

***JE* mRNA Accumulates Rapidly in Aortic Injury and in Platelet-Derived Growth Factor–Stimulated Vascular Smooth Muscle Cells**

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The early response to vascular injury is characterized by migration of inflammatory cells, including monocytes, and platelets to the damaged vessel wall. These inflammatory cells may serve as a source of growth factors and cytokines that stimulate vascular smooth muscle cell (VSMC) migration and proliferation associated with intimal hyperplasia. *JE* is a platelet-derived growth factor (PDGF)-inducible “early” gene that encodes a monocyte chemoattractant and, as such, could play an important role in inflammation. We now report that *JE* mRNA levels are increased in intact aorta after balloon injury. The time course of this increase, with maximal levels at 4 hours, is similar to that seen in PDGF-treated cultured rat aortic VSMCs. The accumulation of *JE* mRNA in cultured VSMCs is accompanied by a marked increase in the secretion of *JE* protein. The elevation of *JE* mRNA levels in VSMCs shows specificity for PDGF, because angiotensin II, α -thrombin, and epidermal growth factor fail to increase *JE* mRNA levels. In contrast to 3T3 fibroblasts, the accumulation of *JE* mRNA in VSMCs in response to PDGF is predominantly due to an increase in *JE* mRNA stability. The accumulation of *JE* mRNA in VSMCs stimulated by PDGF appears to occur via a novel pathway(s) independent of Ca^{2+} mobilization, $\text{Na}^{+}\text{-H}^{+}$ exchange, protein kinase C activation, or elevation in cAMP levels. These findings suggest that VSMCs may take part in the early inflammatory response after injury through the production of *JE*, a potent monocyte chemoattractant. Finally, our data suggest that *JE* may be a marker for PDGF-specific effects on VSMCs, both in vitro and in vivo. Thus, in addition to direct effects on VSMC growth and migration, PDGF may play a role in the early inflammatory response after vascular injury by inducing chemoattractants, such as that encoded by *JE*. (*Circulation Research* 1992;70:314–325)

The response to vascular injury is characterized by an early inflammatory reaction involving migration of circulating white blood cells and platelets to the site of damage. The early response is followed by migration of vascular smooth muscle cells (VSMCs) from the media to the intima. This VSMC migration and subsequent proliferation are major factors in the intimal hyperplasia seen both in experimental models of vessel injury and in restenosis after balloon angioplasty of the coronary arteries.^{1–4} The

stimulus for VSMC migration and proliferation is thought to be due in part to the release of growth factors, such as platelet-derived growth factor (PDGF), from platelets and inflammatory cells, including monocytes and macrophages.⁵ PDGF is also secreted by cells present in the vessel wall, including endothelial cells⁵ and VSMCs.^{6–8} PDGF is both mitogenic^{9,10} and chemotactic for cultured VSMCs.¹¹

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Although its role in vascular injury has not been clearly established, PDGF has been implicated particularly in the migration of VSMCs into the intima.¹² In addition to PDGF, a variety of other agents, most notably fibroblast growth factor, vasoconstrictor hormones, such as angiotensin II (Ang II), and clotting factors, such as α -thrombin, have been implicated in the response of the blood vessel to injury.^{1,4,5}

To elucidate some of the molecular mechanisms underlying the response of the vessel wall to injury, we have examined the expression of a number of PDGF-inducible genes both in vivo and in cultured VSMCs. *JE* is one of the most abundant mRNAs induced by PDGF in fibroblasts, with several thousand copies of *JE* mRNA per cell accumulating within 2 hours of induction.¹³ In general, *JE* is not expressed in quiescent growth-arrested cells and is induced by exposure to a variety of mitogens and growth factors.^{14–17} Activation of *JE* appears to involve different transmembrane signals from those that activate *c-fos* and *c-myc*, and *JE* is not coordinately regulated with these genes.^{18,19} The *JE* gene encodes a low molecular weight glycoprotein^{20,21} whose amino acid sequence is homologous to that of a monocyte-specific chemotactic factor, referred to as MCP-1^{22,23} or MCAF.^{24,25} At least one function of the *JE* protein is that of a monocyte chemoattractant.²¹ *JE* thus serves as a marker for the “early” response to PDGF and may play a critical role in attracting monocytes to sites of inflammation or injury.

We now report that levels of *JE* mRNA increase rapidly in intact aorta after balloon injury with a time course similar to that seen in VSMC culture after treatment with PDGF. The accumulation of *JE* mRNA in VSMCs in response to PDGF is different from that reported for 3T3 fibroblasts, in that it is predominantly due to an increase in *JE* mRNA stability rather than transcription. In addition, the elevation of *JE* mRNA levels in VSMCs shows specificity for PDGF: Ang II, α -thrombin, and epidermal growth factor, all of which induce other early genes, fail to elevate *JE* mRNA levels. The accumulation of *JE* mRNA in rat aortic VSMCs in response to PDGF appears to occur via a novel pathway(s) independent of Ca^{2+} mobilization, Na^+ - H^+ exchange, protein kinase C activation, or elevation in cAMP levels. *JE* may thus serve as an important tool for elucidating PDGF-specific pathway(s) in VSMCs.

Materials and Methods

Growth Factors and Other Reagents

Recombinant *c-sis* protein (Amgen Biologicals, Thousand Oaks, Calif.) was used as a source of PDGF B-chain homodimer. Mouse epidermal growth factor (receptor grade) was purchased from Collaborative Research Inc., Bedford, Mass. Actinomycin D, Ang II, bovine insulin, cycloheximide, dibutyryl cAMP, indomethacin, phorbol 12,13-dibutyrate (PDBu), and phorbol 12-myristate 13-acetate (PMA) were obtained from

Sigma Chemical Co., St. Louis, Mo. Forskolin, human α -thrombin, and quin-2 AM were obtained from Calbiochem Corp., San Diego, Calif.

Cell Culture

VSMCs were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic dissociation as previously described.²⁶ Cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin and serially passaged before reaching confluence. Aortic VSMCs modulate from a contractile to a more fibroblast-like growth phenotype during multiple passaging in culture.^{27,28} This change in phenotype is associated with a loss of actin-myosin filaments as well as a marked decrease in the expression of smooth muscle α -actin mRNA.²⁹ The rapidity and extent of these phenotypic changes appears to vary somewhat with the method used to isolate VSMCs. Thus, Owens and colleagues^{30–32} have shown that, by modifying the isolation procedures, VSMC cultures can be produced that maintain relatively high levels of α -actin and myosin heavy chain expression through multiple subculture passages. The VSMCs used for this study were prepared using similar methods. To confirm that they behaved like differentiated VSMCs, Northern blots from representative passages were hybridized with a probe encoding the 3' untranslated end of rat smooth muscle α -actin (gift of Gary K. Owens, University of Virginia School of Medicine, Charlottesville). The VSMCs expressed smooth muscle α -actin as well as mRNA, encoding smooth muscle α -tropomyosin for up to 15 subculture passages (M.B. Taubman and B. Nadal-Ginard, unpublished observations, February 1991). These cells also maintained high levels of functioning receptors for α -thrombin³³ even at later (>20) passages. In addition, fluorescent analyses of fura-2-loaded, Ang II-stimulated VSMCs were performed to demonstrate that the cells responded to Ang II with a rise in $[\text{Ca}^{2+}]_i$ typical of VSMCs but not of fibroblasts. Most experiments were performed with subculture passages 9–11; some (indicated in the text) were performed as early as passage 3 or as late as passage 15. To produce quiescence, cells were incubated in DME with 0.5% calf serum for 48–72 hours. Under these conditions, incorporation of [^3H]thymidine into DNA was <15% of that seen with 10% serum. For most experiments, agonists and antagonists were added directly to the culture medium of quiescent VSMCs. Unless indicated, experiments were performed three times.

To measure levels of *JE* mRNA in the absence of Na^+ - H^+ exchange and intracellular alkalinization, quiescent VSMCs were washed twice with buffer containing 130 mM choline chloride, 5 mM KCl, 1.5 mM CaCl_2 , 1.0 mM MgCl_2 , 0.25 mg/ml bovine serum albumin, 10 mM glucose, and 20 mM HEPES, pH 7.4, and then equilibrated in the same buffer at 37°C in room air for 30 minutes. PDGF was then added directly to some

cultures and incubated under the same conditions for the times indicated. Under these conditions, agonist-mediated $\text{Na}^+\text{-H}^+$ exchange and intracellular alkalization are completely inhibited.^{34,35}

To measure levels of *JE* mRNA in the absence of a change in $[\text{Ca}^{2+}]_i$, quiescent VSMCs were washed twice with TBSS buffer (130 mM NaCl, 5 mM KCl, 1.0 mM MgCl_2 , 0.25 mg/ml bovine serum albumin, 10 mM glucose, and 20 mM HEPES, pH 7.4) and then equilibrated for 30 minutes at 37°C in room air in the same buffer containing 10 μM quin-2 AM and 4 mM EGTA. PDGF was then added directly to some cultures and incubated under the same conditions for the times indicated. Measurements of $[\text{Ca}^{2+}]_i$ were made on duplicate cultures plated on 12×27-mm glass coverslips and loaded with the Ca^{2+} -sensitive fluorescent dye, fura-2 AM (2 μM), as previously described.³⁶ Under these conditions, agonist-mediated Ca^{2+} mobilization was completely blocked (References 35 and 36 and current results).

To measure cAMP levels, VSMCs (5×10^5 cells per 35-mm well) were growth-arrested by a 48-hour incubation in DME with 0.5% serum. The media were aspirated, and agonists were added in fresh DME with 0.5% serum. After 30 minutes, the cells were washed twice with TBSS, and cellular protein was precipitated by a 30-minute treatment (on ice) with 10% trichloroacetic acid. Cells were then scraped with a rubber policeman, and protein was removed by centrifugation at 1,000g for 15 minutes in glass tubes. The supernatant was extracted four times with ether. Samples were then lyophilized and dissolved in assay buffer. cAMP was determined using the radioimmunoassay kit (New England Nuclear, Boston, Mass.) exactly as described by the manufacturer.

Balloon Injury and Isolation of Rat Aortas

Balloon injury was induced in adult New Zealand White rabbits using techniques described by Consigny et al.³⁷ Rabbits were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). A 4F balloon embolectomy catheter was introduced into the aorta via the femoral artery and advanced to the level of the aortic valve. The catheter was then withdrawn along the full length of the aorta with the balloon inflated; this procedure was repeated three times. The rabbits were killed at varying time points after the procedure, and the aortas were dissected, cleaned, and immediately placed in liquid nitrogen. For light microscopy, portions of some aortas were fixed in freshly prepared 2% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) at 4°C overnight. After fixation, samples were immersed in 10% sucrose/isotonic phosphate-buffered saline for 3 hours at 4°C as a cryoprotectant and then embedded in optimal cutting temperature compound (O.C.T., Miles Scientific) blocks and stored at -80°C. Tissues were sectioned at a thickness of 8 μm with a cryostat, thaw-mounted onto polylysine-coated microscope slides, and immediately frozen at -80°C. Samples were stained with hematoxylin and eosin.

RNA Preparation and Blot Hybridization

Total RNA was extracted from VSMCs or from rabbit aorta by the guanidinium isothiocyanate/CsCl procedure.³⁸ For most experiments, 15 μg total RNA was size-fractionated by electrophoresis on 1% agarose gels in 20 mM MOPS, pH 7.4, 1 mM EDTA, and 3% formaldehyde. For analysis of aortic tissue, 4 μg poly(A)⁺ RNA, purified by oligo(dT)-cellulose chromatography,³⁹ was used instead of total RNA. Transfer to nitrocellulose and hybridization to [³²P]-labeled DNA were as described.³⁵ Prehybridization and hybridization were performed at 42°C. Final washes for all blots were in 0.1× standard saline citrate (SSC) (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7) and 0.1% sodium dodecyl sulfate (SDS) at 65°C for 1 hour. *JE*, a 650-base pair (bp) *Eco*RI fragment of pc*JE*-1,²⁰ was nick-translated to a specific activity of $>10^8$ cpm/ μg and used at 3×10^6 cpm/ml. A 4.5-kilobase pair *Bam*HI/*Hind*III fragment of mouse plasmid pc-*fos*-3⁴⁰ was labeled by random oligomer priming. As a control, filters were also hybridized with cDNA encoding a myosin regulatory light chain (MLC) isolated from rat aortic VSMCs.⁴¹ MLC mRNA is constitutively expressed in VSMCs, and levels were the same in all lanes (not shown). In some experiments, rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH⁴²) was used as a control instead of MLC (indicated in the text or figure legends). To quantify levels of *JE* mRNA, samples were scanned with an LKB Ultrosan XL densitometer, and the area under the appropriate peaks was calculated.

Nuclear Runoff

Nuclear runoff transcription analysis was performed as described by Groudine et al.⁴³ with modifications. Quiescent cells (5×10^7 per sample) were stimulated as described in the text. At 40 minutes, the cells were rinsed in phosphate-buffered saline, lysed in 0.5% Nonidet P-40 solution, and harvested by centrifugation. Runoff reactions were performed at room temperature for 30 minutes with [³²P]UTP. The nuclei were treated with DNase I, added to a solution of 4 M guanidinium isothiocyanate, layered on a 5.7-M CsCl cushion, and centrifuged at 40,000 rpm for 18 hours. The labeled RNA was hybridized at 65°C to nitrocellulose filters on which 10 μg linearized, denatured plasmid DNAs had been blotted. Blots were washed at 68°C in 0.1× SSC. The same number of total counts per minute were hybridized to each filter. In addition to pc*JE*-1, each blot contained MLC plasmid (see above) and the pGEM vector as positive and negative controls, respectively. To verify that pc*JE*-1 identified a single mRNA species under the hybridization and wash conditions used in these assays, an RNA blot containing quiescent and PDGF-stimulated VSMCs was hybridized to ³²P-labeled *JE* cDNA under identical conditions (Figure 1). To quantify levels of *JE* transcript, samples were scanned as described above. The levels of

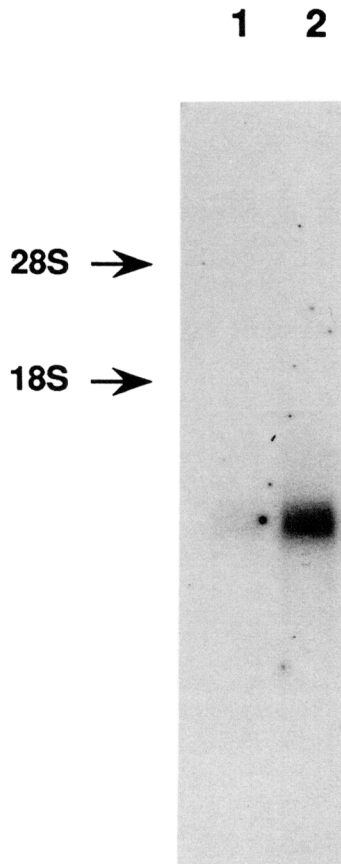


FIGURE 1. RNA blot analysis of *JE* mRNA in rat aortic vascular smooth muscle cells: Probe specificity. Blot contains total RNA (10 μ g) from quiescent vascular smooth muscle cells (grown in 0.5% calf serum for 48 hours) (lane 1) and from vascular smooth muscle cells stimulated with platelet-derived growth factor (10 ng/ml recombinant *c-sis*) for 3 hours, hybridized, and washed as described for nuclear runoff assays (lane 2). Photograph is of the entire gel. Location of the 28S and 18S ribosomal RNAs is shown (arrows).

JE transcript were normalized to those of the constitutively expressed MLC.

Immune Precipitation of *JE* Protein

Rat aortic VSMCs were grown to 60% confluence in three 100-mm dishes and then made quiescent by incubation in 0.5% calf serum for 48 hours. Cultures were then treated with 10^{-7} M Ang II, 20 ng/ml PDGF (*c-sis*), or were left untreated for 20 minutes. Medium was withdrawn from each plate such that 0.5 ml remained, and 0.5 mCi [35 S]methionine (specific activity, 1,169 Ci/mmol) was added. After an additional 4-hour incubation, the medium was removed, phenylmethylsulfonyl fluoride (PMSF; final concentration, 2 mM) was added, and cellular debris was removed by centrifugation. An equal volume of cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, and 1 mM PMSF) was added to 0.25 ml radio-labeled medium, and anti-*JE* antiserum²¹ or normal rabbit serum was added. Incubation continued with

rotation at 4°C for 1 hour, after which 50 μ l of a 50% slurry of *Staphylococcus* protein A-agarose beads (BioRad Laboratories, Richmond, Calif.) was added. Tubes were rotated at 4°C for an additional hour. Beads were washed twice with cold RIPA buffer and once with cold 1 M NaCl/50 mM Tris-HCl, pH 7.5, and resuspended in Laemmli sample buffer.⁴⁴ The slurry was boiled for 1 minute, the beads were centrifuged, and the supernatant was analyzed by electrophoresis on a 17.5% SDS-polyacrylamide gel.⁴⁴

Results

Differential Effects of PDGF and Ang II on JE mRNA

JE encodes a monocyte chemoattractant that is, in general, not expressed in quiescent growth-arrested cells but is rapidly inducible by growth factors.¹⁴⁻¹⁷ To determine whether *JE* mRNA was constitutively expressed in cultured rat aortic VSMCs, cells were made quiescent by incubation in 0.5% calf serum, total RNA was purified, and 10- μ g aliquots were analyzed by RNA blot hybridization. Hybridization of the *JE* cDNA probe to these RNA blots identified a single species of $\sim$ 750 bp, typical of *JE* mRNA (Figure 1). VSMCs constitutively expressed *JE* mRNA (Figure 2A); the levels varied with both the degree of confluence and the time maintained at quiescence. Thus, the levels of *JE* mRNA in VSMCs incubated for 24 and 48 hours in 0.5% calf serum were $152 \pm 6\%$ and $131 \pm 7\%$, respectively, of those measured in cells quiescent for 72 hours ($n=3$). In addition, the levels of *JE* mRNA in cells made quiescent at 50% confluence were $156 \pm 12\%$ of those at 90% confluence ($n=3$). Accordingly, most of the experiments were done on cells made quiescent for 72 hours at 90% confluence (e.g., Figures 3-5 and 7-9). This produced reproducibly low levels of constitutive expression of *JE* mRNA. Regardless of the protocol used for inducing quiescence, the magnitude of the effects seen with PDGF or Ang II was not altered (see below).

When quiescent VSMCs were exposed to PDGF (10 ng/ml recombinant *c-sis*, Amgen), there was a rapid accumulation of *JE* mRNA above the constitutive (control) levels, beginning at $\sim$ 1 hour, peaking at 2-4 hours, and remaining elevated for as much as 9 hours (Figure 2A). In contrast, Ang II (10^{-7} M) failed to increase *JE* mRNA levels. The apparent decrease in *JE* mRNA levels seen 1-2 hours after treatment with Ang II was not statistically significant by densitometric analysis (lowest level was 0.69 ± 0.19 arbitrary units at 1 hour; control, 1.0; $n=4$; $p=NS$), nor was there a significant rise above constitutive levels during the 24-hour treatment period (highest level was 1.24 ± 0.05 arbitrary units at 9 hours, $p=NS$). In comparison, a 4-hour exposure to PDGF resulted in a 10.8 ± 3.5 -fold rise in *JE* mRNA levels ($n=10$, $p<0.001$). The increase in *JE* mRNA levels induced by PDGF was concentration dependent, with a threshold response at 1 ng/ml. However, concentra-

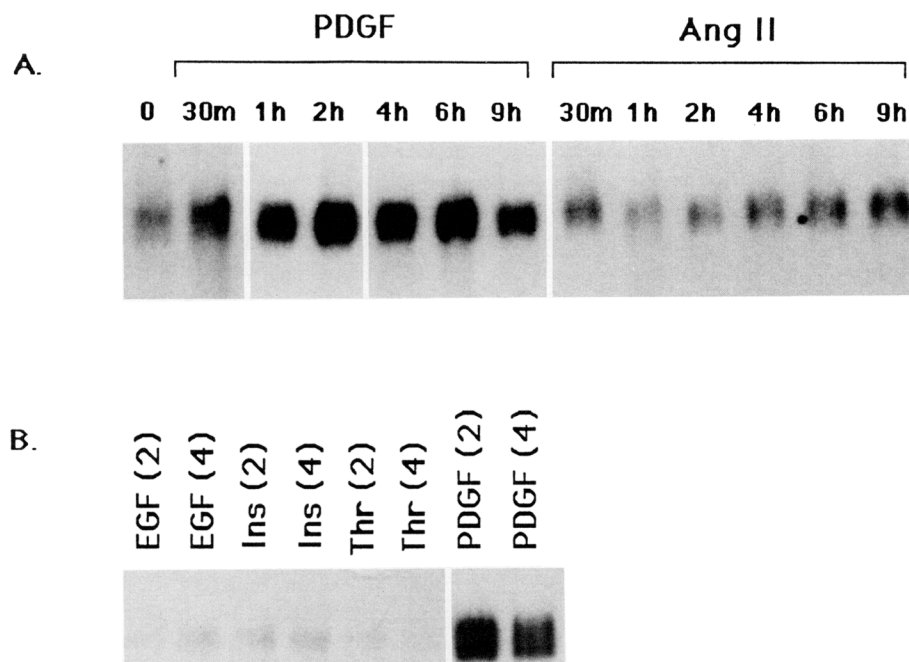


FIGURE 2. RNA blot analysis of JE mRNA in rat aortic vascular smooth muscle cells: Differential effects. Panel A: Time course of JE mRNA induction. Quiescent vascular smooth muscle cells (time 0) were treated for the times indicated with Dulbecco's modified Eagle's medium with 0.5% calf serum containing platelet-derived growth factor (PDGF, 10 ng/ml recombinant c-sis) or angiotensin II (Ang II, 1 μ M). Panel B: Induction of JE mRNA by epidermal growth factor (EGF, 300 ng/ml), insulin (Ins, 25 μ g/ml), α -thrombin (Thr, 39 nM), and PDGF (20 ng/ml recombinant c-sis). Numbers in parentheses represent duration, in hours, of exposure to each agent. Hybridization and wash conditions for this and subsequent RNA blots are as described in "RNA Preparation and Blot Hybridization."

tions of Ang II as high as 10^{-5} M failed to stimulate the accumulation of JE mRNA. The differential expression of JE mRNA in response to PDGF and Ang II was noted at very early passages (passages 3 and 5); in addition, maximum levels of JE mRNA accumulating in the presence of PDGF did not vary significantly in cells of passages 3, 5, 7, 10, and 15 (not shown). Accordingly, VSMCs from passages 9–11 were used for most of the experiments described below.

α -Thrombin (39 nM), an agent that activates many of the same transmembrane signals as Ang II and is also hypertrophic for rat aortic VSMCs,³³ failed to elevate JE mRNA levels (Figure 2B). In addition, neither insulin (25 μ M) nor epidermal growth factor (300 ng/ml, Amgen) increased JE mRNA levels in VSMCs (Figure 2B). Under these conditions, Ang II, α -thrombin, and PDGF (same doses) induced similar levels of c-fos mRNA at 30 minutes.^{33,35}

To elucidate the mechanism underlying the difference in response of JE mRNA to Ang II and PDGF, nuclear runoff assays⁴³ were performed. Under the conditions of this assay, only newly transcribed mRNA is measured. When compared with untreated cells, the amount of newly synthesized JE mRNA after 40 minutes of treatment with PDGF was not significantly increased. In contrast, Ang II produced a marked decrease in the amount of JE mRNA transcribed (Figure 3). Neither PDGF nor Ang II had a significant effect on the transcription of the constitutively ex-

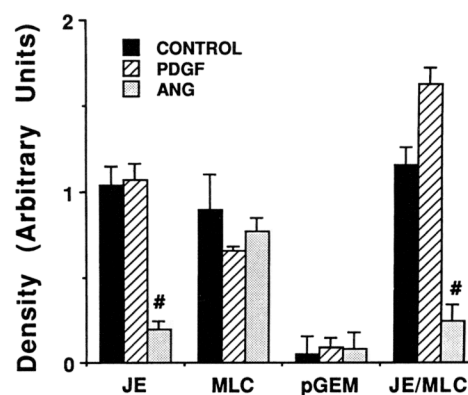


FIGURE 3. Nuclear runoff transcription analysis of JE mRNA. The bar graph depicts the relative rates of JE transcription as determined by scanning densitometry. Nuclei were isolated from untreated quiescent vascular smooth muscle cells (CONTROL) and from those treated for 40 minutes with 1 μ M angiotensin II (ANG) or 20 ng/ml recombinant c-sis (PDGF). Radiolabeled nuclei were then hybridized to slot blots containing 10 μ g pc JE1 cDNA (JE), myosin regulatory light chain (MLC) cDNA, and bacterial plasmid pGEM (pGEM) DNA. Normalized density (JE/MLC) was determined from triplicate experiments and represents the ratio of the density of JE mRNA (determined by laser scanning of autoradiograms) divided by that of MLC mRNA after subtraction of background (pGEM). # $p < 0.05$ by one-way analysis of variance for repeated measures and Tukey's method.

pressed MLC gene. Nonspecific hybridization of the mRNA transcripts, measured using pGEM plasmid DNA, was uniformly low. When compared with untreated cells, the relative rate of newly synthesized *JE* mRNA (normalized to MLC mRNA) after 40 minutes of treatment with PDGF was not significantly increased (1.6 ± 0.1 versus 1.2 ± 0.2 arbitrary units, $p = \text{NS}$). In contrast, Ang II produced an 83% decrease in the rate of *JE* transcription (0.2 ± 0.1 arbitrary units, $p < 0.05$) at 40 minutes.

To evaluate the contribution of mRNA stability to *JE* accumulation, levels were measured in the presence of the transcription inhibitor actinomycin D ($10 \mu\text{M}$). As shown in Figure 4A, there was a rapid decay of *JE* mRNA in the presence of actinomycin D and 0.5% calf serum (closed circles). Addition of 10% calf serum for 2 hours before and during treatment with actinomycin D markedly prolonged the decay of *JE* mRNA (squares). However, when serum-stimulated cells were switched back to 0.5% calf serum concomitant with actinomycin D treatment, the decay in *JE* mRNA levels was virtually identical to that of unstimulated cells (open circles). Thus, the prolongation of *JE* mRNA half-life required persistent exposure to 10% serum. The slopes of the linear regression lines for *JE* mRNA in 0.5% serum and 10% serum were -1.33 ± 0.16 and $-0.33 \pm 0.10 \text{ hr}^{-1}$ ($p < 0.001$), corresponding to half-lives of 0.5 and 2.1 hours, respectively (data pooled from both sets of curves). As shown in Figure 4B, similar to 10% calf serum, both PDGF (20 ng/ml c-sis, squares) and Ang II (10^{-7} M , circles) markedly prolonged the half-life of *JE* mRNA. As was the case in Figure 4A, identical results were obtained whether cells were treated with actinomycin D and either PDGF or Ang II while quiescent (maintained in 0.5% calf serum for 48 hours and during treatment, closed squares and circles) or after a 2-hour stimulation with 10% calf serum (open squares and circles). Accordingly, the linear regression curves shown in Figure 4B were generated from pooled data.

Differential Effects of PDGF and Ang II on *JE* Protein Secretion

Previous studies in fibroblasts^{20,21} have demonstrated that *JE* protein is largely secreted into the culture medium. To determine whether VSMCs secrete *JE* protein, quiescent VSMCs were incubated for 4 hours with [³⁵S]methionine, and radiolabeled *JE* precipitated with specific antibody was examined by SDS-polyacrylamide gel electrophoresis. Quiescent VSMCs constitutively secreted *JE* into the culture medium (Figure 5, lane 1). Ang II (10^{-7} M) had little effect on the level of newly synthesized *JE* protein secreted by VSMCs (Figure 5, lane 2). In contrast, PDGF (20 ng/ml) caused a marked increase in the secretion of *JE* protein (Figure 5, lane 3). Therefore, the differential effects of Ang II and PDGF on *JE* mRNA levels in VSMCs are reflected in the secretion of *JE* protein by these cells. The broad band of

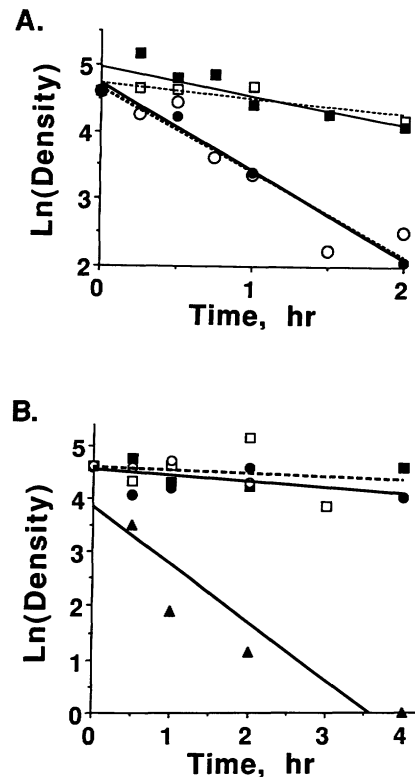


FIGURE 4. RNA blot analysis of *JE* mRNA stability. Natural logarithms of *JE* mRNA levels are plotted at varying time points after actinomycin D ($10 \mu\text{M}$) treatment after determination by scanning densitometry of autoradiograms. Each line is a linear regression plot of the average of duplicate experiments. Panel A: *JE* mRNA response under varying conditions. Actinomycin D was added to quiescent vascular smooth muscle cells (48 hours) kept in 0.5% calf serum ($\bullet$). For the remaining plots, quiescent vascular smooth muscle cells were stimulated for 2 hours with 10% calf serum to maximally induce *JE* mRNA. Cells were then washed and treated with actinomycin D in the presence of 0.5% serum ($\circ$), actinomycin D was added directly to cultures and maintained in 10% calf serum ($\blacksquare$), and cultures were washed and treated with actinomycin D in the presence of fresh 10% serum ($\square$). For each condition, the levels of *JE* mRNA are expressed as the natural logarithm of the percentage of *JE* mRNA measured before addition of actinomycin D [time 0 = $\ln(100) = 4.61$]. Note that the point at time 0 for all four data sets is 4.61. Panel B: Actinomycin D added to quiescent vascular smooth muscle cells (maintained in 0.5% calf serum for 48 hours) alone ($\blacktriangle$) or in the presence of platelet-derived growth factor (20 ng/ml c-sis, $\blacksquare$) or 10^{-7} M angiotensin II ($\bullet$). Quiescent vascular smooth muscle cells were also stimulated for 2 hours with 10% calf serum to maximally induce *JE* mRNA and then washed and treated with actinomycin D in the presence of PDGF ($\square$) or angiotensin II ($\circ$). Note that the point at time 0 for all five data sets is 4.61. Linear regression plots for platelet-derived growth factor (---) and angiotensin II (—) were derived by pooling data from both sets of conditions.

labeled *JE* protein demonstrated by gel electrophoresis also suggests that the molecular size and microheterogeneity of rat *JE* protein is similar to that previously found in mice.²⁰

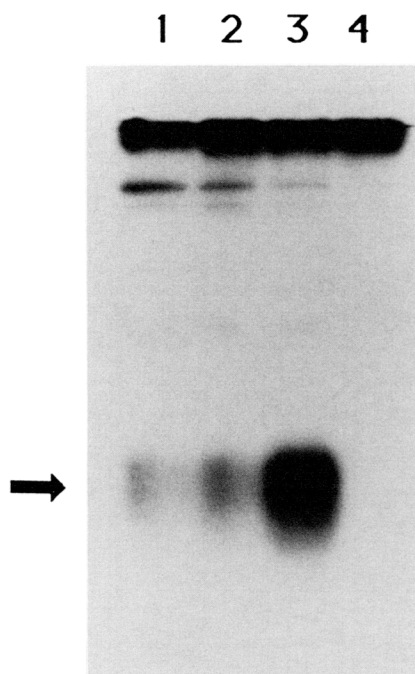


FIGURE 5. Autoradiogram of polyacrylamide gel containing immunoprecipitated JE protein. Quiescent vascular smooth muscle cells were incubated for 4 hours in medium containing [35 S]methionine without additional treatment (lane 1) or in the presence of 1 μ M angiotensin II (lane 2) or 20 ng/ml recombinant c-sis (lanes 3 and 4). Samples were then precipitated with anti-JE antiserum (lanes 1–3) or preimmune serum (lane 4) and run on a 17.5% sodium dodecyl sulfate–polyacrylamide gel. Arrow indicates radiolabeled JE protein.

Induction of JE in Intact Aorta

As noted above, the migration of monocytes to the vessel wall is part of the early inflammatory response to injury. Because levels of JE mRNA and protein increase in VSMCs in response to PDGF and JE are implicated in migration of monocytes, the expression of JE mRNA in response to vascular injury was examined. As shown in Figure 6, levels of JE mRNA were barely detectable in normal rabbit aorta (time 0). Significant amounts of JE mRNA began to accumulate between 1 and 4 hours after balloon injury and returned to baseline by 8 hours. Levels of JE

mRNA were maximal at 4 hours, averaging a 3.8 ± 1.2 -fold increase over control ($n=3$, normalized to GAPDH mRNA levels). As determined by light microscopy, the extent of injury used for these studies completely denuded the vessel of endothelial cells (data not shown). In addition, the adventitia was removed during tissue processing, and the increase in JE mRNA levels preceded the recruitment of blood-borne inflammatory cells (References 2 and 45 and authors' unpublished observations, February 1991). Thus, it is most likely that the VSMC was the predominant source of JE mRNA.

Signal Transduction and JE Expression

PDGF stimulates a variety of transmembrane signals, many of which have been implicated in growth. As noted above, some of these signals are also stimulated by Ang II. The role of several of these signals in the accumulation of JE mRNA in VSMCs was investigated.

Na⁺-H⁺ exchange. Both Ang II and PDGF stimulate Na⁺-H⁺ exchange in VSMCs, resulting in intracellular alkalinization.^{34,46} This pathway has been implicated in the mitogenic response to a variety of agents.^{47,48} Recently, it has been shown in macrophages that induction of JE mRNA by γ -interferon, but not phorbol esters, is dependent on Na⁺-H⁺ exchange.⁴⁹ To determine whether this pathway was important in the increase in JE mRNA levels produced by PDGF in VSMCs, quiescent VSMCs were incubated in a Na⁺-free solution (iso-osmotic replacement with 130 mM choline chloride³⁴). Under these conditions, Na⁺-H⁺ exchange and the resulting intracellular alkalinization are completely blocked.³⁴ However, Na⁺-free medium had no effect on either the constitutive expression of JE mRNA or the elevation in JE mRNA levels by PDGF (Figure 7).

Ca²⁺ mobilization. Both Ang II and PDGF cause a concentration-dependent increase in [Ca²⁺]_i in VSMCs that is due to mobilization of intracellular Ca²⁺ stores.^{5,50} Treatment of VSMCs with the Ca²⁺ chelators, quin-2 AM and EGTA, completely inhibited the change in [Ca²⁺]_i in VSMCs³⁶ and blocked the induction of c-fos by Ang II.³⁵ In contrast, these same agents failed to block the rise in JE mRNA

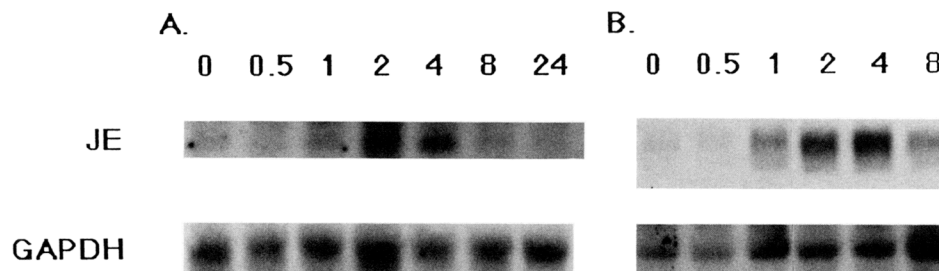


FIGURE 6. RNA blot analysis of JE mRNA in intact aorta. Lanes contain 4 μ g poly(A)⁺ RNA from normal rabbit aorta (time 0) or from aorta harvested at various time points (in hours) after balloon injury. Blots were hybridized to JE cDNA, stripped, and then rehybridized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA. Panels A and B represent two experiments involving separate groups of rabbits.

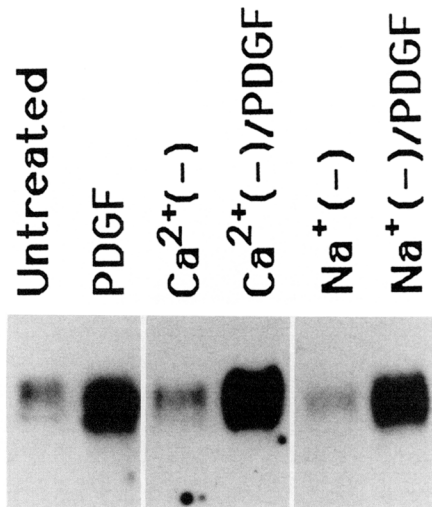


FIGURE 7. RNA blot analysis of *JE* mRNA in vascular smooth muscle cells: Ionic effects. Of six lanes (left to right), lanes 1 and 2 show quiescent vascular smooth muscle cells left untreated or exposed for 2 hours to platelet-derived growth factor (PDGF, 20 ng/ml recombinant c-sis) in Dulbecco's modified Eagle's medium containing 0.5% calf serum. Lanes 3 and 4 show duplicate cultures untreated [$\text{Ca}^{2+}(-)$] or exposed for 2 hours to PDGF [$\text{Ca}^{2+}(-)/\text{PDGF}$] in Ca^{2+} -free buffer containing 10 μM quin-2 AM and 4 mM EGTA. Lanes 5 and 6 show duplicate cultures untreated [$\text{Na}^{+}(-)$] or exposed for 2 hours to PDGF [$\text{Na}^{+}(-)/\text{PDGF}$] in Na^{+} -free buffer.

levels produced by PDGF (Figure 7). These results suggest that $[\text{Ca}^{2+}]_i$ mobilization is not important in the activation of *JE* by PDGF in VSMCs.

Protein kinase C activation. Activation of protein kinase C plays an important role in the induction of

a number of growth-related genes, such as *c-fos* and *c-myc*.^{18,51-54} Both PDGF and Ang II are potent stimulators of protein kinase C. PMA (250 nM), a direct activator of protein kinase C,⁴⁹ stimulated *JE* mRNA in rat aortic VSMCs, but at a time significantly later than that seen with PDGF (Figure 8). This delayed activation of *JE* by phorbol esters is similar to that shown in BALB/c 3T3 fibroblasts.¹⁸ To test whether protein kinase C played a role in the accumulation of *JE* mRNA in PDGF-treated cells, VSMCs were pretreated for 24 hours with PDBu. As previously reported,³⁵ this pretreatment decreases cytosolic protein kinase C activity by 80–90% when measured by phosphorylation of histone III-S. In addition, it abolishes PMA-stimulated and Ang II-stimulated phosphorylation of the 76,000-d protein kinase C substrate.³⁵ Pretreatment of VSMCs with PDBu completely abolished the rise in *JE* mRNA levels in response to PMA (Figure 8) but had minimal effect on *JE* in response to PDGF (Figure 8), suggesting that protein kinase C was not important in the regulation of *JE* mRNA by PDGF.

Effects of cAMP levels on *JE* mRNA. Activation of phospholipase A_2 and the resultant increase in cAMP levels have been implicated in cell growth.^{55,56} It has previously been shown that Ang II alone does not cause a rise in cAMP levels in VSMCs from rat aortic explants.⁵⁷ To determine whether differential induction of cAMP might be important in distinguishing the response of VSMCs to PDGF and Ang II, cAMP levels were measured in quiescent VSMCs before and after a 30-minute exposure to a variety of agonists. The levels of cAMP in untreated quiescent VSMCs was 5 ± 1 pmol/mg. After exposure to 10% calf serum or PDGF (25 ng/ml recombinant c-sis), cAMP levels

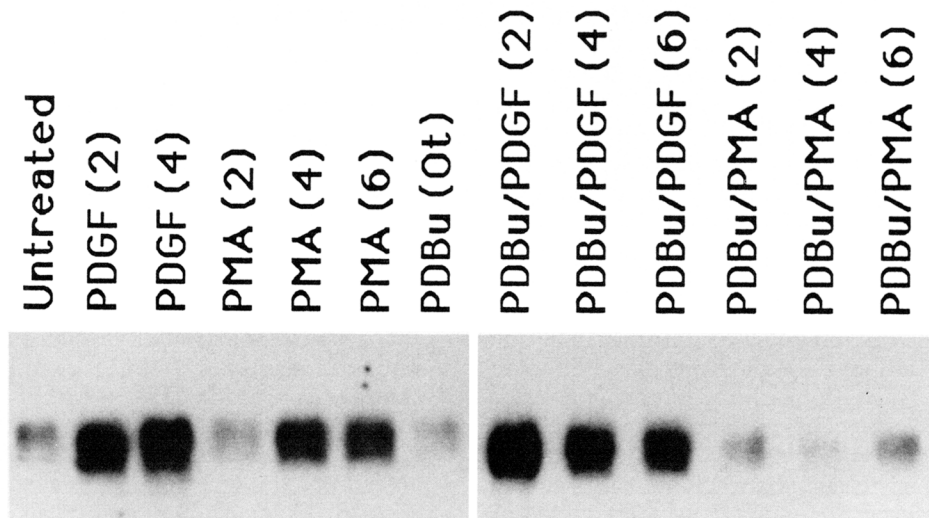


FIGURE 8. RNA blot analysis of *JE* mRNA in vascular smooth muscle cells: Protein kinase C effects. Of 13 lanes (left to right), lanes 1–6 show quiescent vascular smooth muscle cells left untreated or exposed for the times indicated (in hours) to platelet-derived growth factor (PDGF, 20 ng/ml recombinant c-sis) or phorbol 12-myristate 13-acetate (PMA, 250 nM). Lanes 7–13 show quiescent cells pretreated for 24 hours with 1 μM phorbol 12,13-dibutyrate [PDBu (0t)] in Dulbecco's modified Eagle's medium plus 0.5% calf serum and then exposed for the times indicated to either PDGF (PDBu/PDGF) or PMA (PDBu/PMA).

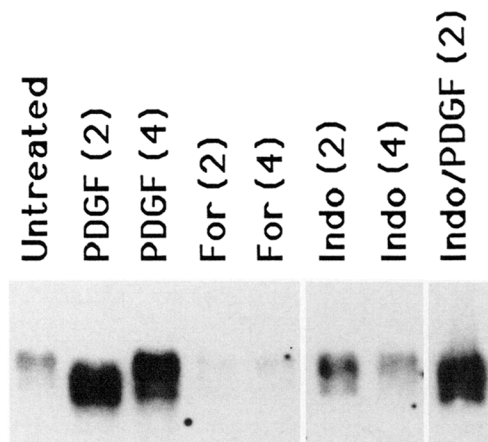


FIGURE 9. RNA blot analysis of *JE* mRNA in vascular smooth muscle cells: cAMP effects. Quiescent vascular smooth muscle cells were left untreated or exposed for the times indicated (in hours) to the following agents: platelet-derived growth factor (PDGF, 20 ng/ml recombinant c-sis), forskolin (For, 2 μ M), both PDGF and forskolin (For/PDGF), indomethacin alone (Indo, 10 μ M), and both indomethacin and PDGF (Indo/PDGF).

rose to 27 ± 4 and 113 ± 21 pmol/mg, respectively. In contrast, Ang II (10^{-6} M) had no effect on cAMP levels (5 ± 1 pmol/mg). To determine whether this pathway might play a role in regulating *JE* mRNA, VSMCs were treated with indomethacin. At a dose (10 μ M) that blocks the PDGF-induced rise in phospholipase A_2 activity,⁵⁸ and thus the rise in cAMP, indomethacin had no effect on either the constitutive levels of *JE* mRNA or on its elevation by PDGF (Figure 9). In addition, forskolin, at a dose (2 μ M) that causes a 10-fold increase in cAMP levels,³⁵ failed to increase levels of *JE* mRNA (Figure 9). Isoproterenol (1 μ M), which induced a rise in cAMP levels to 611 ± 44 pmol/mg, and dibutyryl cAMP (1 μ M) also failed to increase *JE* mRNA levels (data not shown).

Discussion

The migration of monocytes to the vessel wall is an early event after vascular injury.¹⁻⁴ Monocytes play an important role in the inflammatory response after injury and may serve as a source of growth factors and cytokines. These factors, along with those produced by platelets and cells within the vessel wall, may provide the stimulus for VSMC migration and proliferation, leading to intimal hyperplasia. In addition, monocytes may be a source of the foam cells seen in atherosclerotic lesions (see Reference 59 for review). Recent work²¹ has demonstrated that *JE* encodes a monocyte chemoattractant that is analogous to the monocyte chemotactic factor secreted by smooth muscle cells, SMC-CF.^{60,61} In this report, we demonstrate that *JE* mRNA accumulates rapidly in the vessel wall in response to balloon injury with a time course similar to that seen in PDGF-stimulated VSMC culture. Although *JE* mRNA is found in a variety of cell types, it is most likely that, in this

balloon injury model, the VSMC is the predominant source of *JE* mRNA, because the injury completely denudes the vessel of endothelial cells, the adventitia is removed during tissue processing, and the accumulation of *JE* mRNA precedes the recruitment of blood-borne cellular elements (References 2 and 45 and authors' unpublished observations, February 1991). Therefore, these findings suggest that VSMCs take part in the early inflammatory response after injury. This may be of particular importance in balloon angioplasty models in which the endothelium is denuded at the time of injury and the VSMC is thus exposed to circulating blood.

Because of the potential importance of *JE* in the early response to vessel injury, we have used the rat aortic VSMC culture system to further study its expression. Although it is well known that aortic VSMCs modulate from a contractile to a more fibroblast-like growth phenotype during multiple passaging in culture,^{27,28} we found no differences in the maximum levels of *JE* mRNA after treatment with PDGF in early passages (3–5) versus late passages (10–15) of VSMCs. In addition, the differential expression of *JE* mRNA in response to PDGF and Ang II was seen in both very early and late passages. These results suggest that the cells used here (see "Material and Methods") are a valid model for the analysis of *JE* gene expression, despite their modulation of some differentiated functions. Our results in the in vivo model lend further support to the notion that the increase in *JE* mRNA levels seen in VSMC is not an artifact of subpassaged cell cultures. Most important, the rat aortic VSMCs behave differently from the 3T3 fibroblasts previously used to characterize the expression of *JE* in that the major mechanism by which *JE* expression is regulated by PDGF in the VSMC system appears to be posttranscriptional, whereas it is largely transcriptional in 3T3 fibroblasts. The rat aortic VSMC culture system thus appears to be a good in vitro model for studying the regulation of *JE* and, in particular, for studying posttranscriptional regulation of *JE* mRNA expression by PDGF.

Both transcriptional and posttranscriptional mechanisms have been implicated in the accumulation of mRNAs encoding many of the early growth-response genes, including *JE*.^{19,62} In contrast to 3T3 fibroblasts,^{18,19} accumulation of *JE* mRNA in VSMCs in response to PDGF or serum is predominantly due to changes in mRNA stability rather than transcription. The prolongation of *JE* mRNA half-life requires continuous exposure to growth factors: switching stimulated cultures to 0.5% serum rapidly restores the half-life of *JE* mRNA to that seen in quiescent, unstimulated cells. Ang II shares the stabilizing effect on *JE* mRNA with PDGF and calf serum. However, Ang II differs from PDGF in markedly decreasing the rate of transcription of *JE* at 40 minutes. The net effect of the increase in mRNA stability and decrease in transcription is to maintain *JE* mRNA levels at close to baseline. The differential expression of *JE* mRNA in response to PDGF and Ang II is accom-

panied by a similar large difference in the amount of newly synthesized *JE* protein secreted into the culture medium over 4 hours. The slight increase in *JE* protein seen with Ang II may be due in part to the marked stimulatory effect of Ang II on total protein synthesis in these cells.^{63,64}

The accumulation of *JE* mRNA in VSMCs shows unusual specificity for PDGF: epidermal growth factor, α -thrombin, and Ang II, all of which induce other early growth-related genes such as *c-fos*,³⁵ *c-myc*, and *KC* (M. Poon and M.B. Taubman, unpublished observations, April 1991), fail to induce *JE*. *JE* may thus be a marker for a PDGF-specific pathway in VSMCs. Studies in fibroblasts have suggested that the transmembrane signals involved in *JE* induction differ from those involved in induction of *c-fos* or *c-myc*. In particular, protein kinase C and Ca^{2+} mobilization do not appear to be important in *JE* induction in BALB/c 3T3 fibroblasts.¹⁸ The present results indicate that this is also true for the regulation of *JE* mRNA by PDGF in VSMCs. In addition, stimulation of Na^+ - H^+ exchange does not appear to be important for the accumulation of *JE* mRNA in response to PDGF. These findings are not surprising, given that Ang II and α -thrombin, potent activators of these pathways, do not induce *JE* mRNA. Thus, it would appear that other transmembrane pathways activated by PDGF, and not by Ang II, must be required for stimulation of *JE* mRNA. Both cAMP accumulation and tyrosine phosphorylation have been implicated in growth-related processes.^{55,56,65} The present data demonstrate that PDGF but not Ang II causes an elevation in cAMP levels in rat aortic VSMCs. At high concentrations (10^{-5} M), which block activation of phospholipase A_2 , and elevation of cAMP levels,⁵⁸ indomethacin had no effect on the accumulation of *JE* mRNA. In addition, concentrations of forskolin that caused a 10-fold increase in cAMP levels³⁵ or high concentrations of dibutyryl cAMP failed to increase *JE* mRNA levels. These results suggest that the increase in *JE* mRNA levels by PDGF in VSMCs is not mediated solely through its effects on cAMP. The role of tyrosine phosphorylation in the accumulation of *JE* mRNA remains to be determined. Insulin and epidermal growth factor also stimulate tyrosine kinases but do not increase *JE* mRNA levels. Thus, the elevation in *JE* mRNA levels either requires activation of a PDGF-specific tyrosine kinase or stimulation of a pathway(s) not involving tyrosine phosphorylation. Therefore, *JE* may be a product of a novel signaling pathway induced by PDGF, but not by Ang II or even epidermal growth factor, and may serve as a marker for PDGF-specific actions on VSMCs.

The differential expression of *JE* mRNA in response to PDGF and Ang II is intriguing. Both agonists activate many of the same cellular responses, including stimulation of phospholipase C, mobilization of intracellular Ca^{2+} , and activation of amiloride-sensitive Na^+ - H^+ exchange. In addition, PDGF, like Ang II, is capable of causing smooth muscle contrac-

tion.⁶⁶ Despite these similarities, PDGF and Ang II differ in some of their effects on VSMCs in culture. PDGF is hyperplastic for VSMCs, stimulating both DNA and protein synthesis as well as increasing cell number.^{9,10} In contrast, several investigators have reported that Ang II is hypertrophic, stimulating protein synthesis but not DNA synthesis or cell division in normal adult rat aortic VSMCs.^{63,64} PDGF and Ang II also differ in their ability to stimulate migration of cultured VSMCs: PDGF is a potent VSMC chemoattractant,¹¹ whereas Ang II has little effect on migration of rat aortic VSMCs (B.C. Berk and M. Mitsuka, unpublished observations, September 1990.). Initial attempts at elucidating the molecular genetic events that might differentiate the responses of VSMCs to PDGF and Ang II demonstrated that both agents induced the accumulation of mRNAs encoding *c-fos*, *c-jun*, and *c-myc*, three of the earliest events associated with growth factor stimulation.^{13,35,67-69}

The present results demonstrate that there is at least one gene, *JE*, whose mRNA levels are increased by PDGF but not by Ang II. Given the known function of the *JE* protein, it would appear that, in addition to having different effects on VSMC growth and migration in cell culture, PDGF and Ang II differ in their ability to stimulate the production of cytokines by VSMCs. This may have important implications for the role(s) these two agents play in vascular pathology. It has recently been shown in a rat arterial injury model that thrombocytopenia inhibits VSMC migration into the intima but does not prevent VSMC proliferation,¹² suggesting that PDGF may be necessary for VSMC migration, but not proliferation. The inability of thrombocytopenia to prevent VSMC proliferation may be due in part to the fact that agents other than PDGF that are not released by platelets, such as fibroblast growth factor or Ang II, may be capable of inducing VSMC proliferation in vivo. However, PDGF may be specific in inducing VSMC migration. The present study offers the possibility that, in addition to direct effects on VSMC growth and migration, PDGF may play an early role in the early inflammatory response after vascular injury by inducing chemoattractants, such as that encoded by *JE*. Additional work is ongoing to determine whether, in fact, the accumulation of *JE* mRNA in the vessel wall after injury is mediated specifically by PDGF.

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