Intimal Hyperplasia Recurs After Removal of PDGF-AB and -BB Inhibition in the Rat Carotid Artery Injury Model

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Abstract—Several antagonists specific for platelet-derived growth factor (PDGF) or its receptors have recently been developed and shown to inhibit intimal hyperplasia formation in various animal models, but data investigating the durability of this intervention is limited. The present study was designed to investigate the potency of PDGF B-chain aptamer, a novel type of PDGF-AB and -BB antagonist, in the rat carotid model and to characterize intermediate-term effects on lesion formation. One hundred thirty-four animals were randomized to aptamer treatment or placebo. Daily treatment with the antagonist resulted in a 50% reduction in lesion size at 2 weeks (P<0.001). The beneficial effect involved increased apoptosis and possibly an interference with smooth muscle cell migration. Discontinuing administration 1 week earlier did not give any significant benefit compared with phosphate-buffered saline–treated controls. When the antagonist was administered for 2 weeks and the vessels analyzed 6 weeks later, the beneficial effect was lost and the treated lesions had a higher intima-media and area-cell ratio compared with the treated lesions in the 2-week–endpoint study. Our findings confirm a role of PDGF B-chain in intimal hyperplasia, but the successful use of PDGF antagonists may require either prolonged treatment or combination therapy with other agents. (Arterioscler Thromb Vasc Biol. 2000;20:e89–e95.)

Key Words: balloon dilatation ■ intimal hyperplasia ■ PDGF antagonist

Premature obstruction of the vessel lumen is a major complication after percutaneous transluminal angioplasty, bypass grafting, or heart transplantation. An accelerated proliferative and migratory response of arterial wall smooth muscle cells and subsequent deposition of extracellular matrix are believed to play central roles in restenosis as well as atherosclerosis.1 These processes are regulated by various cytokines and growth factors. Descriptive studies of the expression pattern of growth factors in human atherosclerotic lesions, as well as studies in animal models of restenosis, have suggested that platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and their cognate receptors are major mediators of arterial smooth muscle cell proliferation and migration.2–10

PDGF, a growth factor and chemotaxtractant for cells of mesenchymal origin (reviewed in Reference 11), occurs as 3 dimeric isoforms, PDGF-AA, -AB, and -BB, composed of disulfide-linked A-, B-, and C-chains. PDGF signals are mediated by structurally related α- and β-receptors. In the vascular smooth muscle cell proliferation and migration that occur in intimal hyperplasia, most PDGF effects are mediated by the PDGF-β receptor.12,13,15,16 The β-receptor is activated by PDGF-BB and, when coexpressed with the PDGF-α receptor, also by PDGF-AB.

The development of specific PDGF antagonists has allowed studies on the effects of such compounds in different animal models of intimal hyperplasia formation. Promising results have been obtained in rat, porcine, and nonhuman primate models of arterial injury through the use of, for example, antibodies against PDGF and PDGF receptors, receptor antisense oligonucleotides, low-molecular-weight PDGF receptor tyrosine kinase inhibitors, and local overexpression of PDGF-β receptor extracellular domains.12,14–24 However, one remaining question to be answered is the durability of the observed beneficial effect.

To investigate the in vivo potency of PDGF aptamers in the rat carotid injury model and to investigate the durability of the therapeutic benefit of PDGF antagonists on lesion formation, we characterized the short- and intermediate-term effects on lesion formation of PDGF SELEX aptamers. This is a novel type of PDGF antagonist, ie, aptamers identified by the SELEX (systematic evolution of ligands by exponential enrichment) process (reviewed in Reference 25), that bind to the receptor-binding epitopes of the PDGF B-chain and thereby neutralize the biological effects of PDGF-AB and -BB.26

Methods

Animal Experiments
Sprague-Dawley rats (134 3-month-old animals of 370 to 450 g; Mollegaard Breeding Center, Ry, Denmark) were randomly allocated to treatment substance, control substance, or carrier (sterile...
PBS) groups. The animals were weighed twice a week. The experimental protocol was reviewed and approved by the Ethics Committee according to the Uppsala University guidelines. The left common carotid artery was injured with a 2F embolec-tomy catheter (Baxter Healthcare) basically as described by Clowes et al.27 Animals were anesthetized through intraperitoneal administration of 0.33 mL/100 g body weight of 1 part fentanyl/fluanisone (fluanisone 10 mg/mL, fentanyl 0.2 mg/mL; Hypnorm Vet, Janssen Pharmaceutica), 1 part midazolam (5 mg/mL Dormicum; Roche), and 2 parts sterile water.

Aptamers (NX1975, PDGF B-chain–specific aptamers conjugated to 40-kDa polyethylene glycol, and NX2210, the inverted-sequence analogue used as a control) were dissolved in sterile PBS shortly before intraperitoneal administration. A chemically modified form of the originally described aptamer was used, in which the modifications aimed at increasing nuclease resistance and plasma residence time; the modifications include conjugation to polyethylene glycol, substitution of some of the nucleotides with spacers, and exchange of some deoxyribonucleotides by the corresponding 2'-O-methyl or 2'-fluoro derivatives.28 The first 2 doses were given 14 hours and 2 hours before injury, and the subsequent doses every 12 hours thereafter.

Eight, 14, or 56 days after injury, the animals were anesthetized as above. Twenty minutes before surgery, the animals received an intravenous injection of 1.0 mL of 0.5% Evans blue dye (Sigma) in PBS to allow identification of the deendothelialized vessel segment. The animals were euthanatized with an intravenous overdose of the anesthetic agent and the vasculature was cleared of blood by perfusion with ice-chilled PBS at 100 mm Hg. The distal halves of the right and left common carotid arteries were snap-frozen and stored at –85°C until further processing. Immediately thereafter, the remaining proximal segments were perfused with 2.5% glutaraldehyde in phosphate buffer, pH 7.3, and were then prepared for paraffin-embedding.

**Histopathological and Immunohistological Analyses**

From each fixed vessel beginning from the distal cut end, 5 sections approximately 1 mm apart were stained with Verhoeff’s elastin stain and hematoxylin-eosin for measurements of vessel wall areas; with Harries hematoxylin for determination of the number of nuclei in each vessel compartment; and with Picro-Sirius red collagen stain to visualize the extracellular matrix component. All sections from the left carotid artery were confined to the Evans blue–stained region corresponding the deendothelialized vessel segment. Areas for the lumen, tunica intima, and tunica media and the number of nuclei in these 2 vessel wall layers were measured (KS 400 image acquisition

**Figure 1. Effects of NX1975 on intimal hyperplasia.** The left carotid artery was injured and the rats were treated with carrier, control aptamer NX2210, or PDGF B-chain aptamer NX1975 with various concentrations of aptamers and different treatment times. Intima and media areas of carotid artery sections were determined 2 weeks (A and B) or 8 weeks (C) after injury. Each group is presented with their mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs 2-week endpoint PBS control group. †P<0.001 vs 2-week endpoint NX1975.
and analysis system, Carl Zeiss), and intima-to-media and area-per-nuclei ratios were calculated. The mean value of all 5 sections was used for statistical analysis.

Cryostat sections from a single segment corresponding to the proximal cut end of the frozen specimen were fixed in 4% formaldehyde at 4°C, rinsed in 0.1 mol/L PBS, and stained as follows. Sections of the vessels where proliferating cells were to be quantified were incubated with the primary antibody against nuclear antigen Ki-67 (Ki-67 antigen MIB-5, Immunotech) at a dilution of 1:50 in PBS containing 1% bovine serum albumin and 5% horse serum. Omission of primary antibody was used as a negative control. A rat-absorbed biotinylated anti-mouse IgG antibody (1:200, Vector Laboratories) served as a secondary antibody. For detection of the secondary antibodies, an avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) was applied. The peroxidase reaction was carried out with 3,3’-diaminobenzidine tetrahydrochloride as the substrate. Counterstaining was performed with Mayer’s hematoxylin. The positive nuclei were counted in the tunica intima and tunica media (Q500IW image acquisition and analysis system, Leica), and the fraction of positively stained nuclei was calculated.

After brief formaldehyde fixation, cryosections from animals where apoptotic cells were to be quantified were incubated in 3% H2O2 in PBS at 20°C. The terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) reaction was performed for 1 hour at 37°C in a humidified chamber with 6 U (0.3 U/μL) of terminal transferase (Boehringer Mannheim) per slide in reaction buffer containing 2.5 mmol/L CoCl2 and digoxigenin-UTP. After quenching of the reaction, sections were blocked in reaction buffer containing 2.5 mmol/L CoCl2 and digoxigenin-UTP. After quenching of the reaction, sections were blocked in 2% bovine serum albumin a in PBS. After incubation with anti-digoxigenin antibodies conjugated to horseradish peroxidase (Boehringer Mannheim) and washing of the slides, the color reaction was performed with diaminobenzidine- H2O2 (Sigma). Counterstaining was performed with Mayer’s hematoxylin. The fraction of positively stained nuclei was calculated as above.

All instrumentation and measurements were performed by one individual (O.L.) blinded to treatment allocation. Two observers (M.L., M.-A.C.) controlled the measurements from randomly selected multiple samples without knowledge of the origin of the sections.

Statistical Analysis
All data are presented as mean±SD. Statistical comparisons were determined by unpaired Student’s t test or 1-way ANOVA with the Bonferroni-Dunn test. Statistical significance was accepted at the 95% confidence level.

Results
PDGF B-Chain Aptamers Inhibit Intimal Hyperplasia in the Rat Carotid Artery Balloon Injury Model
To evaluate the efficacy of the PDGF B-chain aptamers in the rat model, a study was performed that followed the design of earlier studies, wherein blocking effects of PDGF-neutralizing antibodies have been observed.17,22 Rats were randomized into 2 groups, subjected to embolectomy catheter injury, and treated for 2 weeks by intraperitoneal injections with either 20 mg kg−1 d−1 PDGF B-chain aptamer (NX1975) in PBS or PBS only. As shown in Figures IA, IIB, and IIC, treatment with NX1975 reduced the intima-media ratio by ≈50% (P < 0.001). This is similar to the 40% to 50% reduction that has been observed with neutralizing antibodies against PDGF or PDGF receptors.15–18,22 We conclude that the NX1975 acts as a PDGF-AB/-BB antagonist in vivo after intraperitoneal administration.

The same protocol was subsequently followed with NX1975 at lower concentrations, ranging between 2 and 12 mg kg−1 d−1. As shown in Figure IA, reduction of the dose to 2 mg kg−1 d−1 did not diminish the effect of NX1975 obtained with the dose of 20 mg kg−1 d−1. The inverted-sequence control aptamer (NX2210) at the dose of 12 mg kg−1 d−1 had no effect (P = NS; Figure IA). There was no statistical difference in body weight within identical observation times, as measured at the time of injury, at harvest, or as reported as a change of body weight, between the B-chain aptamer−, control aptamer−, or PBS-treated animals. Data for animals treated with 12 mg kg−1 d−1 NX1975 are shown in Table I.

To investigate the mechanism whereby treatment with NX1975 reduced the intima-media ratio, sections from lesions of treated and untreated animals were analyzed with regard to numbers of cells in the intima and media, cellular density (reported as area per cell), and percentage of proliferating cells (Table I). In the intimal layer, the number of cells was reduced to 63% in NX1975-treated (12 mg kg−1 d−1) animals compared with control animals (P = 0.016). Neither cellular density nor the fraction of proliferating smooth muscle cells in the lesions, as monitored by Ki-67 staining.
after 2-week treatment, differed significantly between treated and control animals (Table I).

Furthermore, we investigated whether apoptotic cell death was involved in the inhibition of lesion formation by PDGF-B-chain aptamers. Ten animals were randomized into 2 groups, subjected to embolectomy catheter injury, and treated with PBS or NX1975 (5 mg kg\(^{-1}\) d\(^{-1}\)) for 8 days (a time point showing a high level of TUNEL-positive cells in this animal model),\(^{29a}\) and the vessels were analyzed immediately thereafter. A trend toward a reduction in the intima-media ratio and the number of cells in the intima was seen, but these observations were not statistically significant (data not shown). However, the apoptotic index of smooth muscle cells in the intima as well as the media was significantly higher after NX1975 treatment compared with controls, as visualized by TUNEL staining (Table II). Together, these findings suggest that apoptotic cell death of smooth muscle cells is an important mechanism in NX1975-mediated reduction of lesion formation in this animal model.

**Shortening of Treatment to 1 Week Reduces the Inhibitory Effect of NX1975 on Intimal Hyperplasia Formation**

To investigate whether treatment with NX1975 during the first week after injury was sufficient to obtain significant reduction in lesion formation, a study was performed wherein animals received NX1975 (12 mg kg\(^{-1}\) d\(^{-1}\)) or carrier for the first week and carrier for the second week after balloon injury. The vessels were analyzed 2 weeks after the injury was performed (Figure IB). Vessel wall areas, numbers of cells, cellular density, or percentage of proliferating cells did not significantly differ from animals treated with carrier only (Table I).

### Two-Week Treatment With PDGF SELEX Aptamer Fails to Give Sustained Effects on the Development of Intimal Hyperplasia

A critical issue that will determine the therapeutic efficacy of PDGF antagonists for lesion formation is whether treatment leads to a permanent reduction of intimal hyperplasia or rather simply delays the process. Previous studies with PDGF antagonists for lesion formation is whether treatment leads to a permanent reduction of intimal hyperplasia or rather simply delays the process. Previous studies with PDGF antagonists in the rat carotid artery model have used end points that coincided with the end of treatment\(^{17,20–23}\) or when the antagonist still exerted its beneficial effect \(^{12,14,16,18,19,24}\) and have therefore not studied this aspect of the effects of PDGF antagonists. To address this issue, a study was performed in which NX1975 (12 mg kg\(^{-1}\) d\(^{-1}\)) was administered during the first 2 weeks after injury, and the effects of lesion formation were analyzed 6 weeks later.
As shown in Figures IC, IID, and IIE, only a slight reduction in intima-media ratio was observed at 8 weeks after injury compared with control animals at the same time point, and the difference was not statistically significant. Furthermore, in animals that had received the PDGF antagonist for 2 weeks, the intima-media ratio was 2- to 3-fold higher 8 weeks after injury, compared with 2 weeks after injury (Figure IC). This indicates that 2-week treatment with PDGF B-chain antagonists in this animal model fails to permanently block the development of intimal hyperplasia.

As in the other experiments, lesions were further analyzed to investigate the cellular processes underlying lesion formation (Table I). The increase in intima area (P=0.017) and intima-media ratio (P=0.006) in control animals at 8 weeks after injury, compared with that at 2 weeks after injury, was caused predominantly by a statistically significant increase in area-cell ratio (P=0.009) rather than an increase in cell number (P=NS; Table I). This is presumably a consequence of extracellular matrix deposition, also indicated by a more intense collagen staining of the lesions (Figures IIB’ and IID’). Also, the statistically significant difference in cell number observed 2 weeks after injury between the PDGF antagonist and control groups (P=0.016) did not remain after 8 weeks (P=NS; Table I). Finally, a significant increase in the intimal area-cell ratio (P=0.001) rather than cell number (P=NS) was found when the 2-week and 8-week end points in NX1975-treated animals were compared, indicating continued extracellular matrix deposition by the cells in treated lesions after removal of the inhibitor (Table I). Together these observations suggest that the effect of the PDGF antagonist on intima area, intima-media ratio, and intimal cell number at 2 weeks is predominantly achieved through a transient delay of the lesion formation process, and that this process recurs after removal of the inhibitor.

Discussion
We show in the present communication that an antagonistic PDGF SELEX aptamer inhibits intimal hyperplasia in the rat carotid injury model by ~50% when the compound is given throughout the study period of 2 weeks, a common end point in this animal model. Aptamers typically interact with their protein targets with nanomolar and picomolar affinities, and in this regard, they are comparable to antibodies. Compared with antibodies, however, the aptamers have a major advantage of not evoking any detectable immune responses (D. Drolet et al, unpublished data, 1999). In the present study, full effect was observed with as little as 2 mg kg\(^{-1}\) d\(^{-1}\). Aptamers are thus attractive PDGF antagonists with potential clinical interest. Parenterally administered SELEX aptamers against vascular endothelial growth factor and topoisomerase I are currently being studied in clinical trials (data from Reference 29b). By using a 10-times-higher dose in the first set of experiments, we were unable to reach the effect reported in the study by Sirois et al\(^{12}\), in which perivascular delivery of 2 different PDGF \(\beta\)-receptor antisense oligonucleotides resulted in a respective 60% and 80% inhibition of lesion formation in the same animal model and time point. However, the level of reduction in our results is consistent with several other studies in which PDGF antibodies,\(^{17,22}\) PDGF \(\beta\)-receptor antibodies,\(^{15,16,18}\) and PDGF receptor kinase inhibitors\(^{14,19-21,23}\) have been shown to inhibit intimal hyperplasia by 35% to 50% in various models in rats, pigs, and nonhuman primates. Our study shows that the PDGF B-chain aptamer NX1975 can be used as an antagonist in vivo, consistent with recent results by Floege et al,\(^{28}\) who demonstrated that an identical aptamer inhibited glomerulonephritis in a rat model.

At the time points of analysis, we found no significant effect of the PDGF antagonist on smooth muscle cell proliferation or cellular density. In contrast, a significant increase in apoptosis was seen in antagonist-treated animals. Our data thus support the notion that an important effect of PDGF in intimal hyperplasia formation is to prevent apoptosis of smooth muscle cells. Given the fact that PDGF-AB and -BB have a powerful chemotactic effect on smooth muscle cells and other cells in vitro,\(^{30,31}\) and PDGF-BB an in vivo effect after its intravascular infusion,\(^{32}\) it is possible that NX1975 treatment also inhibits migration of smooth muscle cells from the media to the intima layer of the vessel wall. Interestingly, our results with 1-week treatment and a 2-week end point with a PDGF antagonist were similar to the data shown by Bendeck et al,\(^{33}\) who blocked migration by using a matrix metalloproteinase inhibitor. In their study, administration of the drug during the first 7 days after injury also failed to inhibit intimal thickening at 2 weeks and was associated with a trend toward increased smooth muscle cell proliferation.

An important finding in the present study was that the beneficial effect of the 2-week treatment was lost when administration was discontinued and the effect measured 6 weeks later. The extent of intimal hyperplasia in both untreated and treated animals was significantly greater at 8 weeks compared with 2 weeks after injury, largely as a consequence of continued matrix deposition. Moreover, treatment with aptamers for the first week after balloon dilatation is too short a duration to achieve a significant reduction of intimal thickening in this animal model at 2 weeks, consistent with reports showing an increased level of PDGF \(\beta\)-receptor activation during the second week after injury.\(^{13,34}\) The recurrence of intimal hyperplasia after removal of PDGF inhibition, as shown in this communication in rats, may also explain results from a nonhuman primate study in which a short course of therapy with PDGF \(\beta\)-receptor antibodies had only a limited effect on intimal hyperplasia at 30 days after injury.\(^{35}\) Our findings indicate that the durability of therapeutic benefit of various compounds against PDGF, an important molecular target in vascular pathology, needs to be taken into...
consideration in designing future studies in higher species, with models that better resemble human disease.

It is relevant to note in this context that Floege et al. have recently observed better therapeutic effect with an identical aptamer in a rat mesangioproliferative glomerulonephritis model. Together with our observations, these results indicate a more central role for PDGF-B-chain in the pathophysiology of kidney glomeruli compared with that of large blood vessels. Even though PDGF may have different functions during embryogenesis than in disease in adult animals, this notion is in agreement with knockout studies; in PDGF-B-chain and \(\beta\)-receptor knockout mice, mesangial cells (and the mesangium) are absent; in contrast, although blood vessels are dilated in PDGF-B-chain knockout mice, smooth muscle cell hypoplasia is not observed, and major veins and arteries appear normal in PDGF-\(\beta\)-receptor knockout animals.

Together, these results suggest that efficient inhibition of intimal hyperplasia formation by PDGF antagonists may require either prolonged treatment or even combination with other treatments. Interestingly, Rutherford et al. found that the combination of PDGF antibodies and antibodies against bFGF caused dramatically decreased intimal hyperplasia at day 8 after injury, suggesting that both PDGF and bFGF have important roles in the early stages of this process. Whether intimal hyperplasia recurs after this combined intervention is not known. Furthermore, continued matrix deposition in the treated lesions at 8 weeks after injury suggests that inhibition of the agents that induce the synthesis of matrix components (eg, transforming growth factor-\(\beta\)) may be useful adjunctive therapy to PDGF antagonists. It is also possible that combining PDGF antagonists with factors that promote endothelial cell coverage of the injury site will lead to permanent inhibition of intimal hyperplasia.

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References


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