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Rapid Communication

Early Proto-oncogene Expression in Rat Aortic Smooth Muscle Cells Following Endothelial Removal

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To study the mechanism(s) of vascular smooth muscle cell proliferation in vivo, ***mRNA levels of*** c-fos, c-jun, ***and*** c-myc ***were determined by Northern blot analysis following vascular balloon de-endothelialization (BDE). Medial smooth muscle cells (SMC) were separated and studied by enzymatic digestion of the vessel wall. mRNA levels of*** c-fos ***and*** c-jun ***from aortic smooth muscle cells (SMC) were simultaneously induced within 30 minutes of BDE and declined to baseline by 1.5 hours.*** c-myc ***mRNA did not begin to increase until 1 hour after vascular injury. Levels of*** c-myc ***peaked at 2 hours and were sustained for an additional 4 hours before gradually declining. Smooth muscle cells derived from enzyme-treated control aortae that did not undergo BDE expressed*** c-fos ***and*** c-jun, ***but showed no evidence of*** c-myc ***message. In contrast, nonenzymatically treated, non-BDE whole aortae (containing both media and adventitia) demonstrated a prominent*** c-myc ***signal, but failed to express*** c-fos ***and*** c-jun. ***Corresponding examination of adventitia derived from enzyme-treated aortae showed this tissue to be a source of all three proto-oncogenes. The results of this study demonstrate the earliest*** in vivo ***molecular markers of vascular injury reported to date and implicate SMC proto-oncogene expression in the initiation of SMC proliferation. Furthermore these findings suggest two avenues for proto-oncogene induction, that are due to (1) vessel wall manipulation and (2) humoral stimulation. (Am J Pathol 1990, 137:761–765)***

Vascular smooth muscle cell (VSMC) proliferation is a key process in the pathobiology of vascular obstructive diseases.[1] Tissue culture studies have demonstrated the induction of various proto-oncogenes such as *c-fos* and *c-myc* immediately after growth-factor stimulation.[2,3] These studies may have limited applicability to *in vivo* events, however, because cell culture of VSMCs alters their phenotype.[4] *In vivo* studies to date have not examined SMC proto-oncogene expression, nor have they focused on the molecular events preceding the entry of VSMCs into the cell cycle. The purpose of this report is to determine the induction of immediate early growth-related proto-oncogenes (*c-myc*, *c-fos*, and *c-jun*) in *in vivo* vascular SMCs using the balloon de-endothelialization (BDE) model. The results demonstrate the induction of these proto-oncogenes in a temporal sequence analogous to tissue culture and suggest a role for these genes in initiation of SMC proliferation. Furthermore these results implicate a potential mechanistic role for both mechanical and humoral mediation of VSMC proto-oncogene induction.

Methods

Reagents

Filtered type I collagenase was obtained from Worthington Biochemical (Freehole, NJ). Type II elastase, soybean trypsin inhibitor, and Medium 199 (M-199) were purchased from Sigma Chemical Company (St. Louis, MO). All reagents for RNA isolation and Northern blotting were molecular biology grade and purchased from Bethesda

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Animal Experiments

Male Sprague-Dawley rats (weighing 350 to 400 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and allowed to acclimate for 3 days. Food and water were provided *ad libitum*. Balloon de-endothelialization (BDE) of the aorta was performed as described.[5] Rats were anesthetized with sodium pentobarbital (50 mg/kg administered intraperitoneally) and killed by cardiac exsanguination at various times after BDE. A minimum of four animals was pooled per condition. Results represent at least two independent experiments.

Separation of Aortic Media from Adventitia

To prepare medial SMC RNA, an enzymatic method of separating the different layers of the vessel wall was used.[6] Briefly, aortae were perfused with cold phosphate-buffered serum, rapidly excised from the animal, and stripped of adherent tissue in a solution of M-199. Aortae were transferred to a fresh solution of M-199 to rinse away residual blood cells and then incubated at 37°C in M-199 plus 1% collagenase, 0.25% elastase, and 1% soybean trypsin inhibitor for 25 minutes in a humidified shaking incubator (95% air, 5% CO_2). After incubation, the adventitia was quickly peeled from the media and both tissues were flash frozen separately in liquid nitrogen. The time interval from aortic excision to liquid nitrogen was 30 minutes. Thus the 0.5-hour condition represents samples from animals killed immediately after BDE followed by a 25-minute enzyme digestion.

RNA Analysis

Total RNA from either aortic SMC, adventitia, or whole aorta was isolated by the guanidinium isothiocyanate/CsCl method.[7] Samples of total RNA (20 μg) from each condition were fractionated in a 1.2% denaturing agarose gel[8] and transferred overnight to a nylon membrane (Zeta Probe; Biorad, Rockville Centre, NY) by capillary transfer. Membranes were rinsed briefly in 10× SSC (1× SSC is 0.15 mol/l [molar] NaCl, 0.015 mol/l sodium citrate, pH = 7) and RNA was cross-linked to the membrane by short-wave (254 nm) irradiation (Stratagene; La Jolla, CA). Prehybridization occurred at 42°C for 2 to 4 hours in a solution containing 50% deionized formamide, 5× Denhardt's solution, 5× SSPE (1× SSPE is 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate, 0.001 mol/l Na_2EDTA, pH = 7), 6.5% dextran sulfate, 1% SDS, 500 μg/ml heparin and 200 μg/ml sheared and denatured salmon sperm DNA. Hybridizations were carried out for 18 hours at 42°C in the above solution supplemented with 1 to 2 × 10^6 cpm/ml denatured cDNA probe labeled to a specific activity of 1 × 10^9 cpm/μg.[9] Blots were washed for 30 minutes at 55 to 60°C in each of three salt solutions of increasing stringency (2× SSC/1% SDS, 1× SSC/0.5% SDS and 0.2× SSC/0.1% SDS) and exposed to X-ray film (Kodak XOMAT, Rochester, NY) with intensifying screens for 1 to 2 days at −80°C. Densitometric scanning was done using a GS 300 Scanning Densitometer (Hoefer; San Francisco, CA). For each lane, three vertical scans were taken across different regions of the band and an average integration value was determined. All densitometric units were normalized against an internal standard and expressed relative to non-BDE controls.

cDNA Probes

Plasmids containing *c-fos,*[10] *c-jun,*[11] and *c-myc*[12] were provided by Drs. T. Curran, L. Ransone, and W. Lee, respectively. A plasmid containing three glyceraldehyde phosphate dehydrogenase (GAPDH) was purchased from American Type Culture Collection (Rockville, MD) and served as an internal standard to correct for unequal RNA during loading and transfer and to demonstrate specificity in gene expression modulation by BDE.

Light Microscopy

Histologic sections were obtained routinely from vessels after enzymatic digestion, as previously described.[13]

Results

Isolation of Medial SMC

Separation of medial SMC from adventitial cells was achieved by enzymatic digestion of the aorta before RNA isolation. Histologic examination from enzyme-digested vessels confirmed the absence of contaminating adventitial and endothelial cells.

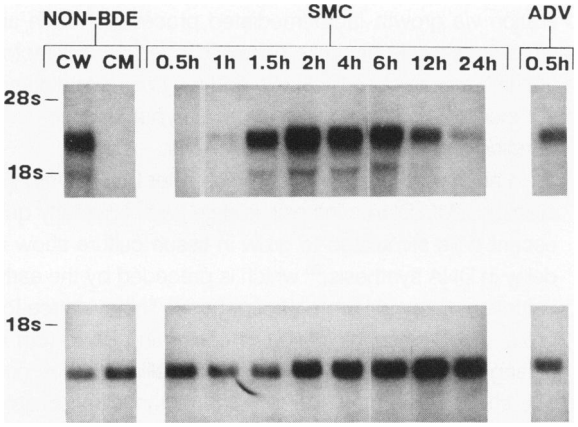

Figure 1. **(top panel)** *This figure shows* c-myc *(2.5 kb) expression by aortic SMCs after BDE. At indicated times, aortae were isolated and incubated in enzymes to isolate SMC. Total RNA (20 μg/lane) was hybridized to a murine* c-myc *cDNA probe. For orientation, 18s and 28s ribosomal RNA markers are shown.* **(bottom panel)** *This figure shows the expression of GAPDH after stripping the above blots and rehybridizing with the GAPDH probe. CW control non-BDE whole aorta (media plus adventitia and endothelium); CM control non-BDE medial SMC (enzyme treated); ADV adventitial cells.*

Effect of BDE on c-myc *expression*

Figure 1 illustrates the pattern of SMC *c-myc* expression after BDE during a 24-hour period. Levels of SMC *c-myc* were first detected 1 hour after BDE and showed a greater than 20-fold increase after 2 hours when compared to non-BDE control SMCs. The induced expression of *c-myc* remained elevated for another 4 hours and then decreased slowly toward baseline. The marked increase in *c-myc* expression was not the result of a generalized increase in gene expression because the mRNA levels of GAPDH increased only slightly, relative to non-BDE controls, during the time course studied (Figure 1).

Smooth muscle cell RNA that was isolated from non-BDE control aortae, which were incubated in enzymes to remove adventitia, showed undetectable *c-myc*. However, RNA isolated from non-BDE whole aortae showed a prominent *c-myc* signal (Figure 1). In contrast to the enzymatically treated control aortae, these vessels were processed immediately for RNA isolation and not subjected to enzyme digestion and separation of media from adventitia. Examination of isolated adventitial RNA showed this tissue to be a source of *c-myc* expression. These results indicate that *c-myc* is differentially expressed across the aortic wall.

Effect of BDE on c-fos *Expression*

Figure 2 shows the pattern of *c-fos* expression in rat aortic SMC following BDE. Although all medial SMC preparations expressed *c-fos* mRNA, a fourfold induction was

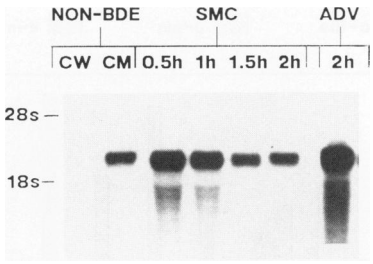

Figure 2. *This figure shows* c-fos *(2.2 kb) expression by aortic SMCs after BDE. The experiment was performed as described in Figure 1.*

noted 30 minutes after BDE. The increase in *c-fos* expression was brief: after 1 hour levels of *c-fos* declined slightly and by 1.5 hours they decreased to baseline. No *c-fos* was detected in non-BDE whole aortae that were processed immediately for RNA without enzymatic separation of the media and adventitia. This was in contrast to the *c-myc* expression observed in these vessels. These data indicate that the presence of *c-fos* in SMCs derived from enzyme-treated, non-BDE control aorta is probably a result of the physical manipulation of the vessel during tissue harvesting and the subsequent delay for enzyme digestion before medial RNA isolation. Enzyme digestion *per se* did not alter proto-oncogene expression after BDE (data not shown).

Effect of BDE on c-jun *Expression*

In light of the close association between the protein products of *c-fos* and *c-jun*,[14] SMC *c-jun* expression was studied following BDE. Both *c-jun* transcripts were increased approximately fourfold 30 minutes after BDE, declined slightly after 1 hour, and returned to control levels by 1.5 hours (Figure 3). In a manner identical to *c-fos, c-jun* transcripts were induced in SMC derived from enzyme-digested non-BDE aortae, but not aortae immediately pro-

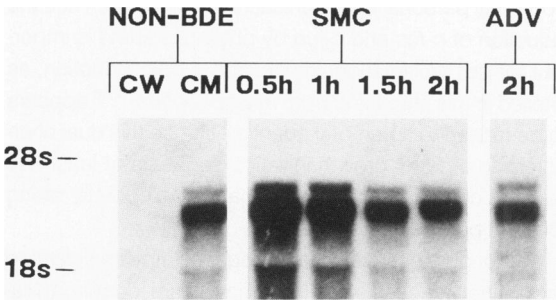

Figure 3. *This figure shows* c-jun *(2.7 and 3.2 kb) expression by rat aortic SMCs after BDE. Experiment was performed as in Figuer 1. Note the nearly identical pattern of* c-jun *expression as compared to* c-fos.

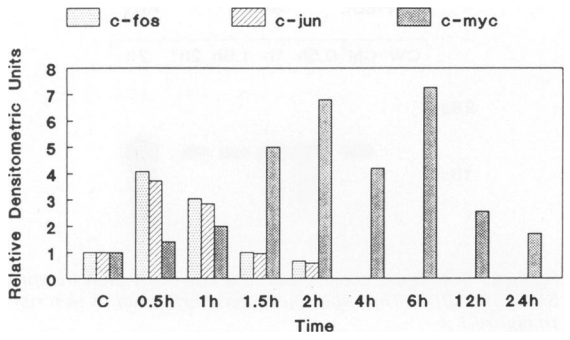

Figure 4. *This figure shows the relative increases in* c-myc, c-fos, *and* c-jun *expression after BDE. All densitometric measurements were normalized against an internal standard (GAPDH). The values are expressed as relative increases over the normalized control non-BDE SMCs. Levels of* c-myc *may be underestimated due to the increase in GAPDH after 2 hours.*

cessed for RNA isolation. Figure 4 summarizes the pattern of *c-myc, c-fos,* and *c-jun* expression following BDE.

Discussion

The results of this study demonstrate, for the first time, the *in vivo* induction of proto-oncogenes in VSMCs. Because VSMCs are the major cells responsible for intimal thickening, which eventually leads to vessel closure and hence clinical symptomatology, understanding the components that influence SMC proliferation has consequences for control of these disease processes. This report indicates that *c-myc, c-fos,* and *c-jun* may be activated by two distinct processes. *c-fos* and *c-jun* appear to be induced by mechanical manipulation of the vessel wall and thus may be important in the initiation of SMC proliferation in iatrogenic diseases such as vascular restenosis following balloon angioplasty. Stretched myocytes[15] and wounded fibroblasts[16] express *c-fos* in the absence of serum. Furthermore wounding the tail of transgenic mice containing the *c-jun* oncogene results in increased *c-jun* expression, which coincides with the development of a fibrosarcoma of the tail (M. Breitman, A. Shuh, F. Monteclaro, and P. Vogt, oral personal communication, April 1990). Thus the induction of *c-fos* and *c-jun* by physical trauma is important for cell proliferation. In contrast, *c-myc* induction, as implied in this study and from *in vitro* reports,[17,18] appears to be related to a humoral agent(s). Hence this dual characteristic of SMC growth initiation suggests at least two distinct control mechanisms that may operate along different pathways.

Although the three proto-oncogenes may be activated by distinct mechanisms, they may share common features that lead to the initiation of SMC growth. One such feature may reside in the expression of growth factors from SMC themselves that can, in turn, autoactivate proliferation *via* growth factor-mediated processes. Such an effect is possible because platelet-derived growth factor (PDGF) is expressed by SMCs and may be released and act locally.[3,19] This process remains hypothetical *in vivo* pending further study of vascular SMCs.

In animals, there is a 24-hour lag after BDE before increased SMC DNA synthesis is detected.[13] Similarly quiescent cells stimulated to grow in tissue culture show a delay in DNA synthesis,[20] which is preceded by the early induction of several proto-oncogenes.[21] The evidence for proto-oncogene participation in converting cells from a resting to a proliferative mode is compelling. Tissue culture studies using antibodies[22] and oligonucleotide antisense RNA[23] have shown an S-phase entry dependence on *c-fos* and *c-myc* expression. Furthermore, micro-injected *c-myc* protein renders cells PDGF independent for entry into the S phase of the cell cycle.[24] However proto-oncogene induction under some circumstances is associated with cell differentiation.[25]

Previous studies examining growth-related gene expression *in vivo* have reported whole vessel preparations.[26-28] In the current report, we isolated SMC RNA from the rat aortic media by an enzymatic digestion procedure.[6] The media of small animals is essentially composed of extracellular proteins and SMCs.[29] No other contaminating cells were found in these preparations. This was confirmed by extensive histologic evaluation of the tissue used in this study and correlates with other reports.[13,30]

In summary, we demonstrated the earliest molecular markers of cell-cycle activity reported to date in vascular tissue. This new information should allow for the careful dissection of initiating events leading to such cell activity. The induction of these and other proto-oncogenes may have general applicability to the development of atherosclerosis and the vascular restenosis that frequently follows balloon angioplasty.

References

1. Liu MW, Roubin GS, King SB III: Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. Circulation 1989, 79:1374–1387
2. Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, Nadal-Ginard B: Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca2+ mobilization and protein kinase C activation. J Biol Chem 1989, 264:526–530
3. Naftilan AJ, Pratt RE, Dzau VJ: Induction of platelet-derived growth factor A-chain and c-myc gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. J. Clin Invest 1989, 83:1419–1424
4. Thyberg J, Palmberg L, Nilsson J, Ksiazek T, Sjolund M: Phenotype modulation in primary cultures of arterial smooth

muscle cells: On the role of platelet-derived growth factor. Differentiation 1983, 25:156–167
5. Stemerman MB: Thrombogenesis of the rabbit arterial plaque; an electron microscopic study. Am J Path 1973, 73: 7–18
6. Cassis LA, Lynch KR, Peach MJ: Localization of angiotensinogen messenger RNA in rat aorta. Circ Res 1988, 1259–1262
7. Chirgwin JM. Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979, 18:5294–5299
8. Davis LG, Dibner MD, Battey JF: Basic Methods in Molecular Biology. New York, Elsevier, 1986
9. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983, 132:6–13
10. Sambucetti LC, Schaber M, Kramer R, Crowl R, Curran T: The fos gene product undergoes extensive post-transcriptional modification in eukaryotic but not in prokaryotic cells. Gene 1986, 43:69–77
11. Lamph WW, Wamsley P, Sassone-Corsi P, Verma IM: Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature 1988, 334:629–631
12. Stanton LW, Watt R, Marcu KB: Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 1983, 303:401–406
13. Goldberg ID, Stemerman MB, Schnipper LE, Ransil BJ, Crooks GW, Fuhro RL:Vascular smooth muscle cell kinetics: A new assay for studying patterns of cellular proliferation *in vivo*. Science 1979, 205–920–921
14. Curran T, Franza Jr BR: Fos and Jun: The AP-1 Connection. Minireview. Cell 1988, 55:395–397
15. Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, Takaku F, Yazaki Y: Stretching cardiac myocytes stimulates protooncogene expression. J Biol Chem 1990, 265:3595–3598
16. Verrier B, Muller D, Bravo R, Muller R: Wounding a fibroblast monolayer results in the rapid induction of the c-fos protooncogene. EMBO J 1986, 5:913–917
17. Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD: Functional role for c-myc in mitogenic responce to platelet-derived growth factor, Nature 1984, 310: 655–660
18. Kelly K, Cochran BH, Stiles CD, Leder P: Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 1983. 35:603–610
19. Libby P, Warner SJC, Salomon RN, Birinyi LK: Production of platelet-derived growth factor-like mitogen by smooth-muscle cells from human atheroma. N Engl J Med 1988, 318: 1493–1498
20. Scher CD, Shepard RC, Antoniades HN, Stiles CD: Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. Biochem Biophys Acta 1979, 560:217–241
21. Lau LF, Nathans D: Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: Coordinated regulation with c-fos or c-myc. Proc Natl Acad Sci USA 1987, 84:1182–1186
22. Riabowol KT, Vosatka RJ, Ziff EB, Lamb NJ, Feramisco JR: Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. Mol Cell Biol 1988, 8:1670–1676
23. Yokoyama K, Imamoto F: Transcriptional control of the endogenous MYC protooncogene by antisense RNA. Proc Natl Acad Sci USA 1987, 84:7363–7367
24. Kaczmarek L, Hyland J, Watt R, Rosenberg M, Baserga: Microinjected c-myc as a competence factor. Science 1985, 228:1313–1315
25. Curran T, Bravo R, Muller R: Transient induction of c-fos and c-myc is an immediate consequence of growth factor stimulation. Cancer Surveys 1985, 4:655–681
26. Majesky MW, Benditt EP, Schwartz SM: Expression and developmental control of platelet-derived growth factor A-chain and B-chain/Sis in rat aortic smooth muscle cells. Proc Natl Acad Sci USA 1988, 85:1524–1528
27. Sarzani R, Brecher P, Chobanian AV: Growth factor expression in aorta of normotensive and hypertensive rats. J Clin Invest 1989, 83:1404–1408
28. Cercek B, Fishbein MC, Forrester JS, Helfant RH, Fagin JA: Induction of vascular insulin-like growth factor-I messenger RNA in rat aorta after balloon denudation. Circ Res 1990, 66:1755–1760
29. Pease DC, Paule WJ: Electron microscopy of elastic arteries; the thoracic aorta of the rat. J Ultrastruct Res 1960, 3:469 – 483
30. Wolinsky H, Daly MM: A method for the isolation of intima-media samples from arteries. Proc Soc Exp Biol Med 1970, 135;364–368

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