Pentraxin 3 Inhibits Fibroblast Growth Factor 2–Dependent Activation of Smooth Muscle Cells In Vitro and Neointima Formation In Vivo

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Objective—The fibroblast growth factor (FGF)/FGF receptor system plays an important role in smooth muscle cell (SMC) activation. Long-pentraxin 3 (PTX3) is a soluble pattern recognition receptor with non-redundant functions in inflammation and innate immunity. PTX3 is produced by different cell types of the vessel wall, including SMCs. PTX3 binds FGF2 and inhibits its angiogenic activity on endothelial cells. We investigated the capacity of PTX3 to affect FGF2-dependent SMC activation in vitro and in vivo.

Methods and Results—When added to human coronary artery SMCs, human PTX3 inhibits cell proliferation driven by endogenous FGF2 and the mitogenic and chemotactic activity exerted by exogenous recombinant FGF2. Accordingly, PTX3 prevents $^{125}$I-FGF2 interaction with FGF receptors on the same cells. Also, PTX3 overexpression after recombinant adeno-associated virus–PTX3 gene transfer inhibits human coronary artery SMC proliferation and survival promoted by FGF2 in vitro. Consistently, a single local endovascular injection of recombinant adeno-associated virus–PTX3 gene inhibits intimal thickening after balloon injury in rat carotid arteries.

Conclusions—PTX3 is a potent inhibitor of the autocrine and paracrine stimulation exerted by FGF2 on SMCs. Local PTX3 upregulation may modulate SMC activation after arterial injury. (Arterioscler Thromb Vasc Biol. 2005;25:1837–1842.)

Key Words: smooth muscle cells ■ arterial injury ■ gene therapy ■ fibroblast growth factor ■ pentraxin 3

Excessive growth of vascular smooth muscle cells (SMCs) is an important component in atherosclerosis and restenosis. Fibroblast growth factors (FGFs), a family of pleiotropic heparin-binding growth factors,1 exert their activity by interacting with tyrosine-kinase receptors (FGFRs) on target cells.2 The FGF/FGFR system plays an important role in SMC activation in vitro and in vivo after arterial injury. Indeed, FGF2 promotes survival, proliferation, and migration of SMCs3–6 that express FGFRs.7 FGFs are expressed in injured arteries and contribute to intimal thickening.8,9 Accordingly, arterial injury leads to FGFR upregulation in SMCs.8,10 Also, most of the cell types found in the restenotic area, eg, endothelial cells, macrophages, T-cells, and SMCs themselves, synthesize FGFs,11 the production and release of which is modulated by inflammatory mediators,12,13 hypoxia,14 and cell damage.15

Pentraxin 3 (PTX3) is the prototypic member of the long-pentraxin family.16 PTX3 is a glycosylated protein whose C-terminal pentraxin domain shares homology with the entire sequence of the classic short-pentraxins C-reactive protein and serum amyloid P (SAP) component, whereas its NH$_2$-terminal portion does not show homology with any other known protein. PTX3 is a soluble pattern recognition receptor with unique non-redundant functions in various physiopathological conditions and may serve as a mechanism of amplification of inflammation and innate immunity.16 Unlike short-pentraxins produced by the liver in response to inflammatory mediators, PTX3 is synthesized at the inflammatory site by monocytes and endothelial cells in response to inflammatory cytokines.16 In endothelial cells, PTX3 upregulates tissue factor expression, possibly acting as an endothelial regulator during thrombogenesis and ischemic vascular disease.17 Also, PTX3 is upregulated during vasculitis,18 and increased plasma level of PTX3 predicts 3-month survival in patients with acute myocardial infarction.19,20 Recent observations have shown that PTX3 is present in atherosclerotic plaques21 and is expressed by SMCs isolated from human arterial specimens.22 Moreover, PTX3 binds FGF2 and inhibits its angiogenic activity on endothelial cells.23 On this basis, we investigated the capacity of PTX3 to affect FGF2-dependent SMC activation in vitro and the impact of recombinant adeno-associated virus (rAAV)-PTX3 gene transfer on neointimal hyperplasia after arterial injury in vivo.
Methods

Chemicals
Human recombinant FGF2 and PTX3 were produced as described.24,25 SAP, epidermal growth factor (EGF), and insulin were from Sigma. Neutralizing anti-FGF2 antibodies were from UBI. The recombinant soluble extracellular domain of FGFR-1 (xcFGFR-1) was provided by A. Isacchi (Pharmacy-Upjohn, Nerviano, Italy).

Cell Cultures and rAAV Transduction
Human coronary artery SMCs (HCASMCs) were grown in complete smooth muscle cell growth medium-2 (PBI International) containing 5% fetal calf serum (FCS). pAAV-PTX3 was constructed by cloning the human PTX3 cDNA in pTR-U6-PS plasmid (provided by N. Muzyczka, University of Florida, Miami, Fla) to replace the GFP and neomycin-resistance genes. pAAV-LacZ was obtained by inserting the LacZ gene from plasmid pCH110 (Amersham) in the pTR-U65 backbone. The cloning and propagation of AAV plasmids were carried out in the JC 8111 Escherichia coli strain. Infectious AAV2 vector particles were generated and titered as described.26 HCASMCs were transduced with AAV-PTX3 or AAV-LacZ, and PTX3 and FGF2 protein levels were evaluated in the cell extracts and conditioned media by ELISA (see supplemental Methods, available online at http://atvb.ahajournals.org).

Cell Proliferation and Caspase-3 Activation Assays
Parental or AAV-infected HCASMCs were seeded at 5000 cells/cm² in complete smooth muscle cell growth medium-2. After overnight incubation, fresh basal medium containing 0.5% FCS was added and cells were incubated at 37°C in the presence of selected molecules. At selected periods of time, cells were trypsinized and counted in a Burker chamber. The experiments were repeated 3 to 6 times in duplicate. Parallel cell cultures were acetonet-fixed and incubated overnight at 4°C with anti-cleaved caspase-3 antibody (Cell Signaling Technology) and then with goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate antibody for 1 hour. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Next, the percentage of cleaved caspase-3 positive cells was assessed under a fluorescence microscope in 10 random 400 fields per sample.

Chemotaxis Assay
HCASMCs (1x10⁶ cells/ml) were seeded in serum-free medium in the upper compartment of a Boyden chamber containing a gelatin-coated 5 μm PVP free polycarbonate filter (Costar). FGF2s with or without PTX3 or SAP were placed in the lower compartment. After 4 hours at 37°C, cells that migrated to the lower side of the filter were stained with Diff-Quik (Dade-Behring) and 5 random fields were counted for each experimental condition. The experiments were repeated 3 times in triplicate.

125I-FGF2 Cell Binding and Solid Phase Binding Assays
Confluent HCASMCs were incubated at 4°C in serum-free medium containing 125I-FGF2 (0.11 nmol/L, specific radioactivity of 800 cpm/nmol), 0.15% gelatin, and 20 mmol/L Hepes buffer (pH 7.5), with or without PTX3. After 2 hours, the amount of 125I-FGF2 bound to FGFRs was evaluated.27 For solid phase binding assay, ELISA microplates were coated with PTX3 and incubated with 125I-FGF2 (1.6 nmol/L) in the presence of increasing concentrations of soluble extracellular domain of FGFR-1 (xcFGFR-1), PTX3-bound radioactivity was then measured (see supplemental Methods). The experiments were repeated 3 times in duplicate.

Reverse Transcription-Polymerase Chain Reaction Assay
Total RNA was isolated by the TRIzol method from HCASMCs (3 x 10⁶ cells/well in 24-well plates). Total RNA (2 μg) was retrotranscribed with Ready-To-Go You-Prime First Strand Beads (Amersham). Then, 1/100th of the reaction was amplified in a final volume of 25 μL using the primers for human PTX3, FGF2, or FGFR-1 (see supplemental Methods). After polymerase chain reaction, 5-μL aliquots were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Balloon Injury and rAAV Vector Application
A 2F Fogarty catheter was introduced into the common carotid artery of anesthetized male Wistar rats (Harlan, Italy), inflated to 1.5 to 2.0 atmospheres, and a 10-mm injury was induced (see supplemental Methods). After balloon removal, 100-μL aliquots of phosphate-buffered saline containing no viral particles (n=6), AAV-LacZ (1x10¹¹ particles, n=11), or AAV-PTX3 (1x10¹² particles, n=12) were injected through a cannula and allowed to incubate in the injured segment for 40 minutes. The external carotid was then tied and the blood flow was restored. Animals were euthanized after 2 and 4 weeks. Media and neointima areas of injured arteries were quantified and intima-to-media ratios were calculated. PTX3 immunostaining of paraffin-embedded carotid sections was performed 4 weeks after injury using a rabbit polyclonal anti-PTX3 antibody (see supplemental Methods).

Statistical Analysis
Results are expressed as mean±SEM. Student t test was used for statistical analysis and P<0.05 was considered significant.

Results
PTX3 Inhibits FGF2-Induced Proliferation and Chemotaxis in SMCs
SMCs produce FGF2 that, in turn, stimulates their proliferation and migration by interacting with FGFRs.28 Accordingly, HCASMCs express FGF2 and FGFR-1 transcripts (Figure 1A, inset), and anti-FGF2 antibodies (60 nmol/L) inhibited the proliferation of HCASMCs when grown in 0.5% FCS (Figure 1A). These data demonstrate the existence of a FGF2-dependent autocrine loop of stimulation in HCASMCs. Under the same experimental conditions, addition of 220 nmol/L of PTX3 to HCASMCs caused a similar inhibitory effect (Figure 1A). SAP, which shares a high homology with PTX3, inhibited the binding of 125I-FGF2 to HCASMCs (Figure 2B). These data suggest that PTX3 inhibits FGF2-driven stimulation by preventing FGF2 interaction with HCASMCs. Indeed, PTX3 inhibits the binding of 125I-FGF2 to FGFRs expressed on HCASMCs at doses similar to those required to inhibit the mitogenic and chemotactic activity of FGF2 (Figure 3A). To confirm that the interactions of FGF2 with PTX3 or FGFRs are mutually exclusive, plastic-immobilized...
PTX3 was incubated with $^{125}$I-FGF2 in the presence of xFGFR-1. As anticipated, xFGFR-1 prevented the binding of $^{125}$I-FGF2 to immobilized PTX3 (Figure 3B).

AAV-PTX3 Transduction Inhibits FGF2-Induced SMC Proliferation and Survival

PTX3 is expressed by vascular SMCs under defined conditions.\(^{21,22}\) To assess the effect of endogenous PTX3 on SMC behavior, HCASMCs were infected with an rAAV harboring the human PTX3 cDNA, generating AAV-PTX3 HCASMCs. Preliminary experiments performed with cells transduced with LacZ-AAV (AAV-LacZ HCASMCs) showed that the infection efficiency was higher than 70% (data not shown).

AAV-PTX3 infection resulted in the upregulation of PTX3 mRNA in AAV-PTX3 HCASMCs when compared with AAV-LacZ HCASMCs (Figure 4A, inset). This was paralleled by an increase of PTX3 protein produced and released by AAV-PTX3-infected cells (PTX3 levels in the conditioned medium of AAV-PTX3 and AAV-LacZ HCASMCs were equal to 100 pmol/L and 8 pmol/L, respectively). AAV infection and consequent PTX3 upregulation did not affect FGF2 expression. Indeed, AAV-PTX3 and AAV-LacZ HCASMCs showed similar levels of cell-associated (4.4 versus 5.0 fmol/μg) and released (2.2 versus 2.7 pmol/L) FGF2 protein. Nevertheless, AAV-PTX3 transduction blocked the proliferation of HCASMCs maintained in 0.5% FCS when compared with AAV-LacZ HCASMCs (Figure 4A, open symbols). Also, AAV-PTX3 HCASMCs were unable to respond to exogenously added recombinant FGF2. The inhibitory effect was specific because the growth factor maintained its mitogenic activity in control AAV-LacZ HCASMCs (Figure 4A).

FGF2 represents an important SMC survival factor.\(^5,6\) Deprivation of endogenous FGF2 after PTX3 upregulation caused caspase-3 activation in AAV-PTX3 HCASMCs that was drastically reduced by recombinant FGF2 treatment to values similar to those observed in AAV-LacZ HCASMCs (Figure 4B and 4C; Figure I, available online at http://...
Accordingly, cell number was reduced over time in PTX3-overexpressing HCASMCs (Figure 4A). Similarly, PTX3 protein treatment, like anti-FGF2 antibodies, caused caspase-3 activation in AAV-LacZ HCASMCs (Figure II, available online at http://atvb.ahajournals.org).

**Efficacy of AAV-PTX3 in the Reduction of Intimal Hyperplasia**

To explore the effects of PTX3 expression in vivo, we evaluated its ability to prevent intimal hyperplasia after arterial injury. Rat carotid arteries infected with AAV-LacZ immediately after balloon injury express β-galactosidase with a peak 2 weeks after infection that persists over the following weeks. The marker gene is expressed throughout the vessel wall, maximally in the neointima (Figure 5D), including infiltrating SMCs. Therefore, the rat carotid artery of 12 rats was exposed to AAV-PTX3 for 40 minutes immediately after balloon injury. A matched group of control animals received either AAV-LacZ or phosphate-buffered saline. Immunostaining of carotid sections demonstrates that PTX3 is expressed in the intima and media of AAV-PTX3-treated animals both at 2 weeks (data not shown) and 4 weeks after infection when compared with control animals (Figure 5C). At these time points, the stenotic response was quantified by measuring the intima and media areas. The thickness of the media of the injured rat carotids was similar in control and AAV-PTX3-treated groups (0.09±0.01 versus 0.11±0.01 mm² at 2 weeks; 0.10±0.01 versus 0.12±0.01 mm² at 4 weeks), whereas the neointimal area of the treated group was significantly reduced both at 2 and 4 weeks (0.13±0.01 versus 0.08±0.02 mm² at 2 weeks, P<0.05; 0.18±0.01 versus 0.15±0.03 mm² at 4 weeks, P<0.05) (Figure 5A and 5B).

Accordingly, the intima-to-media ratio, a more sensitive parameter for assessing relative changes in intima and media thickness, was significantly reduced in the AAV-PTX3-treated group compared with controls (55% and 44% inhibition at 2 and 4 weeks, respectively) (Figure 5E and 5F).

**Discussion**

The FGF/FGFR system plays a key role in SMC proliferation, migration, and survival in vitro and neointimal thickening after arterial injury. Accordingly, in vivo abrogation of FGF2 activity or FGFR signaling inhibits SMC proliferation after intimal injury. Here we demonstrate that PTX3 exerts a potent FGF2 antagonist activity by suppressing cell proliferation, survival, and migration in HCASMCs and by inhibiting intimal thickening after carotid injury.

HCASMCs express both FGF2 and FGFR-1 transcripts. Anti-FGF2 antibodies suppress the proliferation of HCASMCs maintained under low serum concentrations,
demonstrating the existence of an autocrine FGF2 signaling in these cells, as already shown for SMCs of different origin.5 Exogenously added PTX3 and endogenously overexpressed PTX3 both inhibit this autocrine loop of stimulation. Also, PTX3 fully abolishes the activity of exogenous recombinant FGF2 protein. The short pentraxin SAP, however, does not affect FGF2-dependent cell proliferation and chemotaxis, and PTX3 does not inhibit HCASMC proliferation triggered by insulin, EGF, and serum, underlining the specificity of the FGF2-antagonist effect exerted by PTX3.

The antagonist activity of PTX3 depends on its capacity to bind FGF2 with high affinity,23 thus acting as a “FGF2 decoy” that sequesters the growth factor in an inactive form. Accordingly, PTX3 prevents the binding of 125I-FGF2 to FGFRs expressed on HCASMCs. Also, the interactions of FGF2 with PTX3 or soluble xc-FGFR-1 are mutually exclusive. Thus, PTX3 affects the activity of both endogenous and exogenous FGF2 by binding the growth factor and preventing FGFR occupancy in SMCs.

SMCs are permissive to rAAV transduction.31 Accordingly, we successfully infected HCASMCs with an AAV-PTX3 vector. This resulted in high levels of expression and secretion of PTX3, with the consequent inhibition of the activity exerted in vitro by endogenous and exogenous FGF2. Noticeably, PTX3 produced endogenously after rAAV transduction exerts a more potent FGF2-antagonist activity when compared with PTX3 administered as a single bolus of purified protein (the FGF2-antagonist activity being observed at 100 pmol/L versus 220 nmol/L of PTX3, respectively). This indicates that the continuous production of PTX3 and its release in the microenvironment surrounding the FGF2-target cell, an experimental condition that mimics more closely the in vivo situation, results in an increased ability to interact with the growth factor and to antagonize its activity, further supporting the efficacy of the rAAV gene transfer approach.

In injured rat carotid arteries transduced in vivo with AAV-LacZ, expression of β-galactosidase peaks 2 weeks after infection and persists for several weeks. The marker gene is expressed throughout the vessel wall, maximally in the neointima, with an overall efficiency of transduction equal to approximately 10% of the entire vascular wall (Figure 5).30 Consistently, in vivo AAV-PTX3 transduction results in PTX3 protein expression in the vessel wall. This was paralleled by a significant decrease in the intima-media ratio at 2 and 4 weeks after injury. FGF2 is produced by SMCs and other cell types in the restenotic area, including endothelial cells, macrophages, and T-cells.12 Thus, FGF2 may exert autocrine and/or paracrine functions on FGFR-expressing SMCs of the injured vessel.5,32 The bulk of our observations strongly suggests that the inhibition of neointimal thickening exerted by rAAV-delivered PTX3 is attributable to its FGF antagonist activity, in keeping with previous studies about the capacity of FGF2 antisense or dominant negative FGF gene transfer to prevent restenosis.9,10

PTX3 is produced at the inflammatory site in response to cytokines and bacterial components.29,33,34 Several cellular components of the blood vessel wall express PTX3, including endothelial cells,29 macrophages,34 dendritic cells,35 adipocytes,36 fibroblasts33 and myoblasts.37 Also, limited levels of PTX3 mRNA and protein are detected in HCASMCs in vitro (present work). Interestingly, PTX3 expression is upregulated by modified atherogenic lipoproteins in SMCs isolated from human arterial specimens.32 Our observations suggest that PTX3 may exert a modulatory function on SMCs after blood vessel injury by limiting the activity of FGF2. However, this does not seem to be sufficient to prevent the pro-stenotic action of the growth factor, and higher levels of PTX3 expression, like those achieved after rAAV gene transfer, are required to limit neointimal thickness.

Twenty-three members of the FGF family exist that interact with the products of 4 FGFR genes, underlying the complexity of the FGF/FGFR system.1 Here we have shown that PTX3 affects FGF2/FGFR-1 interaction in HCASMCs. Also, PTX3 can bind FGF8 but not FGF1 or FGF4.23 Recently, FGF9/FGFR-2 interaction has been implicated in the proliferation of neointimal SMCs after arterial injury.8 Further studies are required to assess the capacity of PTX3 to affect the activity exerted by different members of the FGF family on SMCs.

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Figure I. Effect of PTX3 overexpression on caspase-3 activation in HCASMCs. Cytoplasmic immunostaining with anti-cleaved caspase-3 antibody (in green, upper panels) of AAV-LacZ (a) and AAV-PTX3 HCASMCs (b) grown for 2 days in basal SMCGM-2 containing 0.5% FCS. No immunoreactivity was observed in AAV-PTX3 HCASMCs stained with non-immune rabbit IgGs (c). Nuclei were stained with DAPI (in blue, lower panels).

Online publication data Figure I.
Figure II. Effect of PTX3 on caspase-3 activation in AAV-LacZ HCASMCs. Cells were treated for 2 days with vehicle, PTX3 (220 nmol/L), or anti-FGF2 antibodies (60 nmol/L) in basal medium containing 0.5% FCS. After 2 days, cells were immunostained with anti-cleaved caspase-3 antibody and nuclei were stained with DAPI. Next, the percentage of cleaved caspase-3 positive cells was assessed under a fluorescence microscope in 10 random x400 fields per sample.

Online publication data Figure II.
Camozzi: PTX3 inhibits SMCs

METHODS

RNA extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay.

HCASMC total RNA (3x10^4 cells/well in 24-well plate) was isolated by the TRIzol method. Two micrograms were retrotranscribed with Ready-To-Go You-Prime First Strand Beads (Amersham). Then, 1/10th of the reaction was amplified using human PTX3 primers [(+)GTGCTCTCTGGTCTGCAGTG; (-)GCAGCTCGTCCAGAGCAGT], human FGF2 primers [(+)AAGCGGCTGTACTGCAAAAACG; (-)AACTGGGTATTTCCCTTGACCGGTA] or human FGFR-1 primers [(+)GGACAATGTGATGAAGATAG; (-)TCCCCTGAGGACGACGTAGAG]. Standard PCR was performed with different annealing temperatures: 67°C (25 cycles) for human PTX3, 64°C (25 cycles) for human FGF2, and 52°C (25 cycles) for human FGFR-1. Aliquots (5 µl) were separated on a 1.5 % agarose gel and visualized by ethidium bromide staining.

rAAV transduction of HCASMCs.

For rAAV infection, HCASMCs (5,000 cells/cm²) were incubated with medium containing 5% FCS and hydroxyurea (1 mmol/L) for 16 hours at 37°C. Then, cells were transduced with AAV-PTX3 or AAV-LacZ (1x10^{12} viral genome particles/ml) in fresh MEDIUM plus 5% FCS for further 16 hours at 37°C. To evaluate the infection efficiency, AAV-LacZ-infected cells were fixed and stained for β-galactosidase activity. Also, AAV-PTX3 and AAV-LacZ HCASMCs were incubated at 37°C in fresh medium plus 0.5% FCS. After 48 hours, PTX3 and FGF2 protein levels were evaluated in the cell extracts and conditioned media. FGF2 was measured using a commercial ELISA kit (R&D Systems, Minneapolis, USA) according to manufacturer’s instructions. PTX3 was measured with a sandwich ELISA based on an anti-PTX3 monoclonal antibody (MNB4, diluted 1:500 in coating buffer) and rabbit antiserum as described 1.

125I-FGF2 solid phase binding assay.

ELISA microplates were incubated for 16 hours at 4°C with 100 µl of 100 mmol/L carbonate buffer, pH 9.6, containing 440 nmol/L PTX3. Then, wells were washed and incubated for 2 hours at 37°C with 1.0% BSA in carbonate buffer. 125I-FGF2 (1.6 nmol/L) was incubated for 2 hours at 37°C onto PTX3-coated wells with increasing concentrations of soluble xcFGFR-1. Then, PTX3-bound radioactivity was measured.

Balloon injury and rAAV vector application.

Animal care and treatment were conducted in conformity with institutional guidelines issued in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12th 1987). Male Wistar rats (300–400 g) were anesthetized using Zoletil (45 mg/kg body weight), and the right common and external carotid arteries were exposed and isolated. A 2F Fogarty catheter (Baxter-Edwards Healthcare, Irvine, CA) was introduced into the common carotid artery through arteriotomy in the external carotid artery and inflated to 1.5-2 atmospheres. A 10 mm-injury was induced as described 2. After balloon removal, rAAV vectors were injected through an Intramedic PE50 polyethylene cannula (Becton-Dickinson) and allowed to incubate in the injured segment in the absence of flow for 40 minutes. The treated group (n=12) received 1x10^{11}
particles of AAV-PTX3; the control group received 1x10^{11} particles of AAV-LacZ (n=11) or 100 µl of PBS (n=6). The external carotid was then tied and the blood flow was restored through the common carotid artery. Animals were sacrificed after 2 and 4 weeks (PTX3 group, n=6 at each time point; control group, n=11 and n=6 at 2 and 4 weeks, respectively). Media and neointima areas of injured arteries were quantified by morphometric analysis and intima-to-media ratios were calculated. Since no significant differences were observed between PBS- and AAV-LacZ-treated groups, all controls animals were considered together.

Rat carotid PTX3 immunostaining.

Five µm sections of paraffin-embedded carotids were incubated for 10 minutes with 0.006% H_{2}O_{2} in absolute methanol. Then, sections were incubated twice for 5 minutes each with 10 mmol/L sodium citrate, pH 6.0, in a microwave oven, blocked for 10 minutes with 1% BSA plus 1% goat serum in PBS, and incubated overnight at 4°C with 1:2000 dilution of rabbit polyclonal anti-PTX3 antibody in a humidified chamber. Sections were then exposed to biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and biotin-labeled tyramide using the TSA Biotin System (NEN Life Science, Perkin-Elmer, Belgium) according to manufacturer’s instructions. Peroxidase color reaction was developed with 3-amino-9-ethyl-carbazole (Sigma).


Online publication Methods.