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 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...
AGCTCGTATATGCACTGACGCGAGTC-5 binds to free NF-kB. We demonstrated successful treatment of AAA by using chimeric decoy ODN in this study (Figure 1).

NF-kB and ets activation involves in extracellular matrix degradation. Urokinase plasminogen activator, which are proteases involved in the fibrin degradation, have been shown to bind the NF-kB-TCGAGCATATACGTGACTGCGCTCAG-3 and 3'-AACGGCATGGACTGAATCGG-5 genes. Among them, we focused on NF-kB and ets, preventing transactivation of various genes described in schema.

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'-TGGCCCTTCAATATCCCTAARUGGAAGG-5'; scrambled chimeric decoy ODN, 5'-TGGCCCTTCAATATCCCTAARUGGAAGG-3' and 3'-ACCGGAAGTATGAGGGATTTCCCTCC-5'; and scrambled NF-kB decoy ODN, 5'-TGGCCCTTCAATATCCCTAARUGGAAGG-3' and 3'-ACCGGAAGTATGAGGGATTTCCCTCC-5'.

NF-kB decoy ODN have been shown to bind the NF-kB transcription factor (Figure 1), consistent with previous reports.

Immunohistochemical Studies

Anti-NF-kB 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 (NiCl2) 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 1:40 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% NiCl2 solution for 5 minutes.

Procedure of AAA Model

Male Wistar rats (400 to 500 g; Charles River Breeding Laboratories) were anesthetized and underwent laparotomy. 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 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-cm2 thin sheet containing 100 nmol decoy ODN/cm2. 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 eichrome black T solution. Elastic fibers stained dark red and were readily distinguishable from the specific FITC-labeled ODN.

Electrophoretic Mobility Shift Assay

Rats were killed 1 week after the operation, and nuclear extracts were prepared from transfected or untransfected aortic aneurysms. In brief, rat aortas were homogenized with a Potter-Elvehjem homogenizer in 4 volumes of ice-cold homogenization buffer (10 mmol/L HEPES [pH 7.5], 0.5 mmol/L sucrose, 0.5 mmol/L spermidine, 0.15 mmol/L spermin, 5 mmol/L EDTA, 0.25 mmol/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 NaCl and 10% glycerol for 15 minutes at 4 °C. The nucleus was then extracted with homogenization buffer containing 0.05 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% (NH4)2SO4 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’-CCTGAA GGATTITCCCTC-3’: only sense strands are shown) and/or ets binding site 5’-GTGCCGGTTAGGAAATGGCTGGG-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, 32P-labeled ODN were purified by application to a Nick column (Pharmacia). Binding mixtures (10 μL) including 32P-labeled primers (0.5 to 1 ng, 10 000 to 15 000 cpm) and 1 μg polydeoxyinosinic-deoxyctydilic 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 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 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-
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).

### 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...
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
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. MMP-9 expression also correlates with increasing aneurysm diameter and is elevated in the circulating plasma of patients with AAA. 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. Treatment with anti-inflammatory agents or MMP antagonists leads to preservation of medial elastin and a reduction in experimental aneurysm development. Also, targeted deletion of only MMP-9 inhibited AAA progression in gene targeting studies with the use of an elastase-induced mouse AAA model. 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. 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.

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