Percutaneous transluminal coronary angioplasty is a well-established and frequently performed procedure developed for the treatment of advanced coronary artery disease. After an “initial gain” in lumen size, the long-term efficacy of this procedure is limited by the high incidence of restenosis due to intima hyperplasia (response to injury). This excessive neointimal formation is caused mainly by the proliferation and migration of medial smooth muscle cells (SMCs). Cardiovascular risk factors such as hypercholesterolemia, dysfunctional endothelium, and the pressure-induced deformation of the vessel wall are pivotal factors in promoting SMC proliferation. Several growth factors released after vascular injury act as mediators for cell cycle progression with subsequent SMC proliferation and neointimal lesion formation. Arterial injury in the setting of elevated serum cholesterol results in diffuse macrophage infiltration, sustained impairment of endothelial function, and marked intimal proliferation.

Many cytokine genes, including those encoding acute-phase proteins and immunoglobulins, share binding sites for CCAAT/enhancer-binding protein (C/EBP) in their 5′-flanking regions, and C/EBP-related transcription factors regulate cell proliferation during terminal differentiation. Therefore, C/EBP represents an attractive target for inhibiting restenosis after balloon angioplasty. In a rabbit model of restenosis that combines balloon injury of the carotid artery with cholesterol-mediated chronic inflammation, a decoy oligodeoxynucleotide (ODN) capable of neutralizing C/EBP was administered to the site of injury for 30 minutes. Electrophoretic mobility shift analysis confirmed that C/EBP activity in decoy ODN–treated segments was virtually absent after 2 days. Morphometric analysis after 28 days revealed significant reduction (up to 50%) of neointimal formation and intravascular inflammation in decoy ODN–treated segments compared with mutant control ODN or vehicle-treated segments. In addition, de novo synthesis of endothelin-1 and the number of proliferating cell nuclear antigen–positive smooth muscle cells in the vessel wall were markedly attenuated at day 3. These findings suggest that decoy ODN–based neutralization of C/EBP may be a feasible and effective method to limit restenosis after angioplasty brought about, at least in part, by inhibiting the de novo synthesis of endothelin-1. (Arterioscler Thromb Vasc Biol. 2002;22:949-954.)

Key Words: balloon injury • endothelin-1 • decoy oligodeoxynucleotides • CCAAT/enhancer-binding protein • restenosis
chronic inflammation. Compared with neointimal lesions in normocholesterolemic animal models, lipid-rich atherosclerosis-like lesions in hypercholesterolemic rabbits represent an important model for studying experimental therapeutic approaches, including gene therapy, for the inhibition of restenosis after percutaneous transluminal coronary angioplasty.14

Methods

Decoy ODN Technique

Double-stranded decoy ODNs were prepared from the complementary single-stranded phosphorothioate-bonded ODN (Eurogentec) as described13 and dissolved in TEN buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 150 mmol/L NaCl, pH 7.5). The single-stranded sequences of the palindromic ODN were as follows (underlined letters denote phosphorothioate-bonded bases): 5’-TG-CAGATTGCGCAATCTGCA 3’ (consensus decoy ODN), 5’-TG-CAGACTAGTCTCTGCA 3’ (mutant control ODN).

A fluorescent Texas red-labeled consensus decoy ODN with the same sequence (also from Eurogentec) was used for verifying vascular transfer of the ODN in ex vivo organ bath experiments (60 minutes of incubation) as described previously.13

Animal Model of Vascular Injury

Except for those animals used for electrophoretic mobility shift analysis (EMSA), the rabbits were fed a diet containing 1% cholesterol (Altromin) beginning 15 days before the intervention. They were maintained on the diet until harvest of the blood vessels 3 or 28 days after balloon injury. Serum cholesterol was found at a level ranging from 884 to 1473 mg/dL on the day of treatment.

All animal experiments were performed with institutional approval and according to local and international guidelines. Male New Zealand White rabbits (2.8 to 3.5 kg body weight) were intubated and anesthetized by inhalation anesthesia (1.5% to 2% isoflurane). After premedication with a mixture of ketamine (0.25 mg/kg, 10%), midazolam (5 mg/kg), pentobarbital (20 mg/kg), and buprenorphine (0.05 mg/kg). A midline vertical incision was made, and the left common carotid artery was dissected free of surrounding tissue. Side branches were ligated with 5/0 silk suture.

Before the clamping of the common carotid artery (proximally) and the internal and external carotid arteries (distally), heparin (100 U/kg) was administered systemically. Then, a 2F Fogarty embolectomy catheter was introduced via a cutdown of the external carotid artery. The balloon was inflated in the mid common carotid artery and pulled back to the bifurcation as described elsewhere.15 This procedure was repeated twice. After the vessel was injured, the balloon catheter was exchanged to a nylon infusion catheter. After the isolated vessel segment was flushed with saline, 300 μL of the DNA solution (20 μmol/L C/EBP decoy ODN or mutant control ODN) or vehicle (TEN buffer) was infused, avoiding any air bubble entrapment. The treated vessel segment was ∼2 cm long. The ODN solution or vehicle was applied at a physiological pressure of 100 mm Hg and allowed to incubate for 30 minutes. After removal of the infusion catheter and ligation of the external carotid artery, clamps were released, and blood flow was reestablished through the common carotid artery and the internal carotid artery. The wound was closed with absorbable 3/0 sutures.

Morphometric Analysis

At the time intervals indicated, rabbits were euthanized by intravenous injection of 1.2 g pentobarbital. The vessel segments were harvested, flushed with saline, and perfusion-fixed with 4% formaldehyde at 100 mm Hg. The segments were embedded in paraffin, and 3-μm-thick sections were cut and stained with hematoxylin/eosin or elastica van Gieson’s stain. Computerized planimetry (Qwin 500 MC; image processing and analysis system, Leica) was used to measure intimal and medial cross-sectional surface area. Neointima was distinguished by its position relative to the internal elastic lamina, and media was differentiated from adventitia by its own elastic fibers and the pink staining of the adventitial collagen (ie, external elastic lamina). Luminal radius, intimal and medial thicknesses, and the (neo)intima-to-media ratio were derived from these data, and averages for each vessel were calculated (minimum of 3 sections per segment).

Electrophoretic Mobility Shift Analysis

Preparation of nuclear extracts and 32P-labeled double-stranded C/EBP ODNs (Santa Cruz Biotechnology), nondenaturing polyacrylamide gel electrophoresis, autoradiography, and supershift analyses with the corresponding supershift antibodies (also from Santa Cruz Biotechnology) were performed as described.14

Immunohistochemistry

Sections (3 μm) of paraffin-embedded segments were stained with 4 different antibodies by using a biotin-avidin immunoperoxidase procedure with 3-aminoo-9-ethylcarbazole as a chromogen: (1) RAM-11, a monoclonal antibody for rabbit macrophages (DAKO); (2) HHF35, a monoclonal antibody for human smooth muscle α-actin (also from DAKO); (3) PC10, a monoclonal antibody for rat proliferating cell nuclear antigen (PCNA), and (4) a monoclonal anti-ET-1 antibody (Dianova). For analysis of positive RAM-11 or HHF35 immunostaining, the specimens were studied by computerized planimetry. All sections were scanned at equal light conditions with a CCD video camera (Nikon). From each section, 4 representative regions were selected, and antigen-positive cells were automatically determined as the percentage of intimal and medial cross-sectional surface area by using Qwin 500 MC software.

The number of PCNA-positive cells in the media was also counted by using the Qwin 500 MC software and expressed as a percentage of the total number of hematoxylin-stained medial cells.

Evaluation of the more diffuse ET-1 immunoreactivity was performed with up to 4 sections from each segment by 2 investigators blinded to the different treatment regimes. The findings were coded semiquantitatively: 0 indicated lack of immunoreactivity; 0.5, weak staining; and 1, positive staining.

Statistical Analysis

Statistical analysis was performed by using Excel 2000 data sheets (Microsoft) and InStat 3.0 software (Graph Pad). Values are expressed as mean±SEM. Significant group effects of data from immunohistochemical analysis or from morphometric planimetry were determined by ANOVA followed by the Bonferroni multiple comparison test with a significance level at P<0.05.

Results

Uptake and Efficacy of the Decoy ODN

Organ bath experiments using intraluminal vascular application to endothelium-denuded native arteries clearly demonstrated uptake of the Texas red–labeled C/EBP-specific consensus decoy ODN by intimal and medial cells (Figure 1a). There was no autofluorescence detectable in the cross sections under the chosen experimental conditions (not shown).

After balloon injury, the in vivo administration of the decoy ODN was well tolerated by the animals. Patency of the vessels was maintained in >95% of all injured arteries, and this percentage was not affected by the decoy ODN treatment.

EMSA analyses were performed to verify the biological activity of the consensus decoy ODN. As shown in Figure 1b, C/EBP activity was effectively neutralized by the decoy ODN and was virtually absent in nuclear extracts from decoy ODN–treated arterial segments 2 days after the intervention.

Inhibition of Injury-Induced Lesion Formation

Morphometric analysis of C/EBP decoy ODN–treated arteries 28 days after injury revealed a significant reduction in...
neointima formation compared with vehicle or mutant control ODN–treated arteries (Figure 2). In C/EBP decoy ODN–treated arteries, all 3 parameters of restenosis (neointima-to-media ratio, total wall thickness, and lumen loss) were reduced by 30% to 40%. Differences in wall thickness were primarily due to neointima formation, because medial thickness was not influenced by the treatment (Figure 2e).

### Histological and Immunohistochemical Criteria

Four weeks after balloon injury, large numbers of foam cells identified immunohistochemically as macrophages were found throughout the intima of vehicle-treated and mutant control ODN–treated arteries and in subendothelial accumulations that corresponded to areas of macroscopic plaque (please see Figure I in online data supplement, which can be accessed at http://atvb.ahajournals.org). In C/EBP decoy ODN–treated segments, only isolated macrophages in areas of small neointimal accumulation were observed, and there were almost no foam cells organized in plaques. Moreover, planimetry revealed an ≈50% decrease in the macrophage (RAM 11)–positive area in C/EBP decoy ODN–treated segments compared with segments treated with the mutant control ODN (Figure 3a). At 3 days after surgery, no macrophages could be identified in any of the vessel segments (not shown).

Neointimal lesions of injured arteries mainly consisted of myofibroblasts arranged in a chaotic order compared with the circumferential arrangement of the SMCs in the media. Immunohistochemical staining with an antibody against smooth muscle α-actin revealed a 16.4±3.1% actin-positive area in C/EBP decoy ODN–treated segments compared with 12.7±0.5% and 15.4±2.3% in vehicle-treated and mutant control ODN–treated arteries, respectively (no significant differences between the groups, n = 7). Moreover, immunohistochemical analysis showed that the media underwent a transformation between day 3 and 28 after balloon injury. At the third day after injury, the media was nearly unchanged compared with normal arterial walls and, thus, was mainly composed of actin-positive cells and some elastic fibers arranged in circumferential order. However, at this time, C/EBP decoy ODN treatment already resulted in an ≈50% decrease in the number of proliferating (ie, PCNA-positive) SMCs in the media (Figure 3b). No PCNA-positive cells were detected in the noninjured contralateral carotid arterial segments (not shown).

Four weeks after the intervention, the media consisted of macrophages localized in the inner region of the media (approximately half) and of actin-positive SMCs in the outer region adjacent to the adventitia (approximately half). Elastic fibers organized these cells in a circumferential order around the neointima. This architecture was not affected by the C/EBP decoy ODN, the mutant control ODN, or TEN buffer (not shown).

### Effects on ET-1 Synthesis

Typical ET-1 immunohistochemical findings are shown in the data supplement (please see online Figure II, which can be accessed at http://atvb.ahajournals.org). Whereas in control segments (TEN buffer or mutant control ODN) ET-1 synthesis was found throughout the intima and media, this area of C/EBP decoy ODN–treated vessels was almost void of ET-1 immunoreactivity. Figure 3c summarizes the semiquantitative analysis of the immunohistochemistry findings. Compared with vehicle or mutant control ODN treatment, C/EBP decoy ODN treatment significantly reduced ET-1 synthesis. This low level of ET-1 immunoreactivity was similar to that in the contralateral carotid arterial segments not subjected to balloon injury.

### Discussion

In the present study, we demonstrated that a single application of a consensus decoy ODN designed to neutralize the transcription factor C/EBP resulted in reduced neointimal lesion formation after vascular balloon injury in hypercholesterolemic rabbits. The mutant control ODN had no effect on lesion formation. We observed a significant inhibition of vascular inflammatory reaction parallel to the inhibition of lesion formation, as shown by reduced macrophage infiltration and foam cell formation. Besides inhibition of the activity of the transcription factor, immunohistochemical analysis showed a clear reduction of injury-induced ET-1 synthesis and SMC proliferation in the tunica media.
Neointimal hyperplasia of medial vascular SMCs contributes significantly to vessel narrowing after angioplasty. Arterial injury in the setting of elevated serum cholesterol results in diffuse macrophage infiltration, sustained impairment of endothelial function, and marked intimal proliferation. Thus, the hypercholesterolemic rabbit model is ideal for studying genetic engineering approaches aimed at modulating cholesterol-mediated mitogenic factors involved in lesion formation. Although cholesterol values seen in the rabbit model are not typical in the clinical setting, the direct relation of hypercholesterolemia to the increased incidence of restenosis in patients undergoing balloon angioplasty has been established.2

For the successful inhibition of restenosis, only small quantities of molecular-based therapeutic agents seem to be sufficient to inhibit the surge of mitogen activation and vascular SMC proliferation leading to neointimal lesion formation. The immediate activation of mitogens as the response to injury,2 with a maximum of DNA replication at day 3, represents a window of opportunity for decoy ODN treatment.17 Synthetic double-stranded ODNs neutralize transcription factors, thereby blocking their binding to the promoter region of targeted genes, resulting in the inhibition of gene transactivation18 and, as a consequence, exerting beneficial therapeutic effects.17 Usually decoy ODNs mimic the consensus DNA binding site of a given transcription factor. For added stability, we used double-stranded phosphorothioate-bonded decoy ODNs, which are taken up by native and cultured vascular cells without additional reagents for enhancing DNA uptake.13,19

The response to injury2 activates a complex molecular program after vascular injury (denudation and overstretch). Factors contributing to this molecular program, besides others, may be increased activity of ET-1, loss of NO synthase activity, and induction of PDGF. ET-1 is one of the most powerful mitogens for vascular SMCs. In addition to control-
It is noteworthy that a single application of the consensus C/EBP decoy ODN resulted in successful transfer of ODN to almost all cells in the intima and media, as demonstrated by using a fluorescence-labeled decoy ODN. The high uptake of the decoy ODN observed ex vivo was sufficient to cause a prolonged suppression of C/EBP activity in vivo, as demonstrated by EMSA 2 days after balloon injury. Furthermore, immunohistochemical analysis demonstrated significant reduction of ET-1 peptide abundance in the vessel wall 3 days after the intervention. ET-1 is produced not only by endothelial cells and vascular SMCs but also by macrophages and polymorphonuclear leukocytes, suggesting a role for ET-1 also in the proinflammatory response. In addition, the rate of proliferation of the medial SMCs was significantly reduced at this point in time in the decoy ODN–treated arteries.

Although ET-1 is a potent mitogen, other factors contribute to neointimal lesion formation as well. It has been reported that the major regulatory domain in the 5′-flanking region of the PDGF-α receptor gene accounting for the increase in gene expression in response to stretch is a C/EBP-binding site that is functionally important. Thus, therapeutic interventions using C/EBP decoy ODN may interact directly with PDGF-α receptor expression. Unfortunately, suitable immunohistochemical detection tools are currently not available to verify the potential effect of the C/EBP decoy ODN on PDGF receptor expression in the rabbit model.

Localized inflammatory reactions are consistently associated with atherosclerotic lesion formation and, in particular, with plaque rupture. Because plaque rupture is a major contributing factor to sudden cardiac death, the therapeutic option of reducing macrophage recruitment to silencing the plaque by a simple molecular-based therapy using a C/EBP decoy ODN is appealing. Recently, C/EBPβ has been implicated in proinflammatory gene expression in macrophages but not fibroblasts, suggesting an important role for C/EBP in cholesterol-mediated mitogenic effects. Moreover, C/EBP plays a role in intercellular adhesion molecule-1 expression. Intercellular adhesion molecule-1 is expressed by vascular SMCs and has been implicated in the homing of leukocytes to sites of inflammation, infection, or tissue damage. However, the exact mechanism by which the C/EBP decoy ODN interacts with macrophage recruitment warrants further studies.

In summary, in the present study, we show that local application of a C/EBP decoy ODN can inhibit neointimal lesion formation after balloon injury. At least in part, the beneficial effects of this decoy ODN treatment may be mediated by interfering with the de novo synthesis of ET-1, a major mitogenic factor induced after vascular injury. Thus, decoy ODN–based transcription factor neutralization may be useful as an adjunct given after angioplasty to inhibit restenosis.

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References
5. Weinberger FF, McLenachan JM, Cybulsky MI, Fallon JT, Hollenberg NK, Cooke JP, Ganz P. Hypercholesterolemia enhances macrophage

Figure 3. a, Summarized planimetry data comparing the RAM-11–positive (ie, macrophage-positive) area among vehicle-treated arteries (TEN, n = 7), C/EBP decoy ODN–treated arteries (C/EBPc, n = 7), and mutant control ODN–treated arteries (C/EBPm, n = 7) 28 days after intervention. IHC indicates immunohistochemical analysis. b, Statistical summary of the number of PCNA-positive SMCs (expressed as percentage of the total number of cells) in the media 3 days after carotid artery injury in vehicle-treated segments (TEN, n = 4), C/EBP decoy ODN–treated segments (C/EBPc, n = 4), and mutant control ODN–treated segments (C/EBPm, n = 4). c, Summary of the semiquantitative analysis of ET-1 immunoreactivity in the intima and media 3 days after intervention in carotid artery segments treated with vehicle (TEN, n = 2), C/EBP decoy ODN (C/EBPc, n = 5), or mutant control ODN (C/EBPm, n = 6). †P < 0.05 vs TEN; ††P < 0.01 vs C/EBPc.


CCAAT/Enhancer-Binding Protein Decoy Oligodeoxynucleotide Inhibition of Macrophage-Rich Vascular Lesion Formation in Hypercholesterolemic Rabbits
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LEGENDS TO FIGURES

**Figure I**
Representative transverse sections of injured carotid arteries stained with the monoclonal anti-RAM 11 antibody, demonstrating the inhibitory effect of C/EBP decoy ODN treatment (b) as compared to the lack of effect of the mutant control ODN (a) on macrophage infiltration (arrows) at 28 days post intervention. Neointimal hyperplasia is marked by arrows (original x25).

**Figure II**
Representative sections (original x400) of injured carotid arteries revealing the inhibitory effect of C/EBP decoy ODN treatment (b) as compared to the lack of effect of vehicle (a) or mutant control ODN treatment (c) on ET-1 peptide abundance in the vessel wall 3 days post intervention. Positive ET-1 immunoreactivity is characterized by the red 3-amino-9-ethylcarbazole deposit. Panel d depicts the low level of ET-1 immunoreactivity in an untreated contra-lateral arterial segment.