Early Growth Response Factor-1 Induction by Injury Is Triggered by Release and Paracrine Activation by Fibroblast Growth Factor-2

Fernando S. Santiago, Harry C. Lowe,
Fiona L. Day, Colin N. Chesterman, and
Levon M. Khachigian

From the Centre for Thrombosis and Vascular Research, School of Pathology, The University of New South Wales, and Department of Haematology, Prince of Wales Hospital, Sydney, Australia

Cell migration and proliferation that follows injury to the artery wall is preceded by signaling and transcriptional events that converge at the promoters of multiple genes whose products can influence formation of the neointima. Transcription factors, such as early growth response factor-1 (Egr-1), with nucleotide recognition elements in the promoters of many pathophysiologically relevant genes, are expressed at the endothelial wound edge within minutes of injury. The mechanisms underlying the inducible expression of Egr-1 in this setting are not clear. Understanding this process would provide important mechanistic insights into the earliest events in the response to injury. In this report, we demonstrate that fibroblast growth factor-2 (FGF-2) is released by injury and that antibodies to FGF-2 almost completely abrogate the activation and nuclear accumulation of Egr-1. FGF-2-inducible *egr-1*-promoter-dependent expression is blocked by PD98059, a specific inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)-1/2 (MEK-1/2), as well as by dominant negative mutants of ERK-1/2. Inducible ERK phosphorylation after injury is dependent on release and stimulation by endogenous FGF-2. Antisense oligonucleotides directed at *egr-1* mRNA suggest that Egr-1 plays a necessary role in endothelial repair after denudation of the monolayer. These findings demonstrate that inducible Egr-1 expression after injury is contingent on the release and paracrine action of FGF-2. (*Am J Pathol* 1999, 154:937-944)

Endothelial cells provide the semipermeable blood tissue interface that lines the entire cardiovascular network. The integrity of endothelium is fundamental for vascular homeostasis and nonthrombogenicity. Endothelial dysfunction or injury can initiate cellular changes and lead to the development of lesions.^{1,2} During routine percutaneous

transluminal coronary angioplasty (PTCA), the endothelium may be damaged at sites distant from the atherosclerotic plaque as the catheter is manipulated into position.^{3,4} This can generate fibrocellular lesions at sites previously angiographically free of disease.^{3,5,6} The role of endothelium in the response to mechanical injury has not received great attention to date; most investigations have focused mainly on smooth muscle cells within the media.

Deliberate denudation of endothelium in rat arteries without traumatizing underlying smooth muscle cells triggers chemotaxis and proliferation at the site of injury.⁷ The first smooth muscle cells to migrate from the media to the intima in this model do so preferentially at the endothelial wound edge,⁸ suggesting that factors produced by injured endothelium can influence this process. Factors such as fibroblast growth factor-2 (FGF-2) are basally expressed in endothelial cells and smooth muscle cells in the artery wall. The inappropriate release of FGF-2 and/or other endogenous factors during catheterization or other forms of injury may initiate molecular events that lead to lesion formation.

The promoters of many genes whose products can influence cell movement and replication in the vessel wall, such as *FGF-2*,⁹ *PDGF-A*,⁹⁻¹¹ *PDGF-B*,^{10,12} and *TGF-β₁*,^{9,13} bear nucleotide recognition elements for the zinc finger transcription factor, early growth response factor-1 (Egr-1).¹⁴⁻¹⁷ Egr-1 (also known as TIS8, *krox-24*, and *NGFI-A*)¹⁸ is an immediate-early gene product that binds preferentially to GC-rich motifs in DNA.^{19,20} Egr-1 is rapidly induced at the endothelial wound edge before the increased expression of growth factors at the same location.¹⁴ Here, we define signaling pathways triggered by endothelial injury and growth factor release that lead to the paracrine activation of this pleiotropic transactivator.

Supported by grants from the National Health and Medical Research Council of Australia (NHMRC) and National Heart Foundation of Australia. H.C. Lowe is a recipient of a Medical Postgraduate Research Scholarship from the NHMRC. L.M. Khachigian is holder of an R. Douglas Wright Fellowship from the NHMRC.

Accepted for publication November 18, 1998.

Address reprint requests to Dr. Levon M. Khachigian, Centre for Thrombosis and Vascular Research, School of Pathology, The University of New South Wales, Sydney, NSW 2052, Australia. E-mail: l.khachigian@unsw.edu.au.

Materials and Methods

p44/42 ERK1/2 Activity

Bovine aortic endothelial cells (Cell Applications) were grown in Dulbecco's modified Eagles' medium (DMEM; Life Technologies), pH 7.4, containing 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. All cells in the experiments were used between passages 1 and 7. Cells were rendered quiescent by incubation in DMEM, pH 7.4, containing 0.25% plasma-derived serum (PDS) for 24 hours. The cells were injured by scraping with a sterile stainless steel comb or exposed to the agonists indicated. Lysates were prepared by the addition of 10 mmol/L Tris/HCl, 150 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin, pH 7.4, at 4°C, and protein concentrations were assessed using the Biorad protein assay. ERK1/2 activity was assessed using the p44/p42 MAP kinase system (Amersham) in accordance with manufacturer's instructions.

Western Blot Analysis

Lysates were resolved by electrophoresis on denaturing 10% SDS-polyacrylamide gels for 2 hours at 100 V. Proteins were transferred to nylon membranes, nonspecific binding sites were blocked with nonfat skim milk, and the membranes were incubated with polyclonal antibodies raised against Egr-1 (1:250; Santa Cruz) or monoclonal antipeptide antibodies recognizing phosphorylated forms of JNK-1 and JNK-2 (1:250; Santa Cruz) before chemiluminescent detection (NEN-DuPont). Where indicated, the Egr-1 blot was stripped and reprobed with antibodies to Sp1 (1:250; Santa Cruz).

FGF-2 ELISA

FGF-2 release into the supernatant was assessed using a commercial ELISA specific for this growth factor (R&D Systems). This kit does not, according to the manufacturer, recognize FGF-1, FGF-4, FGF-5, FGF-6, keratinocyte growth factor, or β -endothelial cell growth factor. It also fails to recognize interleukin (IL)-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, epidermal growth factor (EGF), granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, HB-EGF, hepatocyte growth factor, insulin-like growth factor (IGF)-I, IGF-II, LIF, β -nerve growth factor, PD-endothelial cell growth factor, transforming growth factor (TGF)- α , TGF- β , tumor necrosis factor (TNF)- α , TNF- β , vascular endothelial growth factor, or platelet-derived growth factor (PDGF)-AB. Values were normalized to the concentration of protein in the supernatant.

Electrophoretic Mobility Shift Analysis

Binding reactions were performed with approximately 10 μ g of nuclear extract²¹ in 20 μ l containing 1 μ g of poly(dI/

dC)-poly(dI/dC) (Sigma), 1 μ g of salmon sperm DNA (Sigma), 10 mmol/L Tris/HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5% glycerol, and the ³²P-labeled *egr*-1SRE (120,000 cpm) for 35 minutes at 22°C. In competition or supershift studies, a 150-fold molar excess of unlabeled oligonucleotide or 2 μ g of affinity-purified rabbit anti-peptide antibody (Santa Cruz) was included in the binding mixture 10 minutes before addition of the probe. Bound complexes were separated from the unbound probe by nondenaturing 6% polyacrylamide gel electrophoresis and 1X Tris-buffered borate/EDTA running buffer at 200 V. Gels were dried and exposed to Hyperfilm-MP (Amersham) overnight at -80°C.

Transient Transfection and Reporter Gene Analysis

Endothelial cells were transiently transfected with 10 μ g of *egr*-1 promoter-chloramphenicol acetyl transferase (CAT) reporter construct using Superfect (Qiagen). FGF-2 (25 ng/ml) (final endotoxin concentration less than 1 pg/ml) was incubated with growth-quiescent cells for 24 hours. CAT activity was assessed as previously described²² and normalized to the concentration of protein in the cell lysates.

Results and Discussion

Mechanical injury to the endothelial lining of the artery wall in animal models initiates altered gene expression followed by migratory and proliferative events that culminate in the formation of lesions.^{7,14,23,24} A large number of genes whose products mediate cell movement and replication after injury are targets of the immediate-early gene product Egr-1.²⁵ Egr-1 is rapidly expressed at the endothelial wound edge within minutes of injury.¹⁴ The mechanisms underlying the rapid activation of this pleiotropic transcription factor after injury have not yet been delineated. This information would shed light on the earliest molecular events triggering the response to injury. In this report, we hypothesized that Egr-1 induction is initiated by the paracrine action of preformed growth-regulatory molecules such as FGF-2.

Paracrine Activation of Egr-1 by FGF-2

FGF-2 lacks a conventional hydrophobic sequence for exocytotic secretion.²⁶ Consequently, it is not efficiently transported outside endothelial cells and is instead localized mainly within the cytoplasm and nucleus *in vitro* and *in vivo*.^{7,27,28} To preempt a role for endogenous FGF-2 in the induction of Egr-1, we loaded endothelial monolayers with ⁵¹Cr before injury by scraping. Maximal release of radiolabel was observed within seconds of the mechanical insult (Figure 1A). To provide direct evidence for the release of FGF-2, we performed a solid-phase ELISA specific for FGF-2 (Figure 1B). FGF-2 antigenic activity was readily detected in the supernatant within 2 minutes of scraping (Figure 1B).

Figure 1. FGF-2 is released after endothelial injury and activates Egr-1. **A:** ^{51}Cr release from preloaded endothelial cells after injury. Confluent endothelial monolayers were incubated with sodium $^{51}\text{chromate}$ (200,000 cpm per 100-mm dish) for 4 hours before injury by scraping. Radiolabel released into the cultured supernatant was assessed by scintillation counter. Values are mean $\pm$ SEM of three independent determinations. $*P < 0.05$ versus control group. **B:** Assessment by solid-phase ELISA of immunoreactive FGF-2 released into the supernatant 2 minutes after injury. FGF-2 levels were normalized to the concentration of protein in the supernatant. Values are mean $\pm$ SEM of two determinations. $*P < 0.05$ versus control group. **C:** Endogenous FGF-2 activates Egr-1. Endothelial monolayers were preincubated with neutralizing rabbit antibodies to FGF-2 (60 $\mu\text{g}/\text{ml}$) or an identical concentration of nonimmune rabbit IgG for 2 hours before scraping. Alternatively, the cells were exposed to PMA (100 ng/ml). Nuclear extracts were prepared after 1.5 hours and assessed for Egr-1 immunoreactivity and binding activity by Western blot analysis. The blot was stripped and reprobed with polyclonal antibodies directed toward Sp1. The data are representative of two independent experiments. **D:** Electrophoretic mobility shift assay using nuclear extracts of endothelial cells 1.5 hours after injury. In supershift studies, the extracts were incubated with 2 μg of antibody before the addition of the probe. Arrows denote the nucleoprotein complexes. S denotes a supershift. The sequence of oligo A is 5'-GGG GGG GGC GGG GGC GGG GGC GGG GGA GGG-3' (sense strand).

To determine whether Egr-1 induction by injury is dependent on endogenous FGF-2, we preincubated monolayers with neutralizing polyclonal antibodies specific for this growth factor. These antibodies virtually abrogated the induction and nuclear accumulation of Egr-1 by injury (Figure 1C). In contrast, an identical concentration of species-

matched IgG had little effect (Figure 1C). Reprobing the blot with antibodies to the related zinc finger transcription factor Sp1²⁹ demonstrated no effect of either injury or the FGF-2 antibody on levels of this nuclear protein (Figure 1C).

An electrophoretic mobility shift analysis (EMSA) was performed using nuclear extracts of injured cells with a

32 P-labeled double-stranded oligonucleotide (32 P-Oligo A) bearing the proximal region (bp -76/-47) of the PDGF-A promoter.³⁰ This region contains overlapping consensus Egr-1 binding sites²² and mediates inducible PDGF-A expression in response to multiple agonists of Egr-1.^{21,22,31} A distinct nucleoprotein complex was observed when extracts from injured, but not uninjured, cells were used in the EMSA (Figure 1D). Formation of this inducible complex was strongly inhibited by incubation of the cells with FGF-2 antibodies, but not with non-specific IgG, before injury (Figure 1D). The complex was completely supershifted with antibodies to Egr-1 (Figure 1D). These data, taken together, indicate that Egr-1 activation, its nuclear translocation, and interaction with the PDGF-A promoter in endothelial cells after injury is contingent on the local release of FGF-2.

SRF Binds *egr-1* Promoter in Endothelial Cells

Inducible Egr-1 transcription is dependent on cooperative interactions between serum response factor (SRF, p67)³² and ternary complex factors (TCFs)³³ at serum response elements (SREs) in the *egr-1* promoter.³⁴⁻⁴⁰ SRF binds as dimers to the central CArG box of the SRE, whereas TCFs (such as Elk-1, SAP-1a, and SAP-2a) are activated by phosphorylation and interact directly with SRFs and/or DNA as monomers or dimers.⁴¹ The *egr-1*SRE located at nucleotides -374/-355 mediates inducible promoter-dependent expression in response to multiple agonists and stresses.^{35,42,43} An EMSA was performed using a 32 P-labeled oligonucleotide bearing the -374/-355 region of the *egr-1* promoter. This resulted in the formation of two distinct nucleoprotein complexes (Figure 2A). A 150-fold molar excess of either unlabeled *egr-1*SRE or an oligonucleotide bearing the *c-fos*SRE that contains recognition elements for Elk-1 and SRF, abrogated formation of these complexes (Figure 2A). In contrast, an irrelevant oligonucleotide, P-mSSRE, had no inhibitory effect (Figure 2A). Both complexes were supershifted (complex S) by antibodies recognizing SRF (Figure 2A), whereas neither complex was affected by antibodies directed against transcription factors PEA-3 (Figure 2A), Egr-1 (Figure 2A), WT-1, or AP2 (data not shown). These findings demonstrate hitherto unreported SRF binding activity in endothelial cells. Surprisingly, positive supershifts were not observed using antibodies directed toward native or phosphorylated Elk-1 (at Ser-383), either before or after exposure to FGF-2 (Figure 2A and data not shown).

FGF-2 Activation of *egr-1* Promoter Is MEK and ERK Dependent

Although the precise TCF(s) mediating inducible *egr-1* transcription is not known, all members of this Ets family of *trans*-acting factors are activated by carboxyl-terminal phosphorylation by extracellular-signal-regulated kinase (ERK), the downstream target of MEK. Transient transfection analysis in endothelial cells using a CAT reporter vector driven by a fragment of the *egr-1* promoter bearing

Figure 2. The *egr-1* promoter is bound by SRF and activated by FGF-2. **A:** Interaction of nuclear proteins with the *egr-1*SRE. Endothelial nuclear extracts were incubated with a 32 P-labeled double-stranded oligonucleotide bearing the -374/-355 SRE of the *egr-1* promoter (5'-AGG ATC CCC CGC CGG AAC CCT TAT TTG GGC AG-3', sense strand). In competition studies, 150-fold molar excess of the unlabeled oligonucleotide was incubated with the extracts for 10 minutes before addition of the radiolabeled probe. In supershift studies, the extracts were incubated with 2 μ g of antibody before the addition of the probe. **Arrows** denote the nucleoprotein complexes. S denotes a supershift. Sequences of P-mSSRE and *c-fos*SRE are 5'-CTC GGC TCT ACA CTG TAG CAT AAG CGC C-3' and 5'-CTA CCG CCA ACC GGA ATA GTC CAT ATA AGG ACT C-3' (sense strands), respectively. **B:** FGF-2-inducible *egr-1*-promoter-dependent expression is MEK and ERK dependent. Subconfluent endothelial cells were transfected with 10 μ g of a CAT reporter plasmid driven by a fragment of the *egr-1* promoter. The cells were exposed to 25 ng/ml FGF-2 for 24 hours before assessment of CAT activity in the lysates. PD98059 (20 μ M) and wortmannin (1 μ M) were added 1 hour before the addition of FGF-2. The cells were co-transfected with 3 μ g of dominant negative mutants of ERK1/2 (1.5 μ g of DN-ERK1 and 1.5 μ g of DN-ERK2 in pcDNA3) or the empty expression vector. Values are mean $\pm$ SEM of two independent determinations. * P < 0.05 versus control group.

the -374/-355 SRE and four additional SREs downstream¹⁸ determined that *egr-1*-promoter-dependent CAT activity was induced on exposure to FGF-2 (Figure 2B). PD98059, a flavone that binds inactive MEK and prevents phosphorylation by Raf without affecting other

known serine/threonine or tyrosine kinases⁴⁴ abrogated FGF-2 activation of the *egr-1* promoter. FGF-2-inducible *egr-1*-promoter-dependent expression was also abolished by the phosphatidylinositol 3-kinase inhibitor, wortmannin (Figure 2B). Inhibition by PD98059 and wortmannin was dose dependent and maximal at 20 μ mol/L and 1 μ mol/L, respectively (Figure 2B and data not shown).

Co-transfection experiments further linked ERK signaling with the *egr-1* promoter in endothelial cells. CAT activity inducible by FGF-2 was blocked by overexpression of dominant negative mutants of ERK-1/2 (Figure 2B). Overexpression of the vector alone was without effect (Figure 2B). Cells transfected with the backbone CAT plasmid or a PDGF-A promoter-CAT reporter construct, f36, which bears 55 bp of PDGF-A promoter sequence and has its 5' endpoint located 3' to the Egr-1 binding site,²² showed no increase in CAT activity in cells incubated with FGF-2 (data not shown). Moreover, neither co-expression of the ERK-1/2 dominant negative construct nor exposure to inhibitors had any effect on reporter activity (data not shown).

Endothelial Injury Activates ERK

To provide direct evidence for the activation of ERK as a consequence of endothelial injury, we performed a [γ ³²P]ATP incorporation assay using a peptide substrate specific for this mitogen-activated protein kinase. ERK-1/2 enzymatic activity increased fivefold within 2 minutes of injury (Figure 3). Recombinant FGF-2 also stimulated ERK-1/2, whereas TNF- α had no measurable effect (Figure 3). Monolayers were incubated with FGF-2 antibodies before injury to determine whether ERK-1/2 activity after scraping was dependent on cellular FGF-2. These antibodies suppressed ERK-1/2 activity 8 minutes after injury (Figure 3). Thus, ERK and Egr-1 activation after injury is mediated in part by the release and paracrine action of endogenous FGF-2.

Endothelial Injury Activates c-Jun Amino-Terminal Kinase/Stress-Activated Protein Kinase-1

TCFs can also be activated by members of the JNK family of mitogen-activated protein kinases.^{45–48} Wortmannin has been reported to inhibit JNK activity without affecting ERK.^{49–52} As wortmannin blocked FGF-2 activation of the *egr-1* promoter (Figure 2B), we determined whether JNK was itself phosphorylated by injury. Using monoclonal antibodies recognizing phospho-Thr-183 and Tyr-185 in JNK-1 and JNK-2, we observed time-dependent JNK-1 activation by injury but could not detect phosphorylated JNK-2 (Figure 4). JNK-1 phosphorylation after 8 minutes was inhibited by preincubation of the monolayers with FGF-2 antibodies (Figure 4). Recombinant FGF-2, like injury, strongly stimulated JNK-1 phosphorylation (Figure 4), consistent with JNK activation via Ras.⁵³ These findings demonstrate the activation of a

Figure 3. ERK1/2 enzymatic activity induced by injury is FGF-2 dependent. Lysates of endothelial monolayers injured by scraping were assessed for p44/p42 ERK catalytic activity. Rabbit antibodies to FGF-2 (60 μ g/ml) were incubated with the monolayers 2 hours before scraping. Alternatively, FGF-2 (25 ng/ml), PMA (100 ng/ml), or TNF- α (200 U/ml) were incubated with uninjured cells for 4 minutes before cell lysis. Values are mean $\pm$ SEM of three independent determinations. * P < 0.05 compared with control group.

second signaling pathway dependent on FGF-2 after endothelial injury.

Endothelial Repair after Injury Is Egr-1 Dependent

Denudation of an endothelial monolayer leads to outgrowth from the wound edge and recoverage of the denuded zone. To determine whether Egr-1 plays a critical role in endothelial repair, monolayers were incubated with an antisense oligonucleotide targeting a specific 15-base sequence unique to Egr-1 mRNA before injury. This oligomer, E11, was synthesized with phosphorothioate rather than phosphodiester linkages to increase resistance to possible exonucleolytic cleavage^{54,55} and does not bear the guanine quartet motif that can reportedly interfere with biological processes by nonspecific means.^{56–59} E11 inhibited the induction of Egr-1 by serum at a concentration of 1 μ mol/L (Figure 5A). Moreover, E11 blocked outgrowth from the wound edge 72 hours after injury (Figure 5B). In contrast, an identical amount of E11C, a size-matched, base-scrambled counterpart of E11, had no effect either on the induction of Egr-1 (Figure 5A) or regrowth after injury (Figure 5B). Trypan blue exclusion experiments revealed that E11 inhibition was not the result of toxicity (data not shown). E11 inhibition was reversible, as withdrawal of E11 from the medium 72

Figure 4. Phosphorylation of JNK1 on endothelial injury. Lysates of endothelial monolayers injured by scraping were assessed for JNK1/2 phosphorylation by Western blot analysis. Alternatively, FGF-2 (10 ng/ml), PMA (100 ng/ml), or TNF- α (200 U/ml) was incubated with uninjured cells for 8 minutes before lysis. The FGF-2 antibody was incubated with the monolayers for 2 hours before injury. Values are mean $\pm$ SEM of two independent determinations. * $P < 0.05$ versus control group. The JNK antibody recognizes phosphorylated Thr-183 and Tyr-185 at the carboxy terminus of JNK1 and the corresponding JNK2 sequence. p-JNK denotes the phosphorylated form of JNK1.

hours after injury resulted in resumption of repair and recovery of the denuded zone (E11F, Figure 5B).

Incomplete inhibition by the Egr-1 oligomer suggests a necessary, but not sufficient, role for this transcription factor in endothelial repair after mechanical injury. Previous studies using dominant negative Egr-1 expression vectors in human tumor cell lines and fibroblasts have implicated a role for Egr-1 in x-irradiation-induced $G_1 \rightarrow S$ transition.⁶⁰ Additionally, antisense oligonucleotides directed at Egr-1 have been found to attenuate rat glomerular mesangial cell proliferation⁶¹ and inhibit excitatory synaptic transmission in rat hippocampi.⁶² The present findings extend these observations by providing the first direct link between Egr-1 and wound healing.

Parallels with Vascular Injury in Vivo

Although mechanical injury of arterial endothelium can lead to local neointima formation in animal models,^{7,8,63}

Figure 5. Reparative response of endothelial cells to injury is Egr-1 dependent. **A:** Inhibition of Egr-1 induction by antisense Egr-1 oligonucleotide, E11.⁷¹ Growth-arrested endothelial cells were incubated with 1 μ mol/L phosphorothioate-protected E11 (5'-ACA CTT TTG TCT GCT-3') or its scrambled counterpart, E11C (5'-TTC TTG CAT CTG TCA-3') for 18 hours before and again on addition of 5% fetal calf serum. Cell extracts were prepared 1 hour subsequently and analyzed by Western blot analysis using antibodies to Egr-1. **B:** Recovery of denuded zone after injury is inhibited by E11. Confluent, growth-arrested endothelial cells were denuded by stroking with a sterile toothpick. Oligonucleotides were added 18 hours before injury and again at injury. Seventy-two hours after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde, and stained with H&E. Alternatively, fresh medium without E11 was added to a subset of wells and harvested after another 72 hours (E11F). The area occupied by cells in the denuded zone was calculated in a blind manner. Values are mean $\pm$ SEM of two independent determinations. * $P < 0.05$ compared with control group.

this process is less well characterized in humans. The frequency of catheter-induced lesions during coronary angiography or balloon angioplasty is rare,^{5,6,64} but its very occurrence suggests an important role for endothelium in the response to injury. Catheterization can denude endothelial cells from the blood vessel wall without disrupting the internal elastic lamina or underlying media⁶⁵ and produce fibrocellular lesions.⁵ Lesion formation at sites of endothelial denudation have been described in dogs undergoing routine catheterization of the LMCA.⁶⁶ As endothelial injury can trigger paracrine signaling and transcriptional activation by FGF-2 (present study), catheter-induced lesions at sites independent of pre-existing lesions may be a consequence of growth factor release from the vessel wall.

Studies using the rat carotid model implicate an early role for endogenous FGF-2. For example, proliferation is blocked if FGF-2 antibodies are administered at the time of injury⁶⁷ but not after 4 days.⁶⁸ Whether neointima formation in response to mechanical injury is compromised in *egr-1*⁶⁹ or *FGF-2*⁷⁰ knockout mice is presently not known. An attenuated response to injury in these mice would complement the present findings using oligonucleotide inhibitors of Egr-1 (Figure 5B). That inducible ERK-1/2 activity after balloon injury to the rat carotid is FGF-2 dependent and is blocked by the presence of PD98059⁷¹ adds weight to this possibility. Our inability to completely inhibit Egr-1 activation by targeting FGF-2 (Figure 1, C and D) nonetheless suggests that factors other than FGF-2 *per se* are involved. Elucidation of specific cell surface events that trigger the induction of these regulatory factors after injury will provide additional insights on the earliest molecular events underlying cellular changes to the vessel wall.

Acknowledgments

We are indebted to Dr. Kathleen M. Sakamoto (UCLA School of Medicine) for her generous gift of *egr-1* promoter-CAT construct, -480-CAT, and Dr. Melanie H. Cobb (Southwestern Medical Center) for dominant-negative ERK-1/2 plasmids.

References

- Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993, 362:801–809
- Gorlin R: Treatment of chronic stable angina pectoris. *Am J Cardiol* 1992, 70:26G–31G
- Mishkel GJ, Marquis JF: Restenosis and accelerated left main coronary artery disease presenting six months after successful percutaneous transluminal coronary angioplasty. *Can J Cardiol* 1989, 5:187–190
- Wayne VS, Harper RW, Pitt A: Left main coronary artery stenosis after percutaneous transluminal coronary angioplasty. *Am J Cardiol* 1988, 61:459–460
- Waller BF, Pinkerton CA, Foster LN: Morphologic evidence of accelerated left main coronary artery stenosis: a late complication of percutaneous transluminal balloon angioplasty of the proximal left anterior descending coronary artery. *J Am Coll Cardiol* 1987, 9:1019–1023
- Hamad N, Pichard A, Oboler A, Lindsay J: Left main coronary artery stenosis as a late complication of percutaneous transluminal coronary angioplasty. *Am J Cardiol* 1987, 60:1183–1184
- Lindner V, Reidy MA, Fingerle J: Regrowth of arterial endothelium: denudation with minimal trauma leads to complete endothelial cell regrowth. *Lab Invest* 1989, 61:556–563
- Lindner V: Expression of platelet-derived growth factor ligands and receptors by rat aortic endothelium in vivo. *Pathobiology* 1995, 63:257–264
- Miano JM, Vlasic N, Tota RR, Stemerman MB: Smooth muscle cell immediate-early gene and growth factor activation follows vascular injury: a putative mechanism for autocrine growth. *Arterioscler Thromb* 1993, 13:211–219
- Lindner V, Reidy MA: Platelet-derived growth factor ligand and receptor expression by large vessel endothelium. *Am J Pathol* 1995, 146:1488–1497
- Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM: PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol* 1990, 111:2149–2158
- Lindner V, Giachelli CM, Schwartz SM, Reidy MA: A subpopulation of smooth muscle cells in injured rat arteries expresses platelet-derived growth factor-B chain mRNA. *Circ Res* 1995, 76:951–957
- Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA: Production of transforming growth factor- β 1 during repair of arterial injury. *J Clin Invest* 1991, 88:904–910
- Khachigian LM, Lindner V, Williams AJ, Collins T: Egr-1-induced endothelial gene expression: a common theme in vascular injury. *Science* 1996, 271:1427–1431
- Biesiada E, Razandi M, Levin ER: Egr-1 activates basic fibroblast growth factor transcription. *J Biol Chem* 1996, 271:18576–18581
- Hu R-M, Levin ER: Astrocyte growth factor is regulated by neuropeptides through Tis 8 and basic fibroblast growth factor. *J Clin Invest* 1994, 93:1820–1827
- Sukhatme VP, Cao X, Chang LL, Tsai-Morris C-H, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T: A zinc-finger encoding gene coregulated with c-Fos during growth and differentiation and after depolarization. *Cell* 1988, 53:37–43
- Gashier A, Sukhatme V: Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Prog Nucleic Acids Res* 1995, 50:191–224
- Nakagama H, Heinrich G, Pelletier J, Housman DE: Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. *Mol Cell Biol* 1995, 15:1489–1498
- Swirnoff AH, Milbrandt J: NGFI-A and related zinc-finger transcription factors. *Mol Cell Biol* 1995, 15:2275–2287
- Khachigian LM, Anderson KA, Halnon NJ, Resnick N, Gimbrone MA Jr, Collins T: Egr-1 is activated in endothelial cells exposed to fluid shear stress and interacts with a novel shear-stress response element in the PDGF A-chain promoter. *Arterioscler Thromb Vasc Biol* 1997, 17:2280–2286
- Khachigian LM, Williams AJ, Collins T: Interplay of Sp1 and Egr-1 in the proximal PDGF-A promoter in cultured vascular endothelial cells. *J Biol Chem* 1995, 270:27679–27686
- Schwartz SM: Smooth muscle migration in atherosclerosis and restenosis. *J Clin Invest* 1997, 99:2814–2817
- Silverman ES, Khachigian LM, Lindner V, Williams AJ, Collins T: Inducible PDGF A-chain transcription in vascular smooth muscle cells is mediated by Egr-1 displacement of Sp1 and Sp3. *Am J Physiol* 1997, 42:H1415–H1426
- Khachigian LM, Collins T: Inducible expression of Egr-1-dependent genes: a paradigm of transcriptional activation in vascular endothelium. *Circ Res* 1997, 81:457–461
- Jaye M, Howk R, Burgess W, Ricca GA, Chiu IM, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN: Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosomal localization. *Science* 1986, 233:541–545
- Lindner V, Reidy MA: Expression of basic fibroblast growth factor and its receptor by smooth muscle cells and endothelium in injured rat arteries: an en face study. *Circ Res* 1993, 73:589–595
- Vlodavsky I, Friedman R, Sullivan R, Sasse J, Klagsbrun M: Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor which is secreted. *J Cell Physiol* 1987, 131:402–408
- Jones KA, Kadonaga JT, Rosenfeld PJ, Kelly TJ, Tjian R: A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. *Cell* 1987, 48:79–89
- Bonthron DT, Morton CC, Orkin SH, Collins T: Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc Natl Acad Sci USA* 1988, 85:1492–1496
- Delbridge GJ, Khachigian LM: FGF-1-induced PDGF A-chain gene expression in vascular endothelial cells involves transcriptional activation by Egr-1. *Circ Res* 1997, 81:282–288
- Norman C, Runswick M, Pollock R, Treisman R: Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. *Cell* 1988, 55:989–1003
- Treisman R: Ternary complex factor: growth factor regulated transcriptional activators. *Curr Opin Genet Dev* 1994, 4:96–101
- Cohen DM: Urea-inducible Egr-1 transcription in renal inner medullary collecting duct (MIMCD3) cells is mediated by extracellular signal-regulated kinase activation. *Proc Natl Acad Sci USA* 1996, 93:11242–11247
- Cohen DM, Gullans SR, Chin WW: Urea inducibility of *egr-1* in murine inner medullary collecting duct cells is mediated by the serum re-

- sponse element and adjacent Ets motifs. *J Biol Chem* 1996, 271: 12903–12908
36. McMahon SB, Monroe JG: A ternary complex factor-dependent mechanism mediates induction of *egr-1* through selective serum response elements following antigen receptor cross-linking in B lymphocytes. *Mol Cell Biol* 1995, 15:1086–1093
37. Thompson MJ, Roe MW, Malik RK, Blackshear PJ: Insulin and other growth factors induce binding of the ternary complex and a novel protein complex to the *c-fos* serum response element. *J Biol Chem* 1994, 269:21127–21135
38. Treisman R: The SRE: a growth factor responsive transcriptional regulator. *Semin Cancer Biol* 1990, 1:47–58
39. Christy B, Nathans D: Functional serum response elements upstream of the growth factor-inducible gene, *zif268*. *Mol Cell Biol* 1989, 9:4889–4895
40. Herrera RE, Shaw PE, Nordheim A: Occupation of the *c-fos* serum response element in vivo by a multi-protein complex is unaltered by growth factor induction. *Nature* 1989, 340:68–70
41. Gille H, Kortenjann M, Strahl T, Shaw PE: Phosphorylation-dependent formation of a quaternary complex at the *c-fos* SRE. *Mol Cell Biol* 1996, 16:1094–1102
42. Rupprecht HD, Sukhatme VP, Lacy J, Sterzel RB, Coleman DL: PDGF-induced *Egr-1* expression in rat mesangial cells is mediated through upstream serum response elements. *Am J Physiol* 1993, 265:F351–F360
43. Alexandropoulos K, Qureshi SA, Rim M, Sukhatme VP, Foster DA: *v-Fps*-responsiveness in the *Egr-1* promoter is mediated by serum response elements. *Nucleic Acids Res* 1992, 20:2355–2359
44. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J Biol Chem* 1995, 270:27489–27494
45. Janknecht R, Hunter T: Activation of the *Sap-1a* transcription factor by the *c-Jun* N-terminal kinase (JNK) mitogen-activated protein kinase. *J Biol Chem* 1997, 272:4219–4224
46. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ: Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 1995, 269:403–407
47. Price M-A, Rogers AE, Treisman R: Comparative analysis of the ternary complex factors *Elk-1*, *SAP-1a*, and *SAP-2* (ERP/NET). *EMBO J* 1995, 14:2589–2601
48. Gille H, Kortenjann M, Thomae O, Moormaw C, Slaughter C, Cobb MH, Shaw PE: ERK phosphorylation potentiates *ELK-1*-mediated ternary complex formation and transactivation. *EMBO J* 1995, 14:951–962
49. Lopez-Illasaca M, Li W, Uren A, Yu JC, Kazlauskas A, Gutkind JS, Heideran MA: Requirement of phosphatidylinositol-3 kinase for activation of JNK/SAPK by PDGF. *Biochem Biophys Res Commun* 1997, 232:272–277
50. Logan SK, Falasca M, Hu P, Schlessinger J: Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the *c-Jun* N-terminal kinase signaling pathway. *Mol Cell Biol* 1997, 17: 5784–5790
51. Ishizuka T, Terada N, Gerwins P, Hamelmann E, Oshiba A, Fanger GR, Gelfand EW: Mast cell tumor necrosis factor α production is regulated by MEK kinases. *Proc Natl Acad Sci USA* 1997, 94:6358–6363
52. Ishizuka T, Oshiba A, Sakata N, Johnson GL, Gelfand EW: Aggregation of the *FcERI* on mast cells stimulates *c-Jun* amino-terminal kinase activity. *J Biol Chem* 1996, 271:12762–12766
53. Derijard B, Hibi M, Wu I-H, Barrett T, Su B, Deng T, Karin M, Davis RJ: JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the *c-Jun* activation domain. *Cell* 1994, 76:1025–1037
54. Cazenave C, Stein C, Loreau N, Thuong N, Neckers L, Subasinghe C, Helene C, Cohen J, Toulme J: Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. *Nucleic Acids Res* 1989, 17:4225–4273
55. Marcus-Sekura C, Woerner A, Shinozuka K, Zon G, Quinnan G: Comparative inhibition of chloramphenicol acetyltransferase gene expression by antisense oligonucleotide analogues having alkyl triester, methyl phosphonate and phosphorothioate linkages. *Nucleic Acids Res* 1987, 15:5749–5763
56. Wang W, Chen HJ, Schwartz A, Cannon PJ, Stein CA, Rabbani LE: Sequence-independent inhibition of in vitro smooth muscle cell proliferation, migration, and in vivo neointimal formation by phosphorothioate oligonucleotides. *J Clin Invest* 1996, 98:443–450
57. Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL, Stein CA: Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J Biol Chem* 1995, 270:2620–2627
58. Krieg AM, Stein CA: Phosphorothioate oligonucleotides: antisense or anti-protein? *Antisense Res Dev* 1995, 5:241
59. Villa AE, Guzman LA, Poptic EJ, Labhasetwar V, D'Souza S, Farrell CL, Plow EF, Levy RJ, DiCorleto PE, Topol EJ: Effects of antisense *c-myc* oligonucleotides on vascular smooth muscle cell proliferation and response to vessel wall injury. *Circ Res* 1995, 76:505–513
60. Hallahan DE, Dunphy E, Virudachalam S, Sukhatme VP, Kufe DW, Weischselbaum RR: *c-jun* and *Egr-1* participate in DNA synthesis and cell survival in response to ionizing radiation exposure. *J Biol Chem* 1995, 270:30303–30309
61. Hofer G, Grimmer C, Sukhatme VP, Sterzel RB, Rupprecht HD: Transcription factor *Egr-1* regulates glomerular mesangial cell proliferation. *J Biol Chem* 1996, 271:28306–28310
62. Szklarczyk A, Blaszczyk J, Kaczmarek L: Antisense oligonucleotides against transcription factor *Zif 268* transiently impair excitatory synaptic transmission in the hippocampus without affecting *Zif 268* immunoreactivity. *Neurosci Res Commun* 1996, 19:125–133
63. Lindner V, Fingerle J, Reidy MA: Mouse model of arterial injury. *Circ Res* 1993, 73:792–796
64. Lowe HC, Chesterman CN, Khachigian LM: Left main coronary artery stenosis after percutaneous transluminal coronary angioplasty: importance of remaining "minimally invasive". *Catheter Cardiovasc Diagn* 1999 (in press)
65. Kraiss LW, Clowes AW: Response of the arterial wall to injury and intimal hyperplasia. *The Basic Science of Vascular Disease*. Edited by Sidawy AN, Sumpio BE, Depalma RG. Armonk, NY, Futura, 1997, pp 289–317
66. Pap JM: Catheter-induced intimal injury during routine coronary catheterization in dogs. *Catheter Cardiovasc Diagn* 1987, 13:57–73
67. Lindner V, Reidy MA: Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci USA* 1991, 88:3739–3743
68. Olson NE, Chao S, Lindner V, Reidy MA: Intimal smooth muscle cell proliferation after balloon catheter injury: the role of basic fibroblast growth factor. *Am J Pathol* 1992, 140:1017–1023
69. Lee SL, Sadovsky Y, Swirloff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J: Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor *NGF-1A* (*Egr-1*). *Science* 1996, 273:1219–1221
70. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C: Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci USA* 1996, 95:5672–5677
71. Koyama H, Olson NE, Dastvan FF, Reidy MA: Cell replication in the arterial wall: activation of signaling pathway following in vivo injury. *Circ Res* 1998, 82:713–721