Smooth Muscle Cell Immediate-Early Gene and Growth Factor Activation Follows Vascular Injury
A Putative In Vivo Mechanism for Autocrine Growth
Joseph M. Miano, Niksa Vlasic, Robert R. Tota, and Michael B. Stemerman

To understand the molecular events governing smooth muscle cell (SMC) proliferation in vivo, immediate-early gene (IEG) expression was assessed and related to growth factor ligand and receptor mRNA and SMC DNA synthesis after aortic injury. Balloon catheter injury evoked increases in SMC c-myc and thrombospondin (tsp) within 2 hours. The induction of these IEGs was followed by elevated transcripts to platelet-derived growth factor-A (PDGF-A), transforming growth factor-β1 (TGF-β1) and a basic fibroblast growth factor (bFGF) receptor. Whereas PDGF type-β receptor mRNA was demonstrated in SMCs from control and balloon-injured aortas, no detectable signal was observed for the PDGF type-α receptor. To explore the potential linkage between IEG products and growth factor mRNA expression, cycloheximide was employed to block early protein synthesis after balloon injury. Induction of PDGF-A and TGF-β1 was attenuated by cycloheximide, but bFGF induction was unaffected. Moreover, cycloheximide superinduced IEGs and revealed PDGF-B transcripts, which were otherwise undetected. Seven days after aortic injury, a spontaneous increase in c-myc and tsp mRNA was noted. This IEG reactivation was followed 12 hours later by a twofold increase in SMC DNA synthesis. These findings corroborate an autocrine mode of SMC proliferation in vivo and suggest that IEG products may control such growth by stimulating growth factor genes. (Arteriosclerosis and Thrombosis 1993;13:211–219)

KEYWORDS • immediate-early gene • growth factors • smooth muscle cell proliferation • vascular injury • autocrine growth

Smooth muscle cell (SMC) proliferation is a major determinant of vascular stenosis, yet the molecular signals governing such growth in vivo remain undefined. The mRNA expression of platelet-derived growth factor A-chain (PDGF-A), transforming growth factor-β (TGF-β1), insulin-like growth factor I (IGF-I), and the PDGF type-β receptor (PDGFR-β) in vascular tissue, as well as the detection of mitogenic activity from vessel-derived SMCs, indicates that SMC proliferation may be controlled in part by a local autocrine loop. However, despite these advances, the nature of the stimulus for autocrine growth factor expression in SMCs is unclear. Moreover, although evidence for SMC autocrine growth has been documented in vitro, the existence of similar mechanisms in vivo remains conjectural.

Immediate-early gene (IEG) activation is a fundamental response to growth-stimulatory agents. These genes are defined by their rapid/transient expression and protein synthesis-independent mechanism(s) of induction. IEGs encode numerous products, including transcription factors, cytokines, structural and extracellular matrix (ECM) proteins, and enzymes. Such functional diversity likely affords requisite flexibility in a cell’s response to growth signals. For example, certain IEG products may participate directly in the stimulation of DNA synthesis, whereas others may function in some capacity independent of direct growth stimulation. IEG activation occurs in response to many cellular events, and there is compelling evidence supporting a role for immediate-early proteins in cellular growth control. Although the notion of IEG products that stimulate growth factor genes has been shown or speculated to occur in culture, whether such a mechanism is operative in an in vivo setting is unclear. Moreover, few studies have examined the expression of IEG in vivo models of cell proliferation. Thus, an analysis of IEG expression in vivo is required to validate findings from cell culture studies.

Recently, the expression of immediate-early proto-oncogenes was demonstrated following balloon deendothelialization (BDE) of the rat carotid artery and aorta. In the present study, we have initiated studies to analyze the in vivo relation between IEG and growth factor mRNA expression and SMC DNA synthesis. The results demonstrate sequential IEG and growth factor mRNA induction before the initiation of SMC DNA synthesis. Furthermore, they suggest a plausible link...
between certain IEG products and subsequent growth factor gene activation. Finally, a second peak in IEG expression is demonstrated, which appears to be associated with a second burst in SMC DNA synthesis. These results support a concept of in vivo SMC autocrine growth whereby IEG products participate in the initiation of SMC DNA synthesis and maintain such cell growth by stimulating growth factor genes.

Methods

Reagents

Type I porcine elastase, soybean trypsin inhibitor, lactate dehydrogenase (LDH), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, Mo. Type I collagenase was obtained from Worthington Biochemical Corp., Freehold, N.J. Medium 199 was purchased from GIBCO, Gaithersburg, Md. The reagents for RNA isolation and Northern blotting were molecular biology grade and were purchased from Bethesda Research Laboratories, Gaithersburg, Md. [Methyl-\(^{3}H\)]Thymidine (20 Ci/mmol), cytidine 5'-[\(^{32}P\)]triphosphate (3,000 Ci/mmol), and \[^{35}S\]methionine (1,100 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Cycloheximide (CHX) was purchased from Boehringer Mannheim, Indianapolis, Ind.

Animals

Approximately 200 male Sprague-Dawley rats (350–375 g) were used for these studies. Animals were obtained from Charles River Laboratories, Wilmington, Mass., and were used in accordance with National Institutes of Health and institutional, approved animal care regulations.

Balloon Deendothelialization

BDE was performed in anesthetized rats as described. A minimum of five animals were pooled per time point, and at least two independent experiments were performed for each study. The following time points after BDE were studied: 2, 6, 12, and 24 hours and 2, 7, and 14 days.

Cell Culture

Two cell types were used as a source of positive or negative control RNA for the genes under study. Human umbilical vein endothelial cells (HUVECs) were propagated as described. BALB/c-3T3 cells (clone A-31) were purchased from American Type Culture Collection and grown according to the manufacturer’s specifications.

RNA Isolation/Northern Blotting/Densitometry

Total RNA was isolated from cultured cells and medial SMCs, which were separated from the aorta by an enzymatic digestion method as described. Briefly, pooled aortas (n=5) were rapidly excised from the animal; rinsed in medium 199; and incubated for 25 minutes in medium 199 plus 1% collagenase, 0.25% elastase, and 1% soybean trypsin inhibitor. After incubation in the enzymes, the adventitia and endothelium were rapidly stripped away from the media, which was immediately snap-frozen in liquid nitrogen and homogenized in 4 M guanidinium isothiocyanate containing 2% β-mercaptoethanol. Homogenates of medial SMCs were sheared three times through a 23-gauge needle and clarified by spinning at 10,000g for 10 minutes. The supernatant was layered over 5.7 M CsCl and the RNA pelleted by ultracentrifugation as described.

Cultured cell RNA was isolated in a similar manner. Equivalent amounts of total RNA (15–20 μg/lane) were fractionated in a 1.2% agarose gel containing 0.66 M formaldehyde and 0.2 M morpholinopropanesulfonic acid (pH 7). After capillary blotting, RNA was cross-linked to a nylon membrane (Zeta Probe, Biorad, Rockville Centre, N.Y.) by UV irradiation (Stratagene, La Jolla, Calif.). cDNA probes were labeled to a specific activity of 10^8 cpm/μg by a random hexamer protocol (Boehringer Mannheim). Hybridizations and washes were performed as described. Blots were exposed to x-ray film (Kodak X-OMAT, Rochester, N.Y.) with intensifying screens at -80°C. A Hoefer GS-300 densitometer was used to semiquantify the changes in steady-state transcript levels. Levels of IEG and growth factor mRNA were normalized to 18S rRNA as previously described. Where reported, results are expressed as fold increases above normalized controls (non-BDE).

Cycloheximide Studies

Animals (n=5) were injected intraperitoneally with 7.5 mg/kg body wt CHX either immediately or 2 hours after BDE. Control BDE animals received 0.9% saline vehicle. Total SMC RNA was isolated 6 hours after BDE as described above and analyzed for IEG and growth factor mRNA expression. The toxicity of CHX was determined by measuring serum LDH 6 hours after the combined BDE/CHX treatment.

The effect of CHX on SMC de novo protein synthesis was determined by measuring the incorporation of \[^{35}S\]methionine into trichloroacetic acid (TCA)–precipitable SMC protein 6 hours after BDE. Briefly, animals underwent BDE in the absence or presence of 7.5 mg/kg body wt CHX as described above. After 5 hours, all animals received an intravenous injection of \[^{35}S\]methionine (0.5 mCi/kg body wt) as described. The radioactive methionine was allowed to circulate for 30 minutes, at which time the animals were killed and SMC protein was isolated. Medial SMCs were isolated by enzymatic disassociation and homogenized in a solution of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 2 mM EDTA. The homogenate was clarified by spinning at 11,000g for 20 minutes and the pellet solubilized with the aforementioned phosphate-buffered saline solution containing 1% sodium dodecyl sulfate. Samples were heated to 95°C and then spun at 14,000g for 20 minutes. The supernatant was collected and stored at -80°C. Samples of liver were collected and processed similarly to further document the effect of CHX on protein synthesis inhibition. Aliquots of stored samples were precipitated with TCA, and the radioactivity was measured in a scintillation counter. Protein concentration was determined by the method of Lowry et al. Results are expressed as TCA-precipitable counts per minute per microgram of total protein.

DNA Specific Activity

The measurement of SMC DNA synthesis was performed as described with minor modifications. One
hour before they were killed, the animals received an intravenous injection of [methyl-\textsuperscript{3}H]thymidine (0.5 mCi/kg body wt). The animals were then killed and vessels subjected to enzyme digestion to isolate medial SMCs. At least five animals were pooled per time point in two independent studies. The following time points were examined: 0, 2, 7, 7.5, and 8 days. Results were analyzed by one-way analysis of variance. Tukey's studentized range test was employed for post hoc multiple comparisons. A statistical difference less than 0.05 was considered significant.

cDNA Probes

The following cDNA probes were obtained from individual investigators and prepared by established methods\textsuperscript{25}: a 0.8-kb EcoRI fragment of human basic fibroblast growth factor (bFGF) in pBR322 (Judith Abraham, CalBio Inc., Mountain View, Calif.); a 1.3-kb EcoRI fragment of human PDGF-A in pUC13 (Christier Betsholtz, Ludwig Institute for Cancer Research, Uppsala, Sweden); a 0.75-kb EcoRI–Acc I fragment of human PDGF type-\(\alpha\) receptor in pUC19 and a 0.75-kb Pst I fragment of human PDGF type-\(\beta\) receptor in pUC19 (Lena Claesson-Welsh, Ludwig Institute for Cancer Research); a 1.0-kb EcoRI fragment of human TGF-\(\beta\)I in pSP64 (Rik Derynck, University of California at San Francisco); a 4.5-kb Sal I fragment of human thrombospondin in pUC18 (Visha Dixit, University of Michigan, Ann Arbor); a 1.4-kb EcoRI–HindIII fragment of murine c-myc in pSP65 (William Lee, University of Pennsylvania, Philadelphia); an 18S rRNA fragment in pHRR118 (Ester Sabban, New York Medical College, Valhalla); a 1.8-kb Sac I fragment of human von Willebrand factor in pUC18 (J. Evan Sadler, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Mo.); a 0.4-kb Sal I–Apa I fragment of chicken bFGF receptor in pBluescript KS+ (Lewis T. Williams, Howard Hughes Medical Institute, University of California at San Francisco); and a 2.0-kb BamHI fragment of human PDGF-B in pBR322 (Randi Zicht, National Cancer Institute, Bethesda, Md.). The c-myb, c-fms, glyceraldehyde phosphate dehydrogenase, and \(\beta\)-actin probes were obtained from American Type Culture Collection, Bethesda, Md., and Oncor Probes, Gaithersburg, Md.

Results

SMC Growth Factor Ligand mRNA Expression After Balloon Deendothelialization

Growth factor transcripts have been demonstrated in rat aortic tissue after vascular manipulations.\textsuperscript{4,6} These studies measured steady-state mRNA levels from a mixed population of cells of the media and adventitia. To ascertain whether growth factor ligand transcripts are expressed specifically in aortic SMCs, we employed a previously described enzymatic digestion procedure.\textsuperscript{27} Figure 1 documents the complete removal of the adventitia and endothelium from a normal aorta treated with enzymes. Such treatment also resulted in partial digestion of the neointima from 7- and 14-day BDE vessels (data not shown). The latter preparations of RNA, therefore, represent both medial and neointimal SMCs. The purity of our SMC preparations was further evaluated by probing the RNA pool with cell-specific markers. For example, von Willebrand factor mRNA could easily be demonstrated in undigested aortas; however, such expression was absent from enzyme-treated vessels (data not shown).

Results in Figure 2 demonstrate BDE-induced expression of PDGF-A, bFGF, and TGF-\(\beta\)I in aortic SMCs over a 14-day period. Expression of PDGF-A was barely detected in SMCs obtained from non-BDE aortas (time zero). However, 6 hours after BDE, the 2.3- and 2.9-kb transcripts were elevated more than threefold. PDGF-A transcripts were seen to decrease after 6 hours but were discernible at 12 and 24 hours and 7 days after BDE. In contrast to PDGF-A, steady-state expression of PDGF-B mRNA was not detected at any time point after BDE, although a prominent signal was obtained from HUVECs (Figure 2, lane C2).

A single 6.0-kb bFGF transcript was observed in SMCs from non-BDE aortas (Figure 2). Levels of bFGF mRNA increased approximately threefold 2 hours after BDE and then gradually declined over the remaining time period. The pattern of TGF-\(\beta\)I mRNA expression was distinct from that of PDGF-A and bFGF. This gene was coinduced with PDGF-A and bFGF 6 hours after BDE; however, steady-state levels of TGF-\(\beta\)I mRNA remained elevated above non-BDE controls at every time point studied subsequently (Figure 2).

The induction of growth factor ligand transcripts in SMCs after BDE was not associated with a generalized
FIGURE 2. Growth factor ligand mRNA expression after balloon deendothelialization (BDE), as shown by autoradiographs from three independent studies showing smooth muscle cell (SMC) growth factor ligand mRNA expression after BDE. At the times indicated, total SMC RNA was isolated and processed for Northern blotting. Note the BDE-induced increase in steady-state transcripts to platelet-derived growth factor-A (PDGF-A, 2.3 and 2.9 kb), basic fibroblast growth factor (bFGF, 6.0 kb), and transforming growth factor-ß1 (TGF-ß1, 2.5 kb). The 1.7-kb transcript to PDGF-A is not shown because it was not easily distinguished from nonspecific binding of the probe to 18S rRNA. Hybridization to an 18S rRNA probe verified equal RNA loading. Lanes C1 and C2 correspond to control RNA from quiescent BALB/c-3T3 cells and human umbilical vein endothelial cells, respectively. Lane C2 in the PDGF-A panel represents RNA from serum-stimulated BALB/c-3T3 cells.

FIGURE 3. Growth factor receptor mRNA expression after balloon deendothelialization (BDE), as demonstrated by steady-state mRNA transcripts of type-α and -ß platelet-derived growth factor (PDGF) receptors (designated PDGF-AR and PDGF-BR, respectively) and a basic fibroblast growth factor receptor (bFGFR) after BDE. No detectable transcripts to the type-α PDGF receptor were observed in two independent experiments, although the 6.5-kb transcript is clearly present in BALB/c-3T3 cells. In contrast, both type-ß PDGF (5.7 kb) and a bFGF receptor mRNA (4.7 kb) were readily observed in aortic smooth muscle cells after BDE. The increase in type-ß PDGF receptor expression at 7 days was not a consistent finding. The increase in bFGF receptor mRNA was approximately 10-fold, as determined by densitometry. Note the absence of PDGF type-α and -ß receptors in human umbilical vein endothelial cells.

increase in RNA, since levels of 18S rRNA (Figure 2) as well as β-actin, c-Ha-ras, c-myc, glyceraldehyde phosphate dehydrogenase, and c-fms mRNA (data not shown) showed little or no change in expression between control and BDE aortic SMCs subjected to BDE. Finally, the effect of enzymes on growth factor ligand mRNA was assessed by measuring steady-state transcripts in whole aortic tissue (i.e., vessels not subjected to enzyme digestion). The induction of growth factor ligand transcripts in such tissue was comparable to that observed in SMCs isolated by the enzyme cocktail (data not shown). Thus, transcripts to at least three growth factors are elevated specifically in aortic SMCs after BDE.

SMC Growth Factor Receptor mRNA Expression After Balloon Deendothelialization

To determine whether growth factor receptor transcripts are coinduced with their ligands, steady-state mRNA levels of PDGF type-α and -ß receptors and a bFGF receptor were measured over the same 14-day period. No signal corresponding to the PDGF type-α receptor transcript was detected in SMCs obtained from non-BDE aortas or at any time point after BDE (Figure 3). On the other hand, BALB/c-3T3 cells showed a notable 6.5-kb transcript corresponding to the PDGF type-α receptor (Figure 3). Since BALB/c-3T3 cells are derived from a mouse and rats are close to mice on the evolutionary ladder, it is unlikely that the absence of the PDGF type-α receptor mRNA in rat aortic SMCs is due to the heterologous probe employed. Moreover, the PDGF type-α receptor probe hybridizes to the highly conserved tyrosine kinase domain. The possibility remains, however, that PDGF type-α receptor transcripts are present in aortic SMCs but are below the level of sensitivity of Northern blotting.

In contrast to the PDGF type-α receptor, transcripts to the PDGF type-ß receptor were observed in SMCs from both control and BDE vessels (Figure 3). Indeed, the 5.7-kb PDGF type-ß receptor transcript was detected at every time point after BDE, indicating little or no modulation of this receptor mRNA. A consistent increase in expression of a bFGF receptor transcript was noted between 2 and 12 hours after BDE (Figure 3). Levels of this bFGF receptor mRNA were detectable over the remaining 14-day period with no further modulation. Thus, at least two growth factor receptor transcripts are expressed in aortic SMCs during arterial repair.

Effect of Protein Synthesis Inhibition on Balloon Deendothelialization-Induced SMC Growth Factor Ligand mRNA Expression

Previously, we demonstrated the rapid induction of several IEGs in SMCs after BDE. To examine the possibility that IEG products may regulate growth factor gene expression in vivo, CHX was used to strategi-
Effect of cycloheximide (CHX) on growth factor ligand mRNA induction. This figure shows attenuation of platelet-derived growth factor-A (PDGF-A) and transforming growth factor-β1 (TGF-β1) transcripts after combined balloon deendothelialization (BDE)/CHX treatment. Animals underwent BDE and received either saline vehicle (lane 1), CHX (7.5 mg/kg body wt, lane 2), or CHX 2 hours after BDE (lane 3). All animals were killed 6 hours after BDE. Note partial induction of PDGF-A and TGF-β1 mRNA levels with delayed CHX delivery (lane 3). No changes in basic fibroblast growth factor message levels were detected when CHX was injected immediately or 2 hours after BDE. Levels of PDGF-B mRNA were detected only after CHX treatment. Lane 4 represents equally loaded RNA from human umbilical vein endothelial cells and serves as a positive control for growth factor ligand mRNA expression. Results were confirmed in one additional experiment.

Phasic Expression of Immediate-Early Genes in SMCs After Balloon Deendothelialization

Growth factor stimulation represents a major route for IEG induction. To determine whether SMC IEG induction recurs after growth factor mRNA expression, which would be consistent with SMC autocrine growth stimulation, we measured steady-state transcript levels of c-myc and thrombospondin (tsp) in aortic SMCs over a 14-day time course after BDE. The results demonstrate a phasic pattern of IEG expression after a single injury at time zero (Figure 5). Levels of SMC c-myc and tsp mRNA showed early (2–6 hours) induction, with levels returning toward baseline at 24 hours. After a general diminution in mRNA expression, both IEG transcripts showed a consistent increase at 7 days. By 14 days, steady-state IEG mRNA levels had returned toward baseline at 24 hours. After a general diminution in mRNA expression, both IEG transcripts showed a consistent increase at 7 days. By 14 days, steady-state IEG mRNA levels had returned toward baseline at 24 hours. After a general diminution in mRNA expression, both IEG transcripts showed a consistent increase at 7 days. By 14 days, steady-state IEG mRNA levels had returned toward baseline at 24 hours. After a general diminution in mRNA expression, both IEG transcripts showed a consistent increase at 7 days.
Spontaneous SMC DNA Synthesis

To determine whether the reactivation of IEG at 7 days predicts SMC growth, SMC DNA specific activity was evaluated at 0, 2, 7, 7.5, and 8 days after BDE. A large increase in DNA specific activity was observed 2 days after BDE (Figure 6). Seven days after vascular injury, DNA specific activity declined but remained above control levels. A twofold increase in DNA specific activity was observed 7.5 days after BDE (p<0.05). This spontaneous increase in SMC tritiated-thymidine incorporation was transient; DNA specific activity at 8 days was equivalent to 7-day measurements. Thus, at least two waves of IEG expression precede elevated levels of SMC thymidine incorporation.

Discussion

Autocrine growth stimulation is postulated to play an important role in the proliferation of SMCs. However, in vivo proof supporting this thesis is incomplete. In this report we show that PDGF-A, bFGF, and TGF-β1 mRNA are induced specifically in aortic SMCs after BDE. The elevation in two PDGF-A transcripts 6 hours after injury is similar to that reported previously in the rat carotid artery. Since we and others have been unable to demonstrate PDGF type-α receptor transcripts in aortic SMCs, the function of PDGF-A during arterial remodeling remains unclear. A recent report, however, indicates that PDGF-AB binds to cells in the absence of detectable type-α receptors. Although PDGF-B mRNA is normally undetected in aortic SMCs, CHX treatment reveals readily detected PDGF-B transcripts. This in vivo finding is consistent with cell culture studies that show superinduction of PDGF-B after CHX treatment. Whether the presence of PDGF-B mRNA after CHX administration reflects stabilization of the message or the inhibition of a labile repressor molecule is not known. Nevertheless, if PDGF-B mRNA is present at low but rapidly turning over levels, then it is conceivable that PDGF-AB heterodimers may form and bind the PDGF type-β receptor, whose mRNA is detected at every time point studied. Such molecules may participate in stimulating SMC proliferation and migration during arterial repair.

Levels of bFGF mRNA are elevated in SMCs early after BDE and are coinduced with a receptor mRNA whose translated product recognizes this growth factor ligand. These findings suggest that SMC-derived bFGF may stimulate SMC growth in an autocrine manner. In support of this concept are the findings of Reidy and colleagues, who showed accelerated intimal thickening after administration of bFGF and a significant reduction in SMC thymidine index with antibodies to this growth factor. Although the extrusion of stored bFGF from SMCs after BDE may account for the subsequent growth of these cells, it is equally plausible that newly synthesized bFGF stimulates autocrine growth, perhaps through an intracrine loop.

Whereas BDE-induced mRNA levels of PDGF-A and bFGF are transient, the expression of TGF-β1 mRNA remains elevated over the time course studied. This finding is consistent with studies in the injured rat carotid artery and suggests that this growth factor may be functional over the entire 14-day period. In addition to being a bifunctional regulator of SMC growth, TGF-β1 acts as a chemoattractant and stimulates ECM protein production. Indeed, the production of ECM in the growing intimal mass may result from autocrine stimulation of SMCs by TGF-β1. Supporting the latter concept is the recent demonstration of sequential TGF-β1 and ECM gene expression in the carotid artery after BDE.

Importantly, mRNA induction of PDGF-A, bFGF, and TGF-β1 in aortic SMCs coincides with the immunodetection of several immediate-early transcription factors. To examine the possibility that such growth factor activation is controlled by IEG products, CHX was administered immediately after BDE, and growth factor mRNA levels were measured 6 hours later. Consistent with cell culture studies, CHX blocked BDE-induced SMC PDGF-A gene activation and superinduced IEGs such as c-myc. Protein synthesis inhibition also attenuated SMC TGF-β1 mRNA levels 6 hours after BDE. Levels of these growth factor mRNAs can be partially restored by postponing CHX administration for 2 hours. Taken together, the results indicate that PDGF-A and TGF-β1 are delayed-early genes whose expression, unlike that of IEG, is dependent on early protein synthesis. Such newly synthesized proteins likely include immediate-early transcription factors that may bind to growth factor genes and stimulate their transcription. This notion is further supported by the complete inhibition of immunoreactive IEG products after CHX treatment.

There is precedence for the notion of IEG products' stimulating growth factor gene induction because regulatory sequences to TGF-β1 and PDGF-A contain canonical cis elements corresponding to AP-1 and Egr-1, respectively. Indeed, the TGF-β1 gene ap-

![Figure 6. Bar graph of smooth muscle cell (SMC) DNA specific activity (SA) after balloon deendothelialization (BDE), showing spontaneous reactivation of SMC DNA SA 7.5 days after BDE. This increase was significantly different *p<0.05 from DNA specific activity measured at 7 and 8 days. Results are expressed as the mean±SEM SA, which is defined as the trichloroacetic acid-precipitable counts per minute per microgram of DNA. Graph represents pooled data from two independent experiments.](http://atvb.ahajournals.org/)

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been reported previously and is now shown to be supporting the work of others. The reactivation of IEG transcripts 7 days after BDE. Similarly, results demonstrating that basic fibroblast growth factor (2a), whose expression appears to be independent of de novo protein synthesis, IEG products (3) diverge into at least two pathways. Secretory IEG products include thrombospondin (TSP) (4); TSP may communicate extracellular matrix signals through the plasma membrane (5).

An alternative route for immediate-early protein transport is the nucleus, where such transcription factors (6) directly or indirectly participate in DNA replication (7) and transregulate GF genes such as platelet-derived growth factor-A and transforming growth factor-B1 (8). Protein products of GF (9) stimulate SMC growth through internal (10) or external (11) autocrine mechanisms. Paracrine growth stimulation is also possible (11a). SMC autocrine growth stimulation may proceed through (2) or (2a) and may account for the reactivation of IEG observed at 7 days. *This figure is not meant to exclude other mechanisms of IEG induction.

Figure 7. Schematic representing a hypothetical model for immediate-early gene (IEG)-mediated smooth muscle cell (SMC) autocrine growth. Dotted lines indicate pathways for which there is little or no experimental evidence. One route of SMC growth stimulation may be activation of membrane receptors* (1) and transmission/amplification of growth signals to the nucleus. Such signals activate rapid expression of IEG (2) and may stimulate growth factor (GF) genes such as basic fibroblast growth factor (2a), whose expression appears to be independent of de novo protein synthesis. IEG products (3) diverge into at least two pathways. Secretory IEG products include thrombospondin (TSP) (4); TSP may communicate extracellular matrix signals through the plasma membrane (5).

Unexpectedly, CHX had no effect on steady-state bFGF mRNA expression. These in vivo results have recently been demonstrated in vascular SMCs in vitro and raise the possibility that bFGF has unique control mechanisms that regulate its transcription. Perhaps bFGF gene expression, like that of IEG, requires only posttranslational modifications of preexisting protein(s). Since bFGF is considered to be a primary growth factor for SMC proliferation in vivo, knowledge pertaining to its transcriptional regulation may be vital to a complete understanding of the molecular mechanisms underlying SMC growth.

We have documented a biphasic expression pattern of two functionally distinct IEGs. Expression of c-myc has been reported previously and is now shown to be reactivated 7 days after BDE. Similarly, results demonstrating tsp mRNA induction support the work of others and imply that the tsp signal detected at 7 days may be the consequence of the same stimulus that evokes c-myc reactivation. Such a stimulus may be the generation of locally derived growth factors. This notion is consistent with the second peak in DNA specific activity observed 12 hours after IEG reactivation (see below).

Monocytes and endothelial cells elaborate paracrine growth factors such as PDGF, which may account for the elevated IEG transcripts at 7 days and the subsequent rise in DNA specific activity. Such an effect seems unlikely for several reasons. First, there are virtually no monocytes present in the aortic wall after BDE. The lack of monocytes in the vessel wall is substantiated by the absence of c-fms expression, which has been used as a marker for macrophages. Second, endothelial cell regeneration is concentrated at the dorsal surface of the rat aorta. In contrast, intimal thickening is greatest at the ventral surface 7 days after BDE. Thus, it is difficult to envisage an endothelial cell–mediated paracrine effect on SMC hyperplasia, although it is possible that endothelial cells release mitogens that may act in an endocrine manner by stimulating SMC growth distally.

The reinduction of SMC c-myc and tsp mRNA at 7 days is most consistent with autocrine growth factor stimulation; these IEGs are induced by growth factors, and their reactivation occurs subsequent to SMC growth factor ligand and receptor mRNA expression. It must be emphasized, however, that it is unclear at this time whether IEG transcripts at 7 days accumulate in proliferating SMCs of the neointima. Nor is it known whether IEG transcripts colocalize with growth factor ligand/receptor proteins. Studies combining in situ hybridization with immunocytochemistry and tritiated-thymidine uptake will clarify these points.

Previous work has established a second wave in SMC DNA specific activity after reinjury to the rabbit abdominal aorta. In the present report, a similar increase in SMC DNA synthetic activity occurs 7.5 days after BDE. This increase arises spontaneously without further experimental manipulation beyond the initial BDE event. We hypothesize that this increase in SMC DNA specific activity represents a cyclic phenomenon of SMC growth during arterial repair. Such growth likely pro-
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ceeds through autocrine mechanisms, since the induction of IEG, whose expression is classically induced by growth factors, ensues before heightened SMC DNA specific activity. Future studies will examine additional time points to further assess the relation between IEG expression and DNA specific activity. In addition, blocking studies will be performed to ascertain whether IEG reactivation at 7 days is necessary for the subsequent rise in SMC DNA specific activity.

In summary, we have shown the mRNA induction of functionally disparate IEGs and growth factor ligands and receptors in aortic SMCs after BDE. Protein synthesis inhibition studies reveal a plausible link between immediate-early transcription factors and subsequent growth factor gene activation. The surge in IEG expression at 7 days followed by increased SMC DNA-specific activity is suggestive of an autocrine mode of SMC growth (Figure 7). Accordingly, control of SMC autocrine growth factor expression may have consequences for the progression of vascular stenosis associated with atherosclerosis and restenosis after angioplasty. Our study implies that such control may be achieved by interrupting immediate-early transcription factor expression. Studies in this laboratory have been initiated to address this potential mechanism of growth factor gene activation.

Acknowledgments

We are grateful to the following individuals for providing us with their cDNA clones: Judith Abraham, Christer Betsholtz, Lena Claesson-Welsh, Rik Derynck, Vishva Dixit, William Lee, Ester Sabban, J. Evan Sadler, L.T. Williams, and Randy Zicht. We also acknowledge Umesh Bhatia, Jane Lin, and Eric Olson for reading this manuscript and Dr. Pal Czobor, Nathan Kline Institute, Orangeburg, N.J., for performing the statistics. We are grateful to the following individuals for providing us with their cDNA clones: Judith Abraham, Christer Betsholtz, Lena Claesson-Welsh, Rik Derynck, Vishva Dixit, William Lee, Ester Sabban, J. Evan Sadler, L.T. Williams, and Randy Zicht. We also acknowledge Umesh Bhatia, Jane Lin, and Eric Olson for reading this manuscript and Dr. Pal Czobor, Nathan Kline Institute, Orangeburg, N.J., for performing the statistics. Finally, the technical assistance of Patricio Villalon is much appreciated.

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doi: 10.1161/01.ATV.13.2.211

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