in the progression of AAA. Interestingly, immunohistochemical study demonstrated that the migration of macrophages was markedly inhibited by the transfection of chimeric decoy ODN (Figure 6). Thus, the effect of chimeric decoy ODN is considered to be mediated by not only reduction of MMP expression but also inhibition of the migration of macrophages followed by suppression of inflammatory change.

Discussion

AAA is a common degenerative condition associated with aging and atherosclerosis.[29] Although elective surgical repair is an effective approach to prevent deaths from AAA rupture, there is a conspicuous absence of alternative therapeutic strategies for this disease.[30] Because human aneurysmal tissues are characterized by destructive remodeling of the elastic media and outer aortic wall, recent investigations have emphasized disease mechanisms involving chronic aortic wall inflammation and the progressive degradation of fibrillar matrix proteins.[31–33] The dissolution of elastic fibers requires the presence of specific proteinases, and several elastolytic MMP are thought to contribute to aneurysm development, including MMP-2 and MMP-9.[5,34,35] MMP-9 has attracted particular interest because it is the most abundant elastolytic

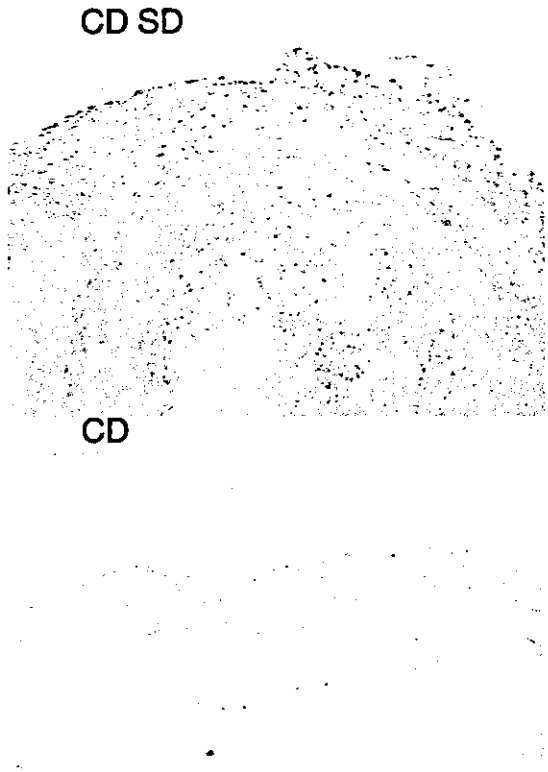

Figure 6. Immunohistochemical staining of macrophages. CD SD indicates aorta of rat AAA model transfected with chimeric scrambled decoy ODN; CD, aorta of rat AAA model transfected with chimeric decoy ODN.

proteinase secreted by human AAA tissue explants in vitro and is actively expressed by aneurysm-infiltrating macrophages located at the site of tissue damage in situ.[36] MMP-9 expression also correlates with increasing aneurysm diameter,[6,37] and it is elevated in the circulating plasma of patients with AAA.[38] In addition, patients with AAA have elevated MMP-2 levels in the vasculature remote from the aorta. These observations have led to speculation that either MMP-1, MMP-2, or MMP-9 might be necessary for aneurysmal degeneration, thereby providing targets for pharmacological therapy.[6-8] Treatment with anti-inflammatory agents or MMP antagonists leads to preservation of medial elastin and a reduction in experimental aneurysm development.[7-10] Also, targeted deletion of only MMP-9 inhibited AAA progression in gene targeting studies with the use of an elastase-induced mouse AAA model.[39] Inhibition of MMPs was also confirmed by the observation that local delivery of TIMP-1, a specific physiological inhibitor of MMP-9, prevented the progression of AAA. The present studies with chimeric decoy ODN also demonstrated a similar inhibitory effect on the progression of AAA through the inhibition of MMP-9. Additionally, it is noteworthy that the contribution of the inflammatory process is also important in the progression of AAA, since macrophages from the intravascular or retroperitoneal space, induced by inflammation, are the main cells secreting MMP-9.[1-3] NF-κB is also known to be a transcription factor for adhesion molecules. Importantly, the present data demonstrated that suppression of NF-κB activation by decoy ODN inhibited the migration of macrophage.

The specificity of the inhibitory effect of chimeric decoy ODN on the progression of AAA is supported by several lines of evidence: (1) In vivo administration of chimeric decoy ODN but not scrambled ODN markedly inhibited dilation of the aorta, accompanied by inhibition of NF-κB and ets binding activity. (2) Reduction of the aortic diameter by chimeric decoy ODN delivered by a peripheral wrapping sheet was more potent than a single transfection of NF-κB decoy ODN. (3) The decrease in matrix degradation activity in adventitia transfected with chimeric decoy ODN was associated with decreased aortic diameter. As NF-κB also regulates ICAM, VCAM, and ELAM, which are well-known adhesion molecules, transfection of chimeric decoy ODN would result in a beneficial effect on macrophage migration. Overall, the suppression of AAA by chimeric decoy ODN could be mediated by 3 pathways: (1) direct inhibition of MMP gene expression driven by either the NF-κB or ets binding site, (2) indirect inhibition of MMP secretion by migrating macrophages and VSMC, and (3) inhibition of migration of macrophages that secrete MMP. In this study, we demonstrated that inhibition of the progression of experimental AAA in rats was achieved by using a new tool: the chimeric decoy strategy against both NF-κB and ets. The present data suggest an important role of NF-κB and ets in the pathogenesis of AAA.

Acknowledgments

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ELSEVIER

Molecular therapy to inhibit NFκB activation by transcription factor decoy oligonucleotides

Ryuichi Morishita[1,*], Naruya Tomita[2], Yasufumi Kaneda[3] and Toshio Ogihara[2]

Molecular therapy is emerging as a potential strategy for the treatment of various diseases for which few known effective therapies exist. One strategy for combating disease processes has been to target the transcriptional process. Two approaches have been used to accomplish this: the use of antisense complimentary to the mRNA of interest and the use of ribozymes, a unique class of RNA molecules that not only store information but also process catalytic activity. Ribozymes are known to catalytically cleave specific target RNA, leading to its degradation, whereas antisense molecules inhibit translation by binding to mRNA sequences on a stoichiometric basis. More recently, small interfering RNA has been shown to inhibit target gene expression. The application of oligonuclotide technology, such as antisense, to regulate the transcription of disease-related genes *in vivo* has important therapeutic potential. Transfection of cis-element double-stranded oligodeoxynucleotides has been reported as a powerful tool in a new class of anti-gene strategies for molecular therapy.

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Abbreviations

ds	double-stranded
ICAM	intracellular adhesion molecule
IL	interleukin
NFκB	nuclear factor-κB
ODN	oligodeoxynucleotide
RA	rheumatoid arthritis
TNF-α	tumour necrosis factor-α
VSMC	vascular smooth muscle cell

Introduction

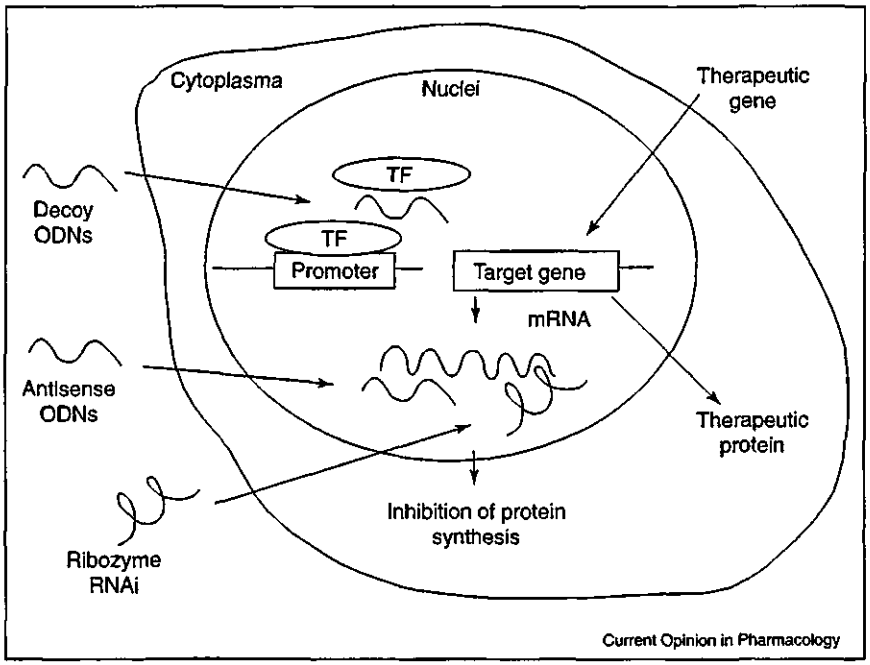
As a consequence of the revolutionary developments in the field of molecular biology and their impact on our understanding of the mechanisms of disease processes, treatment strategies that exploit our expanding knowledge of the structures and functions of molecules are being pursued. Recent progress in molecular biology has provided new techniques to inhibit target gene expression. Particularly, the application of DNA technology, such as the antisense strategy, to regulate transcription of disease-related genes *in vivo* has important therapeutic potential. Antisense oligodeoxynucleotides (ODNs) are widely used as inhibitors of specific gene expression because they offer the exciting possibility of blocking the expression of a particular gene without any changes in functions of other genes (Figure 1). Therefore, antisense ODNs are useful tools in the study of gene function and might be potential therapeutic agents. Indeed, the first approved drug for ODN-based therapy was an antisense drug to treat cytomegalovirus retinopathy in 1999.

The second approach is the use of ribozymes, a unique class of RNA molecules that not only store information but also process catalytic activity. Ribozymes are known to catalytically cleave specific target RNA, leading to degradation of the RNA, whereas antisense molecules inhibit translation by binding to mRNA sequences on a stoichiometric basis. In addition, RNA interference technology is also believed to inhibit target gene expression, potentially providing new therapeutic options. Conversely, we have recently developed a novel molecular strategy in which synthetic double-stranded (ds) DNA with high affinity for a target transcription factor can be introduced into target cells as a 'decoy' cis element to bind the transcriptional factor and alter gene transcription [1]. Transfection of cis-element ds ODNs (a decoy) has been reported as a powerful tool in a new class of anti-gene strategies for molecular therapy. Transfection of ds ODNs corresponding to cis sequences will result in the attenuation of authentic cis-trans interaction, leading to the removal of trans-factors from the endogenous cis-element with subsequent modulation of gene expression (Figure 1). Therefore, the decoy approach might enable us to treat diseases by modulation of endogenous transcriptional regulation. In this article, we review the mechanisms and potential applications of the decoy strategy.

Principles

Correct regulation of gene expression is essential both to normal development and to the correct functioning of the adult organism. Such regulation is usually achieved at the

Figure 1



Target sites for antisense, ribozyme and decoy strategies. Decoy ODNs are targeted to inhibit the binding of transcription factor (TF) to its binding site, whereas antisense ODNs, ribozyme ODNs and RNAi are targeted to the mRNA level.

level of DNA transcription, a process that controls which genes are transcribed into RNA by the enzyme RNA polymerase, although post-transcriptional regulation is also important. The transcription of specific genes is controlled by regulatory proteins known as transcription factors. Transcription factors have been grouped into families on the basis of shared DNA-binding motifs. Other regions of the factors interact with RNA polymerase and its associated proteins to increase or decrease the rate of transcription. The vital role of these transcription factors, together with the fact that a single factor can affect the expression of many genes, suggests that the inactivation of a transcription factor as a result of an inherited mutation is incompatible with survival. Initially, overexpression of TAR-containing sequences (TAR decoys) in a double-copy murine retroviral vector was used to render cells resistant to HIV replication [2]. Currently, TAR decoys (i.e. short RNA oligonucleotides corresponding to the HIV TAR sequence) are used to inhibit HIV expression and replication by blocking the binding of the HIV regulatory protein Tat to the authentic TAR region [2–4]. However, such RNA decoys are difficult to use *in vivo*. In addition, the regulation of decoy expression is problematic. To overcome these issues, we hypothesized that synthetic ds DNA with high affinity for transcription factors could be introduced *in vivo* as a 'decoy' cis-element to bind the transcription factors and block activation of genes, resulting in an effective therapy for treating diseases. Transfection of ds ODNs corresponding to the cis sequence will result in attenuation of the removal of trans-factors from the endogenous cis-element, with subsequent modulation of gene expression. This approach is particularly attractive for several reasons: firstly, the potential drug targets (transcription factors) are plentiful and readily identifiable; secondly, the synthesis of the sequence-specific decoy is relatively simple and can be targeted to specific tissues; thirdly, knowledge of the exact molecular structure of the target transcription factor is unnecessary; and finally, decoy ODNs can be more effective than antisense ODNs in blocking constitutively expressed factors, as well as multiple transcription factors that bind to the same cis element. Although the mechanisms of action of antisense ODNs are still unclear, the principle of the transcription factor decoy approach is simply the reduction of promoter activity as a result of the inhibition of binding of a transcription factor to a specific sequence in the promoter region.

Applications

Restenosis after angioplasty or stenting

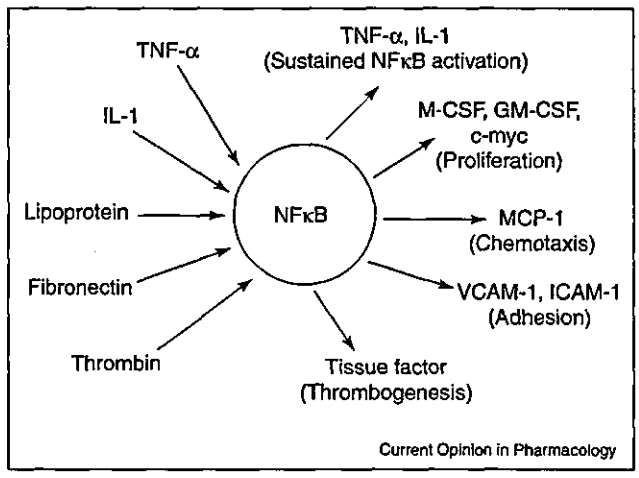
Balloon angioplasty is one of the major therapeutic approaches to coronary artery stenosis. However, restenosis after angioplasty still remains an issue in the field of cardiovascular disease, as the long-term effectiveness of this procedure is limited by the development of restenosis in over 40% of patients [5]. Thus, the possibility of ODN-based therapy for restenosis was expected, and its

effect has been evaluated in many animal models. Most strategies targeted vascular smooth muscle cell (VSMC) proliferation after balloon injury, which is one of the major mechanisms of chronic restenosis. Intimal hyperplasia develops largely as a result of VSMC proliferation and migration induced by a complex interaction of multiple growth factors that are activated by vascular 'injury' [5]. Initially, the effectiveness of antisense ODNs against a proto-oncogene, c-myb, was reported for the treatment of restenosis [6]. Accordingly, inhibition of other proto-oncogenes, such as c-myc, by antisense ODNs was also reported to inhibit neointimal formation in several animal models [7]. Recently, the results from a phase II trial using antisense c-myc to treat restenosis have been reported [8•]. Treatment with 10 mg phosphorothioate-modified ODNs directed against c-myc did not reduce neointimal volume obstruction or the angiographic restenosis rate [8•]. However, that trial utilized intra-coronary infusion of antisense c-myc ODNs without any vectors, and several factors such as low transfection efficiency might limit the efficacy of this strategy.

The process of VSMC proliferation is dependent upon the coordinated activation of a series of cell-cycle regulatory genes that results in mitosis. Our previous data revealed that a single administration of antisense ODNs against proliferating cell nuclear antigen and cdc 2 kinase genes inhibited neointimal formation after angioplasty for at least eight weeks after transfection [9]. Because a critical element of cell-cycle progression regulation involves the complex formed by E2F, cyclin A and cdk 2, it is hypothesized that transfection of VSMCs with a sufficient quantity of decoy ODNs containing the E2F cis element (consensus sequence 'TTTTCGGCGC') would effectively bind E2F, prevent it from transactivating gene expression of essential cell-cycle regulatory proteins, and thereby inhibit VSMC proliferation and neointimal formation. Transfection of E2F decoy ODNs into rat and porcine balloon-injured arteries resulted in significant inhibition of neointimal formation, whereas mismatched ODNs had no effect on neointimal hyperplasia [10]. In 1996, clinical application of E2F decoy ODNs by Dzau at Harvard University was initiated to treat neointimal hyperplasia in vein bypass grafts; this hyperplasia results in failure of up to 50% of grafts within a period of 10 years. Their phase I/IIa study demonstrated successful inhibition of graft occlusion, accompanied by selective inhibition of proliferating cell nuclear antigen and c-myc expression [11]. More recently, similar results were obtained in a double-blind placebo-controlled study to treat cardiac vein graft failure (VJ Dzau, personal communication).

Nuclear factor-κB (NFκB), a transcription factor essential for inflammation, also plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes whose activation has been postulated to be involved in numerous diseases including restenosis. The NFκB complex is a heterodimer of two subunits: p50 and p65. In its inactive form in the cytoplasm, the complex is associated with an inhibitory subunit, IκB. Subunit p65 has the capacity for potent transactivation of target genes and is able to bind IκB. NFκB is activated by a variety of cytokines, endotoxins and oxidative stress. On activation, NFκB dissociates from its inhibitor (IκB), translocates to the nucleus, and initiates transcription of genes for various cytokines and growth and differentiation factors. Numerous cytokines, including interleukin (IL)-1, IL-2, IL-6, IL-8 and tumour necrosis factor-α (TNF-α), are regulated by NFκB. Interestingly, adhesion molecules such as vascular cell adhesion molecule and intracellular adhesion molecule (ICAM) are also upregulated by NFκB. Accordingly, we hypothesized that restenosis could be prevented by the blockade of genes regulating cell inflammation — the final common pathway induced by NFκB binding (Figure 2). The necessity to block cytokine and adhesion molecule genes at more than one point to achieve maximum inhibitory effects might be because of the redundancy and complexity of the interactions of these genes. Importantly, increased NFκB binding activity has been confirmed in balloon-injured blood vessels [12]. Our recent study provided the first evidence of the feasibility of a decoy strategy against NFκB in treating restenosis [12]. Transfection of NFκB decoy ODNs into balloon-injured carotid artery [12] or porcine coronary artery [13•] markedly reduced neointimal formation, whereas no difference was observed between scrambled decoy ODNs and control. In addition to VSMC proliferation, endothelial damage is one of the major mechanisms of restenosis, as endothelial cells play

Figure 2



Role of NFκB. Activation of NFκB by TNF-α, IL-1, lipoprotein, fibronectin and thrombin upregulate genes related to the inflammatory and immunological responses, such as TNF-α, adhesion molecules, macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein (MCP).

an important role as a biological barrier in the suppression of VSMC growth, maintenance of vascular tonus and protection from monocyte and platelet adhesion. Severe damage to endothelial cells by balloon injury induces vascular inflammation and has been assumed to be responsible for restenosis. From this viewpoint, inhibition of NFκB might be ideal. Importantly, transfection of NFκB decoy ODNs inhibited endothelial cell death [14]. Thus, the protective effects of NFκB decoy ODNs against restenosis might be mediated by several mechanisms: firstly, prevention of endothelial damage induced by inflammation; secondly, prevention of macrophage or leukocyte recruitment through suppression of adhesion molecules; thirdly, inhibition of VSMC growth by improvement of endothelial function; and finally, inhibition of VSMC growth by induction of VSMC apoptosis. On the basis of data from preclinical studies, a clinical trial using NFκB decoy ODNs to treat restenosis was started in Japan in 2002. In this trial, decoy ODNs were delivered to the vessel wall through a hydrogel-coated catheter without any viral or non-viral vector.

Other cardiovascular diseases

Myocardial reperfusion injury develops, to a large degree, as a result of severe damage to myocytes and endothelial cells, probably induced by the complex interaction of multiple cytokines and adhesion molecules activated by reperfusion. The process of ischemic reperfusion might be dependent upon the coordinated activation of a series of cytokine and adhesion molecule genes that results in the attachment of leukocytes and release of cytotoxic molecules. Importantly, increased NFκB binding activity was confirmed in hearts with myocardial infarction [15]. Our previous study provided the first evidence of the feasibility of a decoy strategy against NFκB in treating myocardial reperfusion injury [15,16]. Transfection of NFκB decoy ODNs into rat coronary artery before occlusion of the left ascending coronary artery markedly reduced the damaged area of myocytes at 24 hours after reperfusion, whereas no difference was observed between scrambled decoy ODN-treated and untransfected rats. The therapeutic efficacy of this strategy by intra-coronary administration immediately after reperfusion, similar to the clinical situation, was also examined. NFκB decoy ODNs reduced the damage to myocytes resulting from reperfusion. Inhibition of VSMC replication was confirmed by the observation that transfection of NFκB decoy ODNs inhibited the progression of vasculopathy in cardiac transplantation models [17,18].

Glomerulonephritis

The pathophysiology of glomerulonephritis is complicated; numerous cytokines, including IL-1, IL-2, IL-6, IL-8 and TNF-α, regulate this process. These cytokines are also regulated by NFκB in renal cells [19,20]. Thus, intra-renal infusion of NFκB decoy ODNs caused an approximate 50% reduction in urinary protein excretion in a rat model of anti-glomerular basement glomerulonephritis; there was also a substantial reduction in histological damage in the NFκB decoy ODN-treated kidney compared with the scrambled ODN-treated kidney, including a fivefold reduction in glomerular crescent formation [21]. The marked glomerular and interstitial leukocytic infiltration seen in the scrambled ODN-treated animals was also significantly reduced (by approximately 50%) with NFκB decoy ODN treatment. More recently, we hypothesized that transfection of NFκB decoy ODNs into the donor kidney would prevent acute rejection and prolong graft survival, and thus provide effective therapy for renal acute rejection. Using ultrasound exposure with an echocardiographic contrast agent (Optison), graft function (serum creatinine) and histological structure were shown to be well preserved, with significantly decreased expression of NFκB-regulated cytokines and adhesion molecules, including IL-1, inducible nitric oxide synthase, monocyte chemoattractant protein-1, TNF-α and ICAM-1, in allografts transfected with NFκB decoy ODNs [22•]. As a result, animal survival was significantly prolonged in this group compared with controls [22•]. *Ex vivo* gene therapy using NFκB decoy ODNs might be useful to maintain the renal function in dialysis patients.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial proliferation. Overexpression of inflammatory cytokines regulated by NFκB, especially IL-1 and TNF-α, is thought to play an important role in the pathogenesis of joint destruction in the arthritic condition [23]. Cells migrating into the synovium are the major source of these pro-inflammatory cytokines; in particular, the cartilage–pannus junction shows a pronounced expression of IL-1 and TNF-α. Biological agents targeting these cytokines have been successful both in experimental models and human trials [4–7,8•]. These results have indicated the importance of such inflammatory cytokines in the pathogenesis of RA; therefore, blockade of these cytokines or, alternatively, augmentation of anti-inflammatory cytokines can offer an alternative therapy in RA. *In vivo* transfection of NFκB decoy ODNs by intra-articular injection into a rat model of collagen-induced arthritis decreased the severity of hind-paw swelling [24]. Histological and radiographical studies showed a marked suppression of joint destruction following NFκB decoy ODN transfection, accompanied by suppression of IL-1 and TNF-α production in the synovium of arthritic joints. Intra-articular administration of NFκB decoy ODNs prevented the recurrence of streptococcal cell wall-induced arthritis in treated joints [24]. Interestingly, the suppression of synovial cell proliferation was achieved by NFκB decoy ODNs in synovial cells derived from RA patients through inhibition of the production of IL-1β, IL-6, TNF-α, ICAM-1 and matrix metalloproteinase-1. These results prompted us

to evaluate the therapeutic effects of NFκB decoy ODNs on joint destruction in a cynomolgus monkey model of collagen-induced arthritis, which shares various features with human RA as a preclinical model of arthritis. Consecutive radiographic examinations showed the marked suppression of joint destruction by NFκB decoy ODNs. During the observation period, other than inflammatory parameters, no abnormal change in biochemical parameters resulting from ODN administration was recognized. Pathological examinations of brain, heart, lung, liver, kidney and testis also showed no abnormal findings. On the basis of these data, clinical trails to treat RA patients using NFκB decoy ODNs were initiated in 2003 at Osaka University.

Atopic dermatitis

The treatment of atopic dermatitis, a chronic inflammatory disease resulting from complex interactions between genetic and environmental mechanisms, by NFκB decoy ODNs has been considered. Atopic dermatitis afflicts 10–15% of children and adolescents in the Western world. Keratinocytes of patients with atopic dermatitis exhibit a propensity to exaggerated production of cytokines and chemokines, a phenomenon that plays a major role in promoting and maintaining inflammation. NF-κB is of particular interest, as it promotes the transcription of Th2 cytokines (e.g. IL-6), as well as adhesion molecules (e.g. ICAM-1). In addition, the delayed allergic component of atopic dermatitis, which is dependent upon the Th1 dominant pathway, is believed to be regulated by NF-κB-induced cytokines such as interferon-r. Interestingly, topical administration of NF-κB decoy ODNs twice a month resulted in a significant reduction in clinical skin condition score and a marked improvement in histological findings. Reduction of the atopic skin condition by NF-κB decoy ODNs was accompanied by a significant decrease in migration of mast cells into the dermis and an increase in apoptotic cells [25•]. What is the clinical relevance of NFκB decoy ODNs? Currently, the choice of pharmaceutical drugs to treat atopic dermatitis is limited. Despite the rapid and proven efficacy of topical corticosteroids, side effects limit their clinical usefulness. Topically active macrolide immunosuppressants, such as ascomycin and tacrolimus, appear to provide comparable therapeutic potency. Although data from ongoing studies will be crucial in determining both the safety of these agents in the long-term and their place within the current therapeutic armamentarium available for patients with atopic dermatitis, these agents still have potential side effects, such as systemic absorption. In contrast, NF-κB decoy ODNs exerted only local pharmacological effects for the following reasons: firstly, ODNs in serum were rapidly destroyed (within several hours), leading to a lack of systemic effects [26]; and secondly, the toxicity of systemic administration of ODNs was only reported at a concentration over 100-fold higher than that employed in the study. From December 2001, we initiated a Phase I/IIa human clinical trial using NFκB decoy ODNs to treat atopic dermatitis at Hirosaki University. Topical application of NFκB decoy ODNs exhibited marked therapeutic effects on the skin condition of faces of patients with atopic dermatitis (Morishita R *et al.*, unpublished). Because side effects can limit the clinical usefulness of corticosteroids, topically applied NF-κB decoy ODNs appear to provide novel therapeutic potency without significant local or adverse effects.

Cancer

Cancer cachexia, characterized by anorexia, weight loss and progressive tissue wasting, has been postulated to be mediated by various cytokines. However, the precise mechanism of cachexia induction is not fully understood. Transfection of NFκB decoy ODNs into the tumors of mice with adenocarcinoma (colon26) resulted in attenuation of the reductions in body weight, epididymal fat, gastrocnemius muscle mass and food intake, which were all initially induced by the tumor [27]. Unexpectedly, tumor growth of colon26 was not affected by transfection of NFκB decoy ODNs when compared with scrambled decoy ODNs. Cytokines regulated by NFκB could play a pivotal role in the induction of cachexia by colon26, providing a new therapeutic strategy for cancer cachexia. In addition, we employed NFκB decoy ODNs in a model of hepatic metastasis of M5076 reticulosarcoma in mice to evaluate the role of NFκB in the pathogenesis of cancer metastasis. Murine M5076 reticulosarcoma cells, which arise spontaneously in the ovary of a C57BL/6 mouse, are reported to have the ability to form preferentially metastatic colonies in peritoneal viscera such as liver, spleen and ovary, regardless of the site or route of tumor cell injection [28]. Intravenous treatment with NFκB decoy, but not scrambled decoy, ODNs reduced the transactivation of three important genes, IL-1β, TNF-α and ICAM-1, in the liver after intravenous inoculation of M5076, thereby inhibiting the hepatic metastasis of M5076 in mice. Combined treatment of NFκB decoy ODNs with an anti-cancer drug resulted in complete inhibition of hepatic metastasis in half of the mice without affecting myelosuppression induced by the anti-cancer drug. These data suggest NFκB decoy ODNs as a novel molecular therapy.

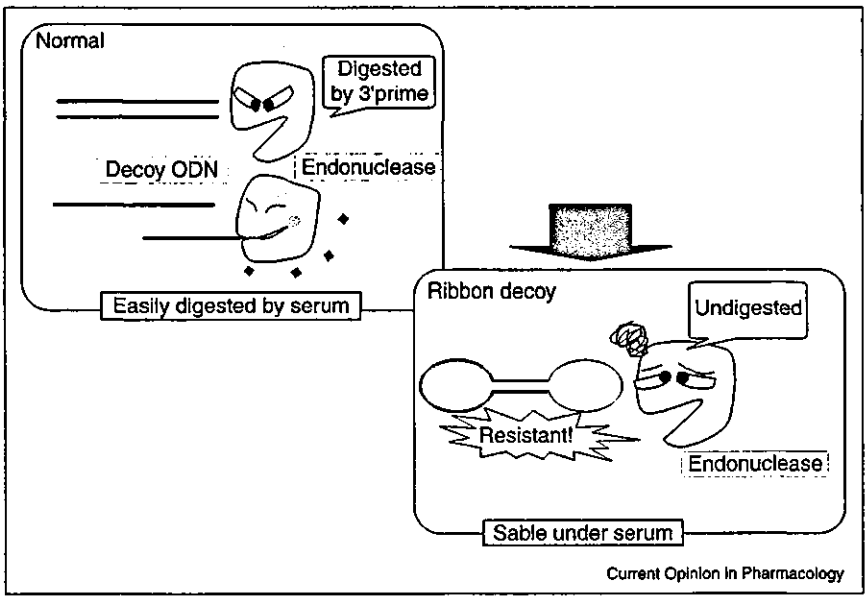
Unresolved issues in ODN-based gene therapy

ODN-based gene therapy still has many unsolved problems such as a short half-life, low uptake efficiency, and degradation by endocytosis and nucleases. Therefore, many groups are currently focusing on modifications of the gel approach using a catheter delivery system. Further modification of ODN pharmacokinetics will facilitate the potential clinical utility of the agents by, firstly, allowing a shorter intraluminal incubation time to preserve organ perfusion; secondly, prolonging the duration of biological action; and thirdly, enhancing efficacy such that the

non-specific effects of high doses of ODNs can be avoided. Although direct transfer of 'naked' decoy ODNs can be achieved through passive uptake, the transfection efficiency seems to be lower than that with single-stranded antisense ODNs. To enhance the transfection efficiency of decoy ODNs, the cationic liposome (hemagglutinating virus of Japan–liposome) method or other vector systems are generally used [29••]. The majority of ODNs is sequestered and degraded in lysosomes and never reaches the nucleus. Because the site of decoy effects is in the nucleus, bypassing the endocytotic pathway and translocation of decoy ODNs from the cytoplasm are extremely important in the practical application of therapeutics.

One of the major concerns about the ODN-based strategy as gene therapy is their non-specific effects, particularly those of phosphorothioate-substituted ODNs. This concern is not only related to the antisense and decoy strategies but also to all ODN-mediated therapy. Non-sequence-specific inhibition might operate through blockade of cell surface receptor activity or interference with other proteins [30]. Concurrently, ODN-containing GC dinucleotides could induce immune activation [31]. In addition, sequence-specific binding of non-transcriptional factor proteins to ODNs has been reported to result in non-specific effects of ODN-based gene therapy [32]. Moreover, Burgess *et al.* [33] reported that the antiproliferative activity of c-myb and c-myc antisense ODNs in VSMCs is caused by a non-antisense mechanism. To overcome these issues, controlled experiments must be performed to eliminate the potential non-specific effects of ODN-mediated therapy. For gene therapy using an ODN-based strategy, the toxicity of phosphorothioate ODNs might also be important. Although low dose administration does not seem to cause any toxicity, bolus infusions could be dangerous. Higher doses over prolonged periods of time can cause kidney damage, as evidenced by proteinuria and leukocytes in the urine of animals [32]. Liver enzymes can also be increased in animals treated with moderate to high doses. Several phosphorothioate ODNs have been shown to cause acute hypotensive events in monkeys [34,35], probably as a result of complement activation [36]. These effects, if managed appropriately, are transient and relatively uncommon. This toxicity can be avoided by using intravenous infusions rather than bolus injections. More recently, prolongation of prothrombin, partial thromboplastin and bleeding times has been reported in monkeys [37]. We recently modified decoy ODNs to increase their stability against nucleases; although chemical modification (e.g. phosphorothioation and methylphosphonation) of ODNs was employed, problems with these modified ODNs became apparent, including insensitivity to RNaseH, lack of sequence-specificity, and immune activation. To overcome these limitations, covalently modified ODNs were developed by enzymatically ligating two identical molecules, thereby preventing their degradation by exonucleases (Figure 3) [38]. Indeed, these modified decoy (ribbon) ODNs possess increased nuclease resistance and are transported more efficiently into cells than were the chemically modified linear ODNs [39,40]. More recently, we reported that transfection of novel activator protein-1 decoy ODNs with a circular ribbon-structure,

Figure 3



Ribbon-type decoy ODNs are resistant to digestion by endonucleases, whereas normal-type decoy ODNs can be digested by these enzymes.

Box 1 Potential targets for gene therapy using NFκB decoy ODNs

Target disease
Cardiovascular disease
Restenosis after angioplasty
Graft failure
Myocardial reperfusion injury
Transplant vasculopathy
Renal disease
Glomerulonephritis
Renal rejection
Cancer
Cachexia
Metastasis
Other diseases
Arthritis
Atopic dermatitis

before the balloon injury procedure, prevented neointimal formation in the rat balloon-injured artery more effectively than did non-modified decoy ODNs [41•].

Conclusions

Molecular therapy founded upon NFκB decoy ODNs could be useful for the treatment of many diseases, including the prevention of restenosis after angioplasty, myocardial infarction and rejection in renal transplantation (Box 1). The first federally approved antisense drug was launched in 1999 for retinopathy patients. Although there are still many unresolved issues, decoy ODN drugs should become a reality.

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8 Gene Therapy vs Pharmacotherapy

Ryuichi Morishita, MD, PhD

CONTENTS

SUMMARY

Recent progress in molecular and cellular biology has developed numerous effective cardiovascular drugs. However, there are still number of diseases for which no known effective therapy exists, such as peripheral arterial disease, ischemic heart disease, restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. Currently, gene therapy is emerging as a potential strategy for the treatment of cardiovascular disease to treat such diseases despite this limitation. The first human trial in cardiovascular disease started in 1994 to treat peripheral vascular disease, using vascular endothelial growth factor (VEGF). Since then, many different potent angiogenic growth factors have been clinically tested to treat peripheral arterial disease. In addition, therapeutic angiogenesis using *VEGF* gene was applied to treat ischemic heart disease from 1997. The results from these clinical trials appear to exceed expectations. Improvement of clinical symptoms in peripheral arterial disease and ischemic heart disease has been reported. Many different potent angiogenic growth factors have been tested in clinical trials to treat peripheral arterial disease or ischemic heart disease. In addition, another strategy for combating disease processes, to target to transcriptional process, has been tested in a human trial. Transfection of *cis*-element double-stranded (ds) oligodeoxynucleotides (ODN) (decoy) is an especially powerful tool in a new class of antigene strategies for gene therapy. Transfection of ds ODN, corresponding to the *cis* sequence, will result in the attenuation of authentic *cis*-trans interaction, leading to the removal of transfactors from the endogenous *cis*-elements with subsequent modulation of gene expression. Genetically modified vein grafts transfected with decoy against E2F, an essential transcription factor in cell cycle progression, revealed apparent long-term potency

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in human patients. This review focuses on the future potential of gene therapy for the treatment of cardiovascular disease.

Key Words: *cis*-element decoy; antisense; angiogenesis; VEGF; HGF.

INTRODUCTION

Somatic gene therapy consists of the introduction of normal genes into the somatic cells of patients to correct an inherited or acquired disorder through the synthesis of specific gene products in vivo. In general, there are three methods of gene modification: (1) gene replacement, (2) gene correction, and (3) gene augmentation. Gene augmentation is the most promising technique for the modification of targeted cells in therapy for cardiovascular disease. For this purpose, many in vivo gene transfer methods have been developed. In vivo gene transfer techniques for cardiovascular applications include: (1) viral gene transfer—retrovirus, adenovirus, adeno-associated virus (AAV) or hemagglutinating virus of Japan (HVJ, or Sendai virus); (2) liposomal gene transfer using cationic liposomes; and (3) naked plasmid DNA transfer. These in vivo gene transfer techniques have different advantages and disadvantages. Although current in vivo methods for cardiovascular gene transfer are still limited by the lack of efficiency and potential toxicity, recent advances in in vivo gene transfer may provide the opportunity to treat cardiovascular diseases, such as peripheral arterial disease by manipulating angiogenic growth factor genes.

Simple comparison of gene therapy vs pharmacotherapy may not be appropriated. The present promising gene therapy is mainly by locally administrated agents, whereas most of pharmacotherapy is based on oral drug administration. To consider the advantage of gene therapy, one might compare it with recombinant therapy, because both concepts are relatively close. There are at least three advantages of gene therapy. First, it has the potential to maintain an optimally high and local concentration over time. This issue may be critical in the case of arterial gene therapy. In order to avoid side effects in the case of therapeutic angiogenesis, however, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressed transgene in the iliac artery, rather than a single or multiple bolus doses of recombinant protein. Indeed, our initial trial using HGF did not demonstrate the increase in serum hepatocyte growth factor (HGF) level. Second, regarding economics, one must consider which therapy would ultimately cost more to develop, implement, and reimburse, particularly for those indications requiring multiple or even protracted treatment. Third, the feasibility of a clinical trial of recombinant protein is currently limited by the lack of approved or available quantities of human-quality recombinant protein. This may be due, in large part, to the nearly prohibitive cost of scaling up from research grade to human quality recombinant protein. Indeed, the report that compared the effectiveness of fibroblast growth factor-2 (FGF-2) as protein and as naked plasmid DNA, in a porcine model of chronic myocardial ischemia, demonstrated that intramyocardial injection of FGF-2 plasmid was more effective than that of FGF-2 protein in improving regional perfusion and contractility compared to untreated ischemia *(1)*. In contrast, gene therapy also has disadvantages, such as safety aspects, and local and limited effects.

GENE THERAPY FOR VASCULAR DISEASES

Gene Therapy to Treat Peripheral Arterial Disease Using Therapeutic Angiogenesis

Critical limb ischemia, which is estimated to develop in 500 to 1000 individuals per million per year, is considered one of most suitable diseases for gene therapy. In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexorable downhill course. Of importance, there is no optimal medical therapy for critical limb ischemia, as the Consensus Document of the European Working Group on Critical Limb Ischemia concluded. Thus, in patients with critical limb ischemia, amputation, despite its associated morbidity, mortality, and functional implications, is often recommended as a solution to the disabling symptoms, particularly in the case of excruciating ischemic rest pain. Indeed, a second major amputation is required in nearly 10% of such patients. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. Therefore, novel therapeutic modalities are needed to treat these patients. In the pathophysiology of the disease, in the presence of obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, ischemia occurs. Recently, the efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischemia *(2–5)*. Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia. Most of the initial studies have used VEGF, also known as vascular permeability factor, as well as a secreted endothelial cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, as endothelial cells represent the critical cellular element responsible for new vessel formation *(2,3)*.

A human clinical trial using *VEGF* gene started in 1994 by Professor Isner at Tufts University. An initial trial was performed using a hydrogel catheter with naked $VEGF_{165}$ plasmid. Although this procedure seems to be effective to stimulate collateral formation in patients with peripheral arterial disease (PAD) *(2–5)*, it is not ideal to treat many patients, as most patients lack an appropriate target vascular lesion for catheter delivery. Thus, his group applied intramuscular injection of naked plasmid encoding $VEGF_{165}$ gene (Fig. 1). Exceeding expectations, this clinical trial demonstrated clinical efficacy for treatment of PAD *(2–5)*. Since then, numerous angiogenic growth factors, such as $VEGF_{121}$, VEGF-2, and basic-fibroblast growth factor (bFGF) have been tested in clinical trials *(6,7)*. In addition to intramuscular injection of naked plasmid DNA, adenoviral delivery, liposomal delivery of angiogenic growth factors, was also utilized in these trials *(2–9)*, despite an unfortunate accident using adenoviral vector reported at the University of Pennsylvania *(10)*. In addition to the success to the intramuscular injection of $VEGF_{165}$ and VEGF2 plasmid DNA, local catheter-mediated $VEGF_{165}$ gene therapy in ischemic lower-limb arteries after percutaneous transluminal angioplasty (PTA) was also success-