Induction of PDGF-Responsive Genes in Vascular Smooth Muscle

Implications for the Early Response to Vessel Injury

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Arterial injury induces vascular smooth muscle cells (VSMC) to modulate from a quiescent to a proliferative state characterized by cell division, migration, and secretion of matrix. These changes have been implicated in the development of intimal hyperplasia after balloon angioplasty. The transition of VSMC to a proliferative state is preceded by the accumulation of platelets and leukocytes, which may release growth factors and cytokines at the site of injury. Platelet-derived growth factor (PDGF) is secreted by platelets and a variety of cellular elements associated with the vessel wall and, as a VSMC mitogen and chemoattractant, has been implicated in the pathogenesis of intimal hyperplasia. We have found that, in addition to its effects on VSMC growth and migration, PDGF induces the expression in VSMC of the *JE* and *KC* genes, which encode monocyte and neutrophil chemoattractants, respectively. The induction of *JE* and *KC* by PDGF in VSMC culture involves several distinct transmembrane signaling pathways. In addition to their induction in VSMC culture, *JE* and *KC* messenger RNA accumulates rapidly and transiently in adult rabbit aorta after balloon dilatation, suggesting a role for these chemoattractants in the early vascular response to injury in vivo. The VSMC may therefore play a dual role in vessel injury, both as a mediator of the inflammatory response through chemoattractant release and as an effector of the hyperplastic response through proliferation. (*Circulation* 1992;86[suppl III]:III-53–III-60)

KEY WORDS • **muscle, smooth** • **vessels** • **growth factors** • **cytokines** • **gene expression**

Vascular smooth muscle is the predominant cell type in the media of the normal mammalian artery and as such provides the vasculature with structural support and vasomotion.[1] The vascular smooth muscle cell (VSMC) performs these functions by maintaining a differentiated contractile phenotype characterized by numerous myofilaments and a quiescent state of growth.[2] In vascular injury, such as that associated with balloon angioplasty, the VSMC proliferates, secretes connective tissue matrix, and migrates from the media to the intima.[2–5] These processes are thought to play a pivotal role in the development of chronic atherosclerosis and in restenosis after angioplasty of human coronary arteries.[3,5]

In balloon-mediated arterial injury, the transition of the VSMC population from a state of quiescence to one of high activity is preceded by platelet deposition and inflammatory cell accumulation at the site of dilatation.[6] Platelets and leukocytes are believed to trigger the activation of VSMCs via the release of growth factors such as platelet-derived growth factor (PDGF)[3] and cytokines such as tumor necrosis factor-α[7,8] and interleukin-1.[9]

PDGF is a dimer consisting of two chains, A and B,[10] each the product of a unique gene. All three dimeric combinations (AA, AB, BB) have been isolated.[11] Several lines of evidence point to PDGF as a central bioregulatory molecule in the response to vascular injury. PDGF is both mitogenic[10,12] and chemotactic[13] for cultured VSMCs. PDGF is synthesized by a variety of cells implicated in intimal hyperplasia, including endothelial cells, macrophages, and VSMCs.[10] The synthesis of PDGF by VSMCs raises the possibility of an autocrine pathway for smooth muscle proliferation. Wilcox et al[14] have demonstrated PDGF A chain messenger RNA (mRNA) in mesenchymal-appearing cells of possible smooth muscle origin in human carotid artery atheroma. Recent investigations using in vivo models have suggested an important role for PDGF in intimal hyperplasia occurring after balloon-mediated arterial injury. The induction of mRNA encoding both the PDGF A chain and the PDGF receptor was demonstrated in the neointima of injured rat carotid arteries.[15] In a study using anti-platelet antibodies to produce thrombocytopenic rats, migration of VSMCs from the media to intima was attenuated after balloon injury.[16] Most recently, the administration of anti-PDGF antibodies was shown to significantly inhibit neointimal VSMC accumulation after angioplasty.[17]

PDGF A and B chains exert their varied biological effects by binding to receptors that are present on VSMCs and fibroblasts but not on endothelial cells. The PDGF receptor exists in two separate forms, called α

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and β,[18,19] each of which has specific affinities for the different dimeric forms of PDGF. The receptor is activated when each chain of the PDGF dimer binds to one receptor subunit to form a receptor dimer.[20] The PDGF receptors are transmembrane proteins that function as tyrosine-specific protein kinases. Activation leads to stimulation of a number of signaling pathways including 1) activation of phospholipase C, which produces inositol trisphosphate, an inducer of intracellular Ca^{2+} accumulation, and diacylglycerol, an inducer of protein kinase C activation; 2) stimulation of Na^+–H^+ exchange, yielding intracellular alkalinization; and 3) activation of phospholipase A_2, which results in increased arachidonate and prostaglandin synthesis and elevation in cyclic AMP levels. Activation of these and other transmembrane pathways by PDGF results in the rapid induction of a variety of genes including proto-oncogenes, cytokine genes, genes encoding enzymes of intermediate metabolism, and genes encoding clotting factors.[10]

KC and *JE* Induction In Vivo

The development of intimal hyperplasia after vascular injury is the result of a wound-healing response that occurs in three overlapping phases: 1) inflammation, which involves platelet aggregation, thrombus formation, and leukocyte infiltration; 2) granulation, which is characterized by smooth muscle proliferation; and 3) extracellular matrix deposition.[6] The initial inflammatory phase is characterized by the accumulation of monocytes and the activation of neutrophils. The migration of monocytes to the vessel wall has been described as an early event after vascular injury.[21–23] Monocytes may serve as a source of growth factors and cytokines and may be responsible for the foam cells seen in atherosclerotic lesions.[24] The monocyte–macrophage cell line may also play a substantial role in chronic atherosclerotic lesions, in which their rate of proliferation appears comparable to that of the VSMC.[25]

In addition to monocytes, neutrophils have been demonstrated in the vessel wall shortly after experimental balloon injury,[26,27] although this observation has been less consistent.[21,28] More recently, a number of reports have documented neutrophil activation after percutaneous transluminal coronary angioplasty in humans.[29–31] An influx of neutrophils into the walls of epicardial coronary arteries in response to ischemia/reperfusion has also been documented.[32] Neutrophils may therefore play an important role in the inflammatory response after injury and, like monocytes, may serve as a source of growth factors, proteolytic enzymes, and cytokines.[33,34] Neutrophils may also participate in the activation of platelets that occurs almost immediately after vessel injury.[35]

We have investigated the expression of two genes, *KC* and *JE,* both in vitro in VSMC culture and in vivo in injured adult rabbit aorta. *JE* encodes a secretory glycoprotein that is homologous to the monocyte-specific chemotactic factor referred to as MCP-1[36] or MCAF.[37] *KC* encodes a glycoprotein that is homologous to that of a neutrophil-specific chemotactic factor referred to as *gro.*[38] These genes were chosen for study because they had been found to be highly inducible in fibroblasts by PDGF[39] and because of their known function as leukocyte chemoattractants.

To examine the expression of *KC* and *JE* in vivo, balloon injury was induced in aortas of adult New Zealand White rabbits.[40] A balloon embolectomy catheter was introduced into the aorta and then withdrawn along its full length with the balloon inflated. Animals were killed at various times after the procedure, the aortas were harvested, and poly $(A)^+$ RNA was isolated and analyzed for *JE* and *KC* mRNA by Northern blot hybridization.[41,42] *JE* and *KC* mRNA levels were quantified by densitometric analysis of autoradiographs of the Northern blots and normalized to the levels of the constitutively expressed enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

As shown in Figure 1, levels of *JE* mRNA were barely detectable in normal rabbit aorta (time 0). Balloon injury induced *JE* mRNA between 1 and 4 hours; levels returned to baseline by 8 hours. These studies have now been extended to *KC.* As shown in Figure 2, levels of *KC* mRNA were barely detectable in normal rabbit aorta (time 0). *KC* mRNA increased rapidly at 4 hours and returned to baseline by 8 hours after balloon injury. Figure 3 is a graphic representation of *JE* and *KC* mRNA levels (normalized to GAPDH mRNA) averaged from three sets of balloon-injured rabbits. As can be seen from this figure, both the time course of expression and the magnitude of increase in mRNA levels (5.4 ± 1.1 for *JE* and 5.0 ± 1.4 for *KC*) were similar for both genes.

KC and *JE* are inducible in a variety of cells including endothelium, fibroblasts, leukocytes, and VSMC (see below). The cellular source of *JE* and *KC* mRNA in the studies depicted in Figure 3 has not yet been determined. However, the VSMC is a likely candidate to be the predominant source. As demonstrated by a number of groups,[2,44] including our own,[41] balloon injury results in a virtually complete loss of endothelial cells. In addition, the adventitia, where fibroblasts predominate, is largely removed during tissue processing. Finally, the time course of *JE* and *KC* induction precedes that described for the recruitment of leukocytes into the vessel wall,[21,28] and we have not observed significant numbers of leukocytes in our samples. Thus, the VSMC appears to be by far the most abundant cell type in the samples processed for mRNA. Additional experiments are under way, using in situ hybridization with complementary RNA probes to establish the exact cell type and the location of cells producing *JE* and *KC* mRNA after balloon injury.

KC and *JE* Induction In Vitro

Because of the potential role of the VSMC as a source of leukocyte chemoattractants after balloon injury, the expression of *JE* and *KC* was studied in detail in cultured adult rat (Sprague-Dawley) aortic VSMCs.[41,45] VSMCs, made quiescent by incubation in serum-deprived medium (Dulbecco's modified Eagle's medium with 0.4% calf serum) for 48 hours, constitutively expressed low levels of both *KC* and *JE* mRNA. When these cells were exposed to 10% calf serum, there was a rapid accumulation of *KC* mRNA above constitutive levels, beginning within 15 minutes, peaking at 1 hour, and falling to below baseline after 4 hours (Figure 4). Levels of *JE* mRNA also markedly increased after serum treatment. The response to serum, however, was more prolonged than that seen for *KC,* beginning at approximately 1 hour, peaking between 2 and 4 hours,

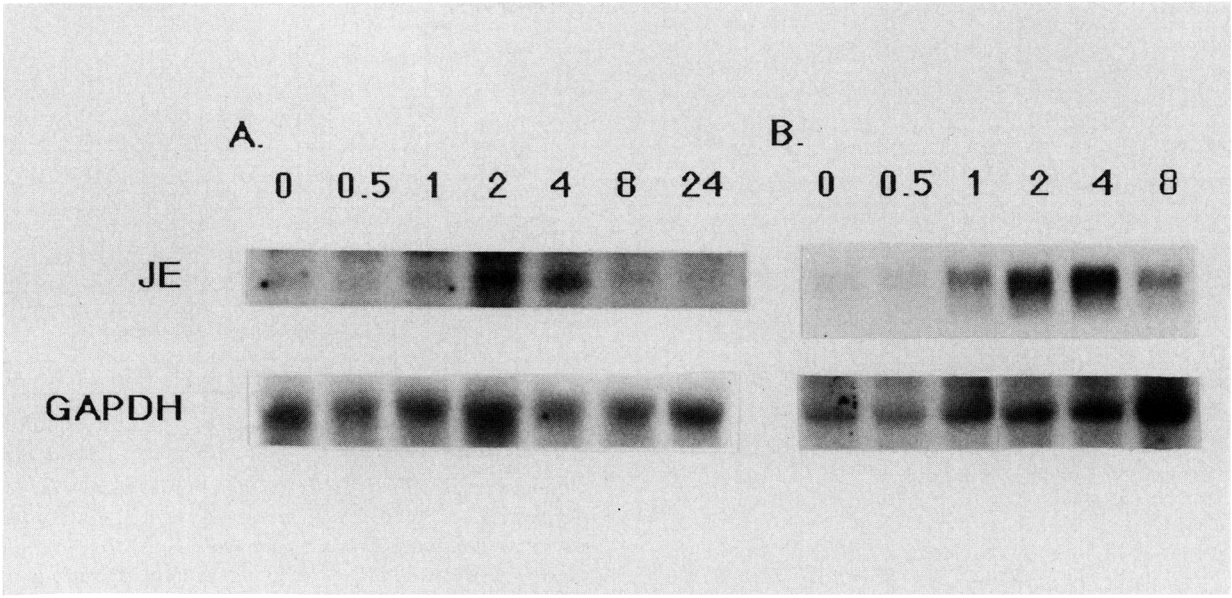

FIGURE 1. *RNA blot analysis of* JE *messenger RNA in intact aorta. Balloon injury was induced in aortas of adult New Zealand White rabbits.*[26] *Rabbits were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). A 4F balloon embolectomy catheter was introduced via the femoral artery and advanced to the level of the aortic valve. The balloon was then inflated and the catheter withdrawn along the full length of the aorta three times. After this procedure, animals were killed at the indicated time points, and the aortas were removed, cleaned of adventitia, and immediately placed in liquid nitrogen. Total RNA was extracted, poly (A)$^+$ RNA was isolated, and 4-μg aliquots were loaded on each lane of a 1% agarose gel. After electrophoresis, RNA was transferred to nitrocellulose and hybridized to [^{32}P]-labeled DNA as previously described.*[41] *The insert from pcJE-1*[43] *was labeled by random oligomer priming. As a control, filters were also hybridized with cDNA encoding rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Final washes for all blots were in 0.1×SSC (1×SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7) and 0.1% sodium dodecyl sulfate at 65°C.* Panels A *and* B *represent two experiments involving separate groups of rabbits. From Reference 41; reproduced with permission.*

and remaining elevated for as much as 9 hours. The differences in the time course of *JE* and *KC* mRNA accumulation depicted in Figure 4 suggest that the molecular mechanisms regulating *JE* and *KC* mRNA levels in culture may be distinct. This has been confirmed (see below) by studying the response of these genes to a variety of mediators of vessel injury.

Effects of PDGF, Angiotensin II, and α-Thrombin on JE *and* KC *mRNA Expression*

In addition to PDGF, vasoconstrictor agonists such as angiotensin II (Ang II) and the clotting factor α-thrombin have been implicated in the vascular response to injury. Both agents induce hypertrophy of cultured adult rat aortic VSMC[47–49] and activate many of the

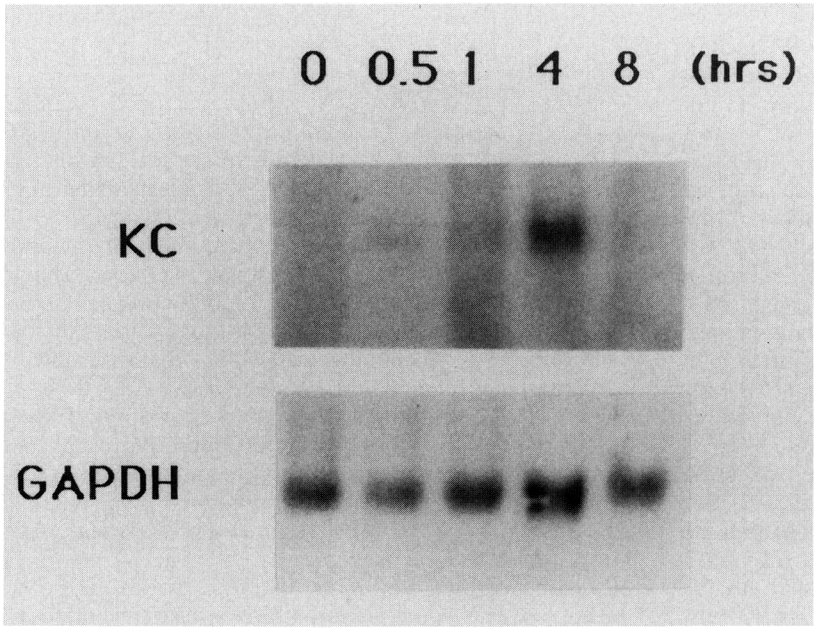

FIGURE 2. *RNA blot analysis of* KC *messenger RNA in intact aorta. Lanes contain 4 μg of poly (A)$^+$ RNA from normal rabbit aorta (time 0) or from aorta harvested at various time points (hours) after balloon injury. The insert from pKC*[38] *was labeled by random oligomer priming. Blots were hybridized to* KC *complementary DNA (cDNA), stripped, and then rehybridized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The RNA blot shown is representative of three complete time courses, each generated from different animals.*

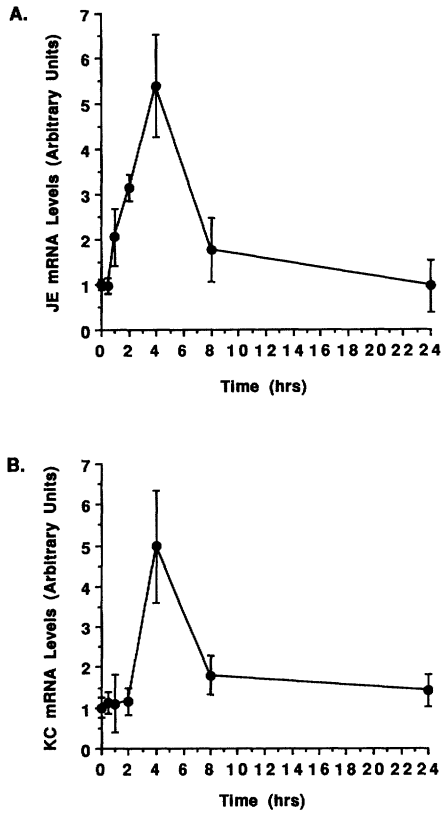

FIGURE 3. *Graphs showing time course of* JE *and* KC *messenger RNA (mRNA) induction in balloon-injured rabbit aorta. Graph depicts relative levels (normalized to glyceraldehyde-3-phosphate dehydrogenase) of* JE (panel A) *and* KC (panel B) *mRNA as determined by densitometric analysis of autoradiograms. To quantify levels of* JE *and* KC *mRNA, autoradiograms were scanned in two dimensions with a Xerox Datacopy GS scanner. Densitometry using 256 gray scales was performed on an Apple Macintosh IIcx computer using IMAGE 1.36 software (Wayne Rasband, National Institutes of Health, Bethesda, Md.). Each time point represents the results from three rabbits; error bars indicate standard deviations.*

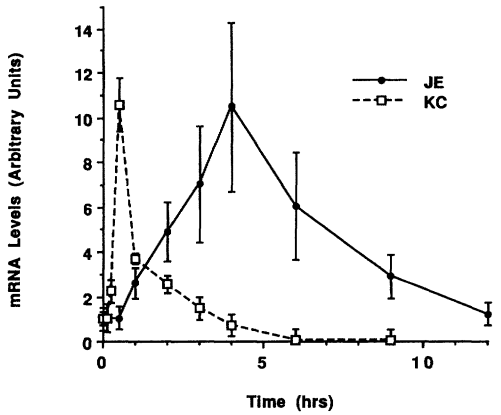

FIGURE 4. *Graph showing time course of* JE *and* KC *messenger RNA (mRNA) induction in cultured vascular smooth muscle cells (VSMCs). Graph depicts relative levels of* JE *and* KC *mRNA in VSMC culture. Quiescent VSMCs (time 0) were stimulated by treatment with Dulbecco's modified Eagle's medium (DME) +10% calf serum. At various time points, RNA was harvested and blots containing 10 μg of total RNA per lane were hybridized to* JE *and* KC *complementary DNA as described in Figure 1.* JE *and* KC *mRNA levels were determined by two-dimensional densitometry of scanned autoradiograms. Levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA and are expressed relative to quiescent VSMCs (time 0=1); error bars indicate standard deviations. VSMCs were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic dissociation.[46] To produce quiescence, cells were incubated in DME with 0.4% calf serum for 48 hours. Under these conditions, incorporation of [^{3}H] thymidine into DNA is <15% of that seen with 10% serum.[41]*

transmembrane signals stimulated by PDGF,[50,51] including protein kinase C, intracellular Ca^{2+} mobilization, and Na^+–H^+ exchange. α-Thrombin stimulates monocyte chemotaxis[52] and enhances adherence of neutrophils to vascular endothelium.[53] Ang II has been reported to induce VSMC proliferation in injured vessels.[54] In addition, angiotensin converting enzyme inhibitors have been found to suppress neointimal formation after experimental balloon injury,[55] although this observation has not been seen by all investigators.[56]

As shown in Figure 5A, PDGF BB (c-*sis* homodimer; Amgen) markedly induced both *JE* and *KC* mRNA in cultured VSMC. In contrast, neither Ang II nor α-thrombin induced *JE* mRNA. The inability of Ang II and α-thrombin to induce *JE* was demonstrated over a full 12-hour time course.[41] In contrast to their effect on *JE,* both agents induced *KC* to a degree similar to that seen with PDGF. Epidermal growth factor also induces *KC* but not *JE* in the VSMC. Thus, unlike *KC* and a number of other early growth-related genes such as c-*fos* and c-*myc, JE* mRNA induction appears to be PDGF-specific in vascular smooth muscle. It should be noted that for the experiments depicted in Figure 5, *KC* mRNA levels were measured in cells harvested at 1 hour after exposure to PDGF; levels of *JE* mRNA were measured in cells harvested at 3 hours. These times correspond to the maximal effect of PDGF on *KC* and *JE* mRNA expression, respectively, in cultured VSMCs.

Signal Transduction of PDGF-Induced KC *and* JE *Expression*

PDGF activates phospholipase C, an enzyme that cleaves the membrane-bound phosphatidylinositol bisphosphate to generate inositol trisphosphate and diacylglycerol. Inositol trisphosphate and diacylglycerol increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and activate protein kinase C, respectively. To test whether protein kinase C played a role in the induction of *JE* and *KC* mRNA, VSMCs were pretreated for 24 hours with 1 μM phorbol 12,13-dibutyrate. This pretreatment downregulates cytosolic protein kinase C activity by 80–90% when measured by phosphorylation of histone III-S.[51] Downregulation of protein kinase C did not prevent the induction of *JE*[41] but did abolish the induction of *KC* by PDGF (Figure 5B). The accumulation of *KC* mRNA after stimulation by Ang II or α-thrombin was also dependent on protein kinase C.

To measure *KC* and *JE* induction in the absence of a change in $[Ca^{2+}]_i$, quiescent VSMCs were incubated in

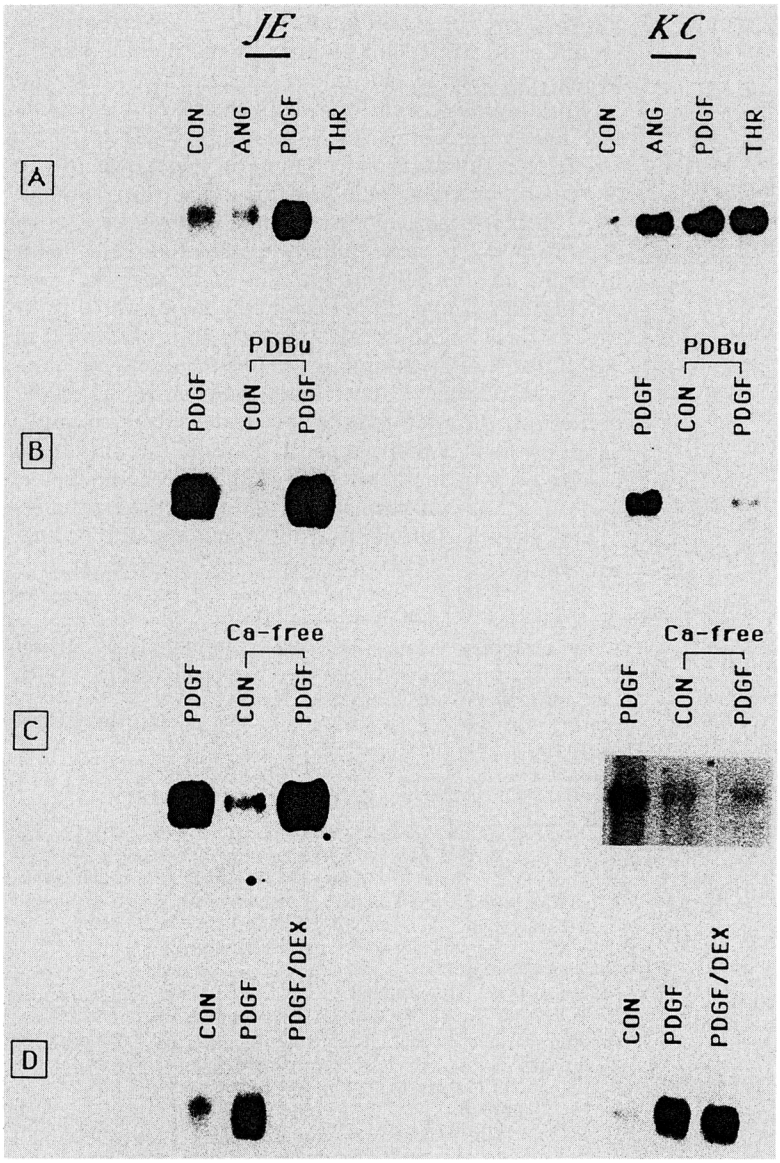

FIGURE 5. *RNA blot analyses of* JE *and* KC *messenger RNA (mRNA) in rat aortic vascular smooth muscle cells (VSMCs). Composite figure derived from eight different experiments (in part in References 41 and 45) representing the differences between* JE *and* KC *mRNA expression in VSMCs. To measure* JE *mRNA levels, cells were harvested at 3 hours after treatment with PDGF or other agonists; in experiments designed to measure* KC *mRNA, cells were harvested at 1 hour.* Panel A: *Specificity of induction: Quiescent VSMCs untreated (CON) or treated with Ang II (10^{-6} M; ANG), platelet-derived growth factor (*JE, *20 and* KC, *5 ng/ml c-*sis *homodimer; Amgen; PDGF), or α-thrombin (39 nM; THR).* Panel B: *Role of protein kinase C: Lane 1: Quiescent VSMCs treated with PDGF. Lanes 2 and 3: Duplicate cultures pretreated for 24 hours with 1 μM phorbol 12,13-dibutyrate (PDBu). This pretreatment decreases cytosolic protein kinase C activity by 80–90%.*[51] *After PDBu pretreatment, VSMCs were then left untreated (lane 2) or treated with PDGF (lane 3).* Panel C: *Role of intracellular Ca^{2+} mobilization: Lane 1: Quiescent VSMCs treated with PDGF. Lanes 2 and 3: Duplicate cultures incubated under Ca^{2+}-free conditions and either left untreated (lane 2) or treated with PDGF (lane 3). To produce a Ca^{2+}-free environment, VSMCs were washed twice in 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. Agonists were then added directly to some cultures and incubated at 37°C in room air. Under these conditions, agonist-mediated Ca^{2+} mobilization is completely blocked.*[51] Panel D: *Glucocorticoid effect: Quiescent VSMCs (CON) were treated with PDGF (PDGF) alone or in combination with 1 μM dexamethasone (PDGF/DEX). All lanes depicted in this figure contain equal amounts of RNA as determined by hybridization with glyceraldehyde-3-phosphate dehydrogenase.*

a Ca^{2+}-free buffer containing the intracellular and extracellular Ca^{2+} chelators Quin 2/AM and EGTA. This treatment has been shown to completely inhibit the rise in $[Ca^{2+}]_i$ induced by vasoactive agonists and growth factors in VSMCs[50] and blocks the induction of c-*fos* by Ang II.[51] As shown in Figure 5C, inhibition of intracellular Ca^{2+} mobilization had no effect on *JE* mRNA induction by PDGF.[41] In contrast, PDGF induction of *KC* mRNA was completely inhibited. The accumulation of *KC* mRNA after stimulation by Ang II or α-thrombin was also dependent on intracellular Ca^{2+} mobilization.

Effects of Corticosteroids on PDGF-Induced Gene Expression

Corticosteroids are known to inhibit VSMC growth in culture.[57,58] In addition, they are potent inhibitors of the inflammatory response.[59,60] In particular, glucocorticoids decrease the number of monocytes in blood and inhibit the accumulation of monocytes and macrophages at sites of inflammation and injury. Because of their known effects on VSMCs and inflammation, the effects of corticosteroids on the induction of *JE* and *KC* in VSMCs were examined. Pretreatment of quiescent VSMCs in culture with dexamethasone completely abolished PDGF-induced *JE* mRNA expression (Figure 5D). This effect was seen with a variety of glucocorticoids, such as hydrocortisone (not shown). Pretreatment of cells with testosterone and progesterone had no effect, suggesting that glucocorticoid activity was responsible for the observed inhibition of *JE* mRNA induction. In keeping with other differences between the two genes, PDGF-induced *KC* mRNA

induction was not inhibited by corticosteroids. We have recently found that, in addition to their induction in balloon injury, *JE* and *KC* mRNA are also induced in the kidney after renal ischemia.[61] Corticosteroids inhibit the induction of *JE* after renal ischemia but have no effect on *KC*.

Because *JE* encodes a secretory glycoprotein that induces monocyte chemotaxis, the inhibition of *JE* mRNA accumulation may be an important mechanism through which glucocorticoids suppress inflammation. In this regard, the lack of inhibition of *KC* mRNA induction suggests that there may be both glucocorticoid-sensitive and -insensitive pathways involved in inflammation. This may have important consequences for designing treatment strategies to inhibit the inflammatory response to vascular injury.

Discussion

One of the earliest molecular genetic events associated with PDGF treatment of VSMCs is the induction of *KC* and *JE*, genes encoding leukocyte chemoattractants. The induction of these chemoattractant genes is complex and appears to involve several distinct transmembrane pathways. In the case of *KC*, the mechanism of induction is typical of that seen for proto-oncogenes, such as c-*fos*, c-*myc*, and c-*jun*, and involves activation of phospholipase C and the subsequent stimulation of protein kinase C and intracellular Ca^{2+} mobilization. In the case of *JE*, the pathway remains to be determined. Given the narrow range of agents involved in inducing *JE* in vascular smooth muscle, this pathway appears to be PDGF-specific and not shared by Ang II, α-thrombin, or epidermal growth factor.[41] The differences in specificity of *JE* and *KC* induction in VSMCs may be important vis-à-vis the known functions of their protein products. Thus, the recruitment of neutrophils may be a generalized phenomenon, which can be triggered by a broad range of agents, such as growth factors, clotting factors, and vasoactive agonists, in part through the induction of *KC*. In contrast, *JE*-mediated recruitment of monocytes may take place under a more specific set of circumstances and thus be regulated by a smaller group of agents.

Balloon injury appears to represent at least one circumstance under which *JE* and *KC* are both induced. The time course of induction is similar for both *JE* and *KC* and in general parallels that seen in cell culture in response to PDGF or serum. The accumulation of both mRNAs (particularly *KC*) after balloon injury does occur later than that seen in VSMC culture. This may reflect the need to accumulate significant amounts of agonists such as PDGF at the site of injury. On the other hand, it may imply that the induction of these genes occurs via a mechanism distinct from that seen in VSMC culture.

As noted above, additional work with in situ hybridization of arterial tissue sections will be necessary to firmly establish the VSMC as the source of *JE* and *KC* mRNA accumulation seen in response to balloon injury. Our findings in cell culture, however, together with light microscopy showing the VSMC to be the predominant cell type present at the time of RNA isolation from injured vessels, implicate the VSMC as the most likely source. Thus, the induction of *JE* and *KC* would involve the VSMC in the early response to vessel injury. The VSMC has long been thought to play a critical role in restenosis by proliferating and migrating from the vessel media to the intima. This intimal hyperplasia results in narrowing of the vessel lumen, which is exacerbated by secretion of extracellular matrix by the intimal VSMC. More recently, it has been demonstrated that the VSMC may play an earlier role in the cascade of events leading to restenosis by producing PDGF.[15] Thus, VSMCs may be involved in stimulating their own growth and migration via an autocrine loop. The work summarized in this article supports the role of the VSMC in an early response to vessel injury, namely, the recruitment of inflammatory cells to the site of injury. We have recently found that tissue factor, the major activator of the extrinsic clotting pathway,[62] is also induced in VSMCs within 20 minutes of exposure to PDGF, Ang II, or α-thrombin.[63] Thrombus formation is one of the earliest events after vessel injury[64] and has been implicated in the short-term[65] and long-term[5] complications associated with angioplasty. Thus, the VSMC may act as a pluripotent cell involved in all phases of vascular injury, including thrombosis, inflammation, and intimal hyperplasia.

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