Fibroblast Growth Factor Receptor-1 Signaling Induces Osteopontin Expression and Vascular Smooth Muscle Cell–Dependent Adventitial Fibroblast Migration In Vitro

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Background—Increased expression of osteopontin (OPN), fibroblast growth factors (FGFs), and their type-1 receptor (FGFR-1) is associated with neointima formation and atherosclerosis. This study tested the hypothesis that ligand activation of FGFR-1 stimulates OPN expression in rat aortic smooth muscle cells (RASMCs), explored the signaling pathway involved, and assessed the functional consequences of activating this pathway on adventitial fibroblast (AF) migration in vitro.

Methods and Results—Exogenous FGF-1 stimulated expression of OPN mRNA and protein in RASMCs in vitro in a dose- and time-dependent manner. OPN mRNA induction by FGF-1 was completely inhibited by either actinomycin D or cycloheximide, selective inhibitors of RNA polymerase and protein synthesis, respectively. OPN mRNA induction by FGF-1 was attenuated by PD 166866, a highly selective and potent FGFR-1 tyrosine kinase inhibitor. Addition of either PP2 or PD98059, specific inhibitors of Src and mitogen-activated extracellular signal–regulated kinase (MEK)/mitogen-activated protein (MAP) kinases, respectively, attenuated FGF-1–stimulated OPN mRNA expression. FGF-1 treatment of RASMCs enhanced RASMC-conditioned medium-stimulated AF migration; this effect was inhibited by pretreatment of RASMCs with either PD166866 or PP2. Immunodepletion of OPN from RASMC-conditioned medium inhibited both basal and FGF-1–stimulated AF migration.

Conclusions—This in vitro study provided a first indication that ligand-activated FGFR-1 plays a significant role in upregulation of OPN expression at the transcriptional level via signaling to Src/MEK/MAP kinases in RASMCs and that this pathway is functionally significant in mediating AF migration via stimulation of OPN expression. (Circulation. 2002;106:854-859.)

Key Words: muscle, smooth ■ signal transduction ■ receptors, fibroblast growth factor ■ osteopontin ■ cells

Expression of osteopontin (OPN), along with its αβ3 integrin receptor, has been shown to be markedly increased in injured blood vessels, atherosclerotic plaques, and arteries of diabetic subjects, although virtually absent from normal arteries.1–4 A role for OPN and its αβ3 integrin receptor in response to vascular injury has been elegantly established in animals and humans. Neutralizing antibodies against OPN and selective αβ3 integrin receptor antagonists significantly suppress neointima formation and reduce restenosis in various vascular injury models.5–7 Furthermore, previous in vitro studies in our laboratory demonstrated that vascular smooth muscle cells (VSMCs) in culture express high levels of OPN mRNA and secreted protein that robustly directs haptotatic migration of VSMCs and adventitial fibroblasts (AFs) in an integrin-β3–dependent manner.8

A variety of growth factors, cytokines, and chemoattractants are released from damaged cells after vascular injury and participate in the formation of neointima and the other cellular responses to injury. Aberrant expression of FGF-1 and FGF-2 may be central to the vascular injury and atherosclerotic disease process. FGF-1 and FGF-2 mediate their biological effects by binding to a family of selective high-affinity cell-surface receptors with protein tyrosine kinase activity. Although FGF-2 has received substantial research attention, FGF-1 has been relatively neglected. High levels of FGF-1 and its type-1 receptor (FGFR-1) are expressed in human atherosclerotic lesions and accelerated coronary atherosclerosis in cardiac transplants characterized by coronary intimal hyperplasia.9–11 Immunohistochemical staining for FGFR-1 reveals a high level of expression in balloon-injured rat carotid arteries 3 (in the adventitia) and 14 (in the neointima) days after injury.12 Upregulation of FGFR-1 has also been associated with neointima formation in vitro in a porcine aortic organ culture model.13 Direct gene transfer of a eukaryotic expression vector encoding a secreted form of FGF-1 induces intimal thickening in porcine arteries.14 Collectively, these studies predict an important role for FGF ligands and FGFR-1 in neointima formation after vascular injury.
injury. The present study tested the hypotheses that FGF ligand activation of FGFR-1 signaling plays an important role in regulating OPN expression in RASMCs and VSMC-mediated AF migration. Furthermore, we used selective kinase inhibitors to delineate the signaling pathway involved.

**Methods**

**Materials**

Recombinant human FGF-1, actinomycin D, and cycloheximide were purchased from Sigma Chemical Co. The mouse monoclonal anti-rat OPN antibody (MPIIIB10) was purchased from American Research Products (Belmont, Mass). The FGFR-1 tyrosine kinase inhibitor PD166866 was provided by Parke-Davis Pharmaceutical Research, Division of Pfizer Inc. The Src family tyrosine kinase inhibitor PP2, the mitogen-activated extracellular signal–regulated kinase (MEK)/mitogen-activated protein (MAP) kinase inhibitor PD98059, the PI3 kinase inhibitor LY294002 were purchased from Calbiochem-Novabiochem Corp. The rat OPN cDNA probe was generated from a plasmid with a 1.1-kb HindIII/BamHI insert (kindly provided by Dr C.M. Giachelli, University of Washington, Seattle). |(β−P)32P|dCTP (3000 Ci/mmol) was purchased from Dupont Co. The random priming DNA labeling kit was purchased from Promega Co.

**Cell Culture**

Primary cultures of RASMCs and AFs were derived from 10-week-old female Sprague-Dawley rats (Charles River), as previously described.8,15 Cells were cultured in complete medium containing DMEM (GIBCO BRL) supplemented with 10% (vol/vol) FBS, 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Experiments were performed with cells from passages 4 through 9.

**Northern Blot**

RASMCs were grown to subconfluence (~95%) in complete medium, serum deprived for 24 hours, and then treated with defined reagents for varying times or concentrations (see figure legends for details). Total RNA (10 to 15 μg/lane) was extracted from cells by the guanidine thiocyanate method, and expression of OPN mRNA was analyzed by Northern blot as previously described.16

**Western Blot**

RASMCs were grown to subconfluence (~95%) in complete medium, serum deprived for 24 hours, and then treated with FGF-1 (10 ng/mL) complexed with 10 U/mL heparin for various times. Protein was extracted and subjected to Western analysis using a mouse monoclonal anti-rat OPN antibody MPIIIB10, (1:1000 dilution) as previously described.8

**Migration Assay**

Haptotaxis migration assays were performed using Transwell migration chambers (Costar Corp) essentially as described.8 Briefly, upper chamber membranes were coated (4°C; 48 hours) with media conditioned by VSMCs that were treated with FGF-1 (10 ng/mL) for 16 hours or pretreated with either PD166866 (1 μmol/L) or PP2 (0.5 μmol/L) for 45 minutes followed by FGF-1 treatment for an additional 16 hours. To exclude direct effects of FGF-1, PD166866, and PP2 on AF haptotactic migration, upper chamber membranes were coated with media that contained each of the reagents and had been conditioned in the absence of VSMCs. AFs (~95% confluence) were trypsinized briefly (~1 minute), centrifuged, resuspended in DMEM plus 1 mg/mL BSA, and incubated (37°C; 30 minutes). A suspension (200 μL) of AFs (3×10⁴ cells) was added to the upper chamber. Migration was allowed to proceed for 6 hours at 37°C in a humidified incubator, and cells that migrated to the bottom of the filter were fixed with methanol and stained with hematoxylin. Migration was quantitated by cell counts of 4 random high-power (×100) fields in each well. Each assay was performed in quadruplicate.

A separate set of experiments tested the effect of FGF-1–stimulated OPN production and release into VSMC-CM on haptotactic migration. Depletion of OPN from media was achieved by immunoadsorption technique.8 Briefly, 5 μg of monoclonal anti-

**Results**

To determine the effects of FGF-1 on OPN mRNA expression in RASMCs, time- and dose-response studies were performed. FGF-1 (10 ng/mL) stimulated the expression of OPN mRNA in a time-dependent manner (Figure 1A).
Compared with time 0, steady-state OPN mRNA was significantly increased 3.2-, 4.6-, 10-, and 9.0-fold at 4, 8, 12, and 24 hours (all \( P < 0.001 \)), with a peak at 12 hours. FGF-1–induced stimulation of OPN mRNA expression was also dose-dependent, with a peak effect at 5 ng/mL (Figure 1B). At 12 hours of incubation with FGF-1 (1, 5, and 10 ng/mL), OPN mRNA was increased 6.7-, 10.6-, and 10.4-fold, respectively, compared with the untreated control (all \( P < 0.001 \)).

OPN mRNA induction by FGF-1 was completely inhibited by pretreatment of RASMCs with either the RNA polymerase inhibitor actinomycin D (20 \( \mu \)g/mL) or the protein synthesis inhibitor cycloheximide (40 \( \mu \)g/mL) 30 minutes before either a 4-hour or a 12-hour exposure to 10 ng/mL FGF-1 (Figures 2A and 2B, respectively). This result indicates that FGF-1 induction of OPN mRNA expression is mediated through activation of FGFR-1 in RASMCs in vitro and suggests the possibility that there is constitutive activation of OPN mRNA by secreted FGF ligands in these cells.

To elucidate the signaling pathways involved in regulation of OPN mRNA expression in response to activation of FGFR-1 by FGF-1, RASMCs were pretreated with the indicated kinase inhibitors for 45 minutes and then incubated with 10 ng/mL FGF-1 for an additional 12 hours. Under basal conditions, the MEK/MAP kinase inhibitor PD98059 (25 \( \mu \)mol/L) and the Src family kinase inhibitor PP2 (1 \( \mu \)mol/L) suppressed OPN mRNA expression by 47.7% \(( P < 0.001)\) and 41.7% \(( P < 0.001)\), respectively (Figures 4B and 4C). Under FGF-1–stimulated conditions, PD98059 (25 \( \mu \)mol/L) and PP2 (1 \( \mu \)mol/L) inhibited OPN mRNA expression by 55.5% \(( P < 0.001)\) and 38.4% \(( P < 0.001)\), respectively (Figures 4B and 4C). In contrast, the P38 MAP kinase inhibitor SB203580 (5 \( \mu \)mol/L) had a minimally detectable inhibitory effect on both basal and FGF-1–stimulated OPN mRNA expression (Figure 5A). Furthermore, the PI3 kinase inhibitor LY294002 (10 \( \mu \)mol/L) did not affect OPN expression under either basal or FGF-1–stimulated conditions (Figure 5B). These results suggest that the FGF-1 induction of OPN mRNA expression...
is mediated mainly through activation of a Src-like kinase-dependent and MEK kinase-dependent, but P38 MAP kinase-independent and PI3 kinase-independent, signaling pathway in RASMCs in vitro.

A subsequent experiment tested the hypothesis that FGFR-1–mediated effects of FGF-1 on VSMCs are functionally significant in directing the migration of AFs. Haptotatic migration directed by VSMC-CM was evaluated after treatment of VSMCs with indicated reagents under the defined conditions (Figure 6A). VSMC-CM robustly stimulated AF haptotatic migration (140 cells/field). FGF-1 treatment increased VSMC-CM–directed AF migration by 56%. PD166866 treatment markedly attenuated AF migration directed by VSMC-CM under both basal and FGF-1–stimulated conditions, suggesting that the VSMC-CM–directed AF migration is mediated via FGFR-1–dependent mechanism. Under basal and FGF-1–stimulated conditions, PP2 treatment inhibited AF migration by 35.2% and 52%, respectively. Moreover, direct treatment of the membrane of the migration chamber with FGF-1, PD166866, or PP2 did not alter AF migration (data not shown).

To determine the OPN dependence of FGF-1–induced AF migration, OPN was immunodepleted from VSMC-CM (Figure 6B). Under basal conditions, OPN immunodepletion caused an 18% decrease in AF migration (P < 0.01) compared with the control group (bar 1 in Figure 6B), suggesting a small but significant contribution of constitutive expression of OPN to VSMC-mediated AF migration. Under FGF-1–stimulated conditions, FGF-1 enhanced AF migration by 58% (P < 0.01) compared with the control group. OPN immunodepletion caused an 83% decrease in FGF-1–enhanced AF migration (P < 0.01) compared with the FGF-1–treated control (bar 2 in Figure 6B). These results suggest that OPN contributes to VSMC-mediated AF migration under both basal and FGF-1–stimulated conditions and that FGF-1–induced AF migration is mediated mainly via an OPN-dependent mechanism.
Injury of rat carotid arteries leads to an increase in FGF receptors and activation of the OPN-3 integrin pathway.12,17 Together with our recent observation that FGF-1 and FGFR-1 were markedly increased in balloon-injured rat carotid arteries (data not shown), these studies suggest that increased expression of FGF-1 and FGFR-1 may play a role in the pathogenesis of atherosclerosis and the response to vascular injury in vivo.

OPN and its α, β, integrin receptor have also been implicated in atherosclerosis and vascular injury response.1–8 Increased OPN expression has been demonstrated in the early phase after injury. Wang et al16 found a 1.5-fold increase in OPN mRNA at 6 hours that peaked at 1 and 3 days with a 3.1-fold increase in balloon-injured rat carotid arteries. Immunohistochemical examination localized OPN expression to medial SMCs at 1 day after injury and thereafter to the neointima. Similar results were demonstrated by Giachelli et al1 using in situ hybridization and immunohistochemical methods. The early appearance of increased OPN expression in those in vivo studies is consistent with the time course of OPN mRNA expression during FGF-1 stimulation of VSMCs in vitro in our own study.

The present study was the first to demonstrate that FGF-1 stimulates OPN expression in VSMCs in vitro. The mechanism of FGF-induced signal transduction in vascular cells is not well defined. In nonvascular cells, FGF causes rapid autophosphorylation of FGFR-1, which subsequently activates downstream effectors such as phospholipase Cγ, Src, MAP kinase, and PI3 kinase.18–21 FGF-1 may bind to 4 FGF receptors, termed FGFR-1, -2, -3, and -4. FGFR-1 is the predominant form of FGF receptor in VSMCs.22,23 The relative levels of expression of the 4 major types of FGF have been studied in 13 different human arterial smooth muscle cell isolates, demonstrating that FGFR-1 is the major form of FGF receptor mRNA expressed by proliferating human arterial smooth muscle cells in culture.23

PD 166866 is a potent (nanomolar range) and highly selective small molecule inhibitor of FGFR-1 signalling that does not perturb signals induced by PDGFR, EGFR, Src, MEK, PKC, or CDK4.21 In this study, we extended our observations to define an FGF-1–induced signal transduction pathway that is associated with regulation of OPN expression in RASMCs. The present study demonstrated that PD166866 effectively inhibited RASMC expression of OPN mRNA after FGF-1 treatment. This result indicates that FGF-1 stimulation of OPN mRNA expression in RASMCs in vitro is mediated in an FGFR-1–dependent manner.

Furthermore, the present study explored the signaling pathway after FGF-1 activation in RASMCs in vitro. Inhibition of MEK/MAP kinase by PD98059 and the Src-like tyrosine kinase by PP2 significantly inhibited FGF-1–induced OPN mRNA expression. Inhibition of P38 MAP kinase by SB203580 had minimal inhibitory effects on OPN expression, and inhibition of PI3 kinase by LY294002 had no effect on OPN mRNA expression under either untreated or FGF-1–treated conditions, suggesting that the MEK/MAP kinase and the Src-like tyrosine kinase, but not the P38 MAP kinase and the PI3 kinase, are involved in FGF-1–stimulated OPN mRNA upregulation.

Constitutive expression of OPN in VSMCs in vitro under basal conditions also appeared to involve FGF ligand activation.
of FGFR-1 signaling, because inhibition of kinase activities associated with FGFR-1, Src, or MEK/MAP kinase significantly reduced the production of OPN mRNA under these conditions. Admittedly, this basal level response may involve FGFR-1 activation by any of the 23 identified members of the FGF gene family, some of which are expressed in VSMCs.

Growth factors, cytokines, and extracellular matrix proteins secreted by VSMCs may play an important role in regulating vascular cell proliferation and migration in an autocrine/paracrine manner. We and others have demonstrated that AF activation and migration may contribute to neointima formation and vascular remodeling after balloon angioplasty. The present study tested the hypothesis that the VSMC-directed AF migration is mediated via activation of FGFR-1-dependent signaling. Our results demonstrated that FGF-1 treatment of RASMCs markedly enhanced VSMC-mediated AF migration. This FGF-1–enhanced AF migration is mediated via activation of FGFR-1–dependent signaling, related in part to activation of Src kinase and induction of OPN expression.

Admittedly, in addition to Src/MEK/MAP kinase cascade, other signaling pathways participate in regulating OPN expression in response to FGF and possibly other growth factors, because neither PD98059 nor PP2 completely inhibited OPN mRNA expression under basal and FGF-1–stimulated conditions. Likewise, in addition to OPN, other substances in VSMC-CM also contribute to VSMC-mediated AF migration, because OPN immunodepletion did not fully eliminate AF migration under these conditions. However, immunodepletion of OPN from VSMC-CM almost completely eliminated FGF-1–stimulated AF haptotaxis, supporting the role of OPN as the target chemoattractant molecule in this signaling cascade.

In summary, the present study provided a first indication that FGF ligand–activated FGFR-1 plays a significant role in upregulation of OPN expression at the transcriptional level via activation of FGFR-1 and subsequent activation of the Src/MEK/MAP kinase pathway, but not the F38 MAP and PI3 kinase pathway, in RASMCs in vitro. The functional significance of these findings was demonstrated by the observation that VSMC-directed AF migration is mediated via activation of FGFR-1–dependent signaling related in part to activation of Src kinase and induction of OPN.

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