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PDGF-BB Induces Vascular Smooth Muscle Cell Expression of High Molecular Weight FGF-2, Which Accumulates in the Nucleus

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Abstract Basic fibroblast growth factor (FGF-2) and platelet-derived growth factor (PDGF) are implicated in vascular remodeling secondary to injury. Both growth factors control vascular endothelial and smooth muscle cell proliferation, migration, and survival through overlapping intracellular signaling pathways. In vascular smooth muscle cells PDGF-BB induces FGF-2 expression. However, the effect of PDGF on the different forms of FGF-2 has not been elucidated. Here, we report that treatment of vascular aortic smooth muscle cells with PDGF-BB rapidly induces expression of 20.5 and 21 kDa, high molecular weight (HMW) FGF-2 that accumulates in the nucleus and nucleolus. Conversely, PDGF treatment has little or no effect on 18 kDa, low-molecular weight FGF-2 expression. PDGF-BB-induced upregulation of HMW FGF-2 expression is controlled by sustained activation of extracellular signal-regulated kinase (ERK)-1/2 and is abolished by actinomycin D. These data describe a novel interaction between PDGF-BB and FGF-2, and indicate that the nuclear forms of FGF-2 may mediate the effect of PDGF activity on vascular smooth muscle cells. J. Cell. Biochem. 95: 1292–1300, 2005.

Key words: fibroblast growth factor; mitogen-activated protein kinase; platelet-derived growth factor; translational control

Basic fibroblast growth factor (FGF-2), the prototypic member of a large family of proteins with diverse effects on a variety of cell functions [Bikfalvi et al., 1997; Ornitz and Itoh, 2001], is a ubiquitous and pleiotropic growth factor first identified as a basic protein of 18 kDa [Abraham et al., 1986]. The analysis of the human cDNA sequence upstream of the 5′ AUG translation initiation codon has identified at least three (two in rodents) additional CUG initiation sites located on the same mRNA. These alternative initiation codons originate additional, 22, 22.5, and 24 kDa (20.5 and 21 kDa in rodents) FGF-2 forms referred to as high molecular weight (HMW) FGF-2 [Florkiewicz and Sommer, 1989; Powell and Klagsbrun, 1991; Bikfalvi et al., 1997; Delrieu, 2000]. Although all FGF-2 forms have comparable biological activities when added exogenously to cultured endothelial cells [Moscatelli et al., 1987], their endogenous expression results in differential intracellular localization and biological activities, possibly related to different biochemical properties. 18 kDa FGF-2 is mostly cytoplasmic, and is released from viable cells despite the lack of a signal peptide. Conversely, HMW FGF-2 localize predominately to the nucleus [Florkiewicz and Sommer, 1989; Renko et al., 1990; Bugler et al., 1991; Mignatti et al., 1991, 1992; Claus et al., 2003] by means of their N-terminal extension [Quarto et al., 1991a; Patry et al., 1994; Pintucci et al., 1996; Arese et al., 1999]. The expression of the various FGF-2 forms is

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controlled by specific cellular conditions (see below), and their distribution seems to be differentially controlled in different tissues [Coffin et al., 1995]. Overexpression of 18 kDa FGF-2 promotes NIH 3T3 cell proliferation and migration through interaction with specific cell membrane receptor(s); in contrast, overexpression of HMW FGF-2 results in cell transformation with a receptor-independent mechanism, and does not effect cell migration [Bikfalvi et al., 1995, 1997; Arnaud et al., 1999]. We have shown that both cytoplasmic (18 kDa) and nuclear (HMW) FGF-2 induce vascular endothelial growth factor (VEGF) expression [Seghezzi et al., 1998], raising the question whether signaling generated by different FGF-2 forms is mediated by common intracellular pathways. In addition, the observation that a number of growth factors can be targeted to the nucleus with or without their receptors has generated interest in alternative mechanisms through which they exert their biological activities [Maher, 1996; Stachowiak et al., 1997, 2003; Keresztes and Boonstra, 1999; Sun et al., 2001].

FGF-2 controls a variety of biological functions [Bikfalvi et al., 1997]. During injury-induced vascular remodeling, such as after percutaneous transluminal coronary angioplasty and coronary artery by-pass grafting [Schwartz et al., 1995; Motwani and Topol, 1998], a number of growth factors and cytokines control the development of intimal hyperplasia, the major cause of failure of vascular interventions. FGF-2, platelet-derived growth factors (PDGFs), angiotensin II, and thrombin have been implicated in the control of medial smooth muscle cell (SMC) proliferation and migration into the intima, and in the abundant extracellular matrix deposition that characterizes the development of intimal hyperplasia [Schwartz et al., 1995; Waltenberger, 1997; Stouffer and Runge, 1998]. Various pharmacological approaches have been aimed at individual growth factors to limit this pathological response [Kaneda et al., 1997]. FGF-2 activity has been targeted using either blocking antibodies [Lindner and Reidy, 1991; Nguyen et al., 1994] or antisense mRNA [Hanna et al., 1997; Neschis et al., 1998] in vascular injury models. However, the interactions between different growth factors and their effect on the generation of intimal hyperplasia have not been elucidated. PDGF, a growth factor initially described in platelets, occurs as homo- or hetero-dimer of two different gene products, PDGF-A and -B. PDGF-AA, -AB, and -BB interact specifically with homo- and hetero-dimers of PDGF receptors ($\alpha-\alpha$, $\alpha-\beta$, $\beta-\beta$) that possess tyrosine kinase activity. PDGF-BB is the only isoform to interact with all three PDGF receptors. PDGFs significantly effect SMC proliferation and migration [Heldin and Westermark, 1999]. FGF-2 and angiotensin II appear to be rather relevant in controlling muscle cell proliferation [Schwartz et al., 1995]. Although various approaches have significantly reduced intimal hyperplasia in animal models, the elucidation of the mechanism(s) regulating the response of vascular cells to injury is far from complete.

A number of studies have shown that FGF-2 expression is increased in injured vessels, and that PDGF upregulates FGF-2 expression in cultured vascular cells [Lindner and Reidy, 1991, 1993; Sato et al., 1991]. However, blocking antibodies to FGF-2 fail to inhibit the generation of intimal hyperplasia in injured vessels [Koyama et al., 1998], suggesting that this growth factor may exert its activity with an intracellular (intracrine) mechanism. Recently a number of reports have unequivocally implicated HMW FGF-2 as a major respondent to stress, aging, cytokines, and estrogens [Stachowiak et al., 1994; Vagner et al., 1996; Joy et al., 1997; Cowan et al., 2003; Garmy-Susini et al., 2004; Sabbieti et al., 2005]. These observations prompted us to characterize the expression and intracellular localization of the different forms of FGF-2 in SMC treated with PDGF. In this report we show that PDGF-BB, but not PDGF-AA, upregulates HMW FGF-2 expression and accumulation in the nucleus of SMC, and that this effect is mediated by the ERK-1/2 pathway of intracellular signaling.

MATERIALS AND METHODS

Cells

Rat primary aortic smooth muscle cells (RAOSMC) were purchased from Cell Applications, Inc. (San Diego, CA) and grown in RAOSMC growth medium (Cell Applications, Inc.). The cells were used between passage 3 and 10 with comparable results.

Cell Treatment and Western Blotting Analysis

Subconfluent (70%–80% confluency) RAOSMC, starved overnight with RAOSMC basal medium (Cell Applications, Inc.) containing 0.5% fetal

calf serum (FCS), were treated for the indicated times with either 10 ng/ml of human recombinant (hr) FGF-2 (Invitrogen, Carlsbad, CA), or 20 ng/ml of hrPDGF-AA (Intergen, Purchase, NY), or 20 ng/ml of either human or rat recombinant PDGF-BB (R&D Systems, Minneapolis, MN; and Peprotech, Rocky Hill, NJ, respectively), or with control medium. Where indicated, the cells were preincubated for 20 min with either the MEK inhibitor UO126 (10 μM; Promega, Madison, WI), the PI3 kinase inhibitor wortmannin (1 μM; Calbiochem, San Diego, CA), the transcription inhibitor actinomycin D (2 μg/ml; Sigma-Aldrich, St. Louis, MO) or with dimethyl-sulfoxide (DMSO, vehicle) 0.1% as a control. The cells were lysed and their protein content was measured as described [Pintucci et al., 2003]. Equal amounts (20–100 μg) of cell extract protein were loaded onto a sodium dodecyl sulfate (SDS)-10% (for MAPK and Akt activation) or 12% (for FGF-2 expression) polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA) that was probed with the following antibodies: anti-phospho-ERK-1/2 polyclonal antibody (Cell Signaling Technologies, Beverly, MA), and, after stripping, anti-ERK-2 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA); anti-phospho-Akt polyclonal antibody and, after stripping, anti-Akt polyclonal antibody (both from Cell Signaling Technologies); anti-human FGF-2 monoclonal antibody (354FI, a generous gift from Texas Bio-Technologies, Houston, TX). The membranes were then incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG antibodies (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), and antigen-antibody complexes were detected by enhanced chemiluminescence (LumiLight, Roche, Indianapolis, IN).

Immunofluorescence Analysis of FGF-2 Expression and Intracellular Distribution

To study the intracellular localization of endogenous FGF-2 following treatment of the cells with exogenous FGF-2 or PDGF-AA or PDGF-BB, RAOSMC grown to 70%–80% confluency on glass coverslips in 24-well plates were incubated overnight in basal medium supplemented with 0.5% FCS. The following reagents were then added to the culture medium: 10 ng/ml of human recombinant FGF-2, or 20 ng/ml of human PDGF-AA, or 20 ng/ml of rat recombinant PDGF-BB, or an equivalent volume of control medium with no growth factors. Where indicated, the cells were preincubated (20 min) with the MEK inhibitor UO126 (10 μM), or with 0.1% DMSO as a control. After 6 h incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, followed by 5 min permeabilization with 0.5% Triton X-100 in PBS. After three washes with PBS and one wash with PBS containing 0.2% bovine serum albumin (BSA, Intergen), the cells were incubated with 20 μg/ml of anti-FGF-2 antibody 354FI or w/o antibody (negative control) for 1 h at 37°C in a moist chamber. Following three washes with PBS and one wash with PBS containing 0.2% BSA, the cells were incubated with TRITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) for 30 min at room temperature. The coverslips were thoroughly washed with PBS, mounted on microscope slides with one drop of Fluoromount-G mounting medium (Southern Biotechnology Associates, Inc., Birmingham, AL), and observed under a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with the appropriate filter.

RESULTS

Previous studies have shown that treatment of vascular SMCs with PDGF upregulates FGF-2 expression [Sato et al., 1991]. In preliminary experiments we found that the effect of PDGF-BB on RAOSMC expression of FGF-2 peaked after 6 h incubation (data not shown). Therefore we used this time-point to characterize the effect of PDGF-BB on the expression of the different forms of FGF-2. Rat cells produce two HMW FGF-2 forms of 20.5 and 21.0 kDa in addition to 18 kDa FGF-2 [Powell and Klagsbrun, 1991]. 18 kDa FGF-2 is poorly detected by Western blotting of RAOSMC extracts. Treatment of RAOSMCs with PDGF-BB resulted in increased levels of the HMW FGF-2 forms but had no significant effect on 18 kDa FGF-2 expression. In contrast, PDGF-AA did not affect total FGF-2 expression at all, while hrFGF-2 (18 kDa) appeared to increase predominantly the 18 kDa form and, to a minor extent, the HMW forms (Fig. 1B).

To investigate the relative contribution of the PDGF isoforms to intracellular signaling we treated RAOSMCs with either PDGF-AA or

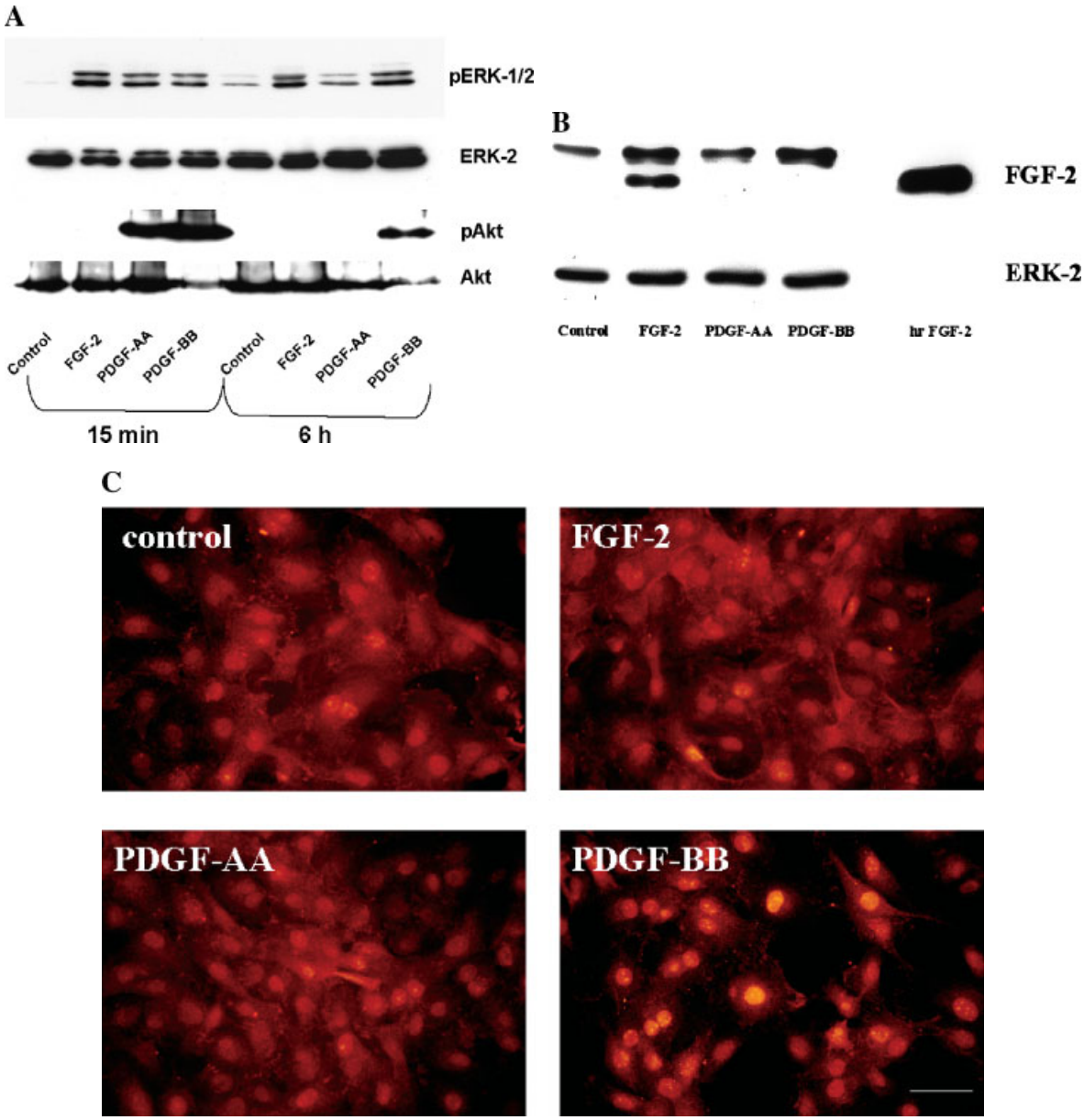

Fig. 1. PDGF-BB activates the ERK-1/2 and PI-3K/Akt pathways, and upregulates HMW FGF-2 expression. **A**: Western blotting analysis of signaling pathway activation. RAOSMC were treated with either FGF-2 (10 ng/ml) or PDGF-AA (20 ng/ml) or PDGF-BB (20 ng/ml) or with control medium (Control) for 15 min or 6 h. Cell lysates were analyzed for ERK-1/2 and PI-3K/Akt activation by Western blotting with antibodies to phosphorylated ERK1/2 (pERK-1/2) or to phosphorylated Akt (pAkt). As loading control, the corresponding blots were also probed with antibodies to total ERK-2 or Akt, respectively. **B**: **Upper panel**: Western blotting analysis of FGF-2 expression in RAOSMC treated with PDGF-BB for 6 h. Human recombinant 18 kDa FGF-2 (hr FGF-2) is shown in the rightmost lane as a control. Lower panel: After stripping, the same membrane was analyzed for ERK-2 expression as a loading control. **C**: Immunofluorescence analysis of FGF-2 expression and localization in RAOSMCs treated for 6 h with either FGF-2 (10 ng/ml) or PDGF-AA (20 ng/ml) or PDGF-BB (20 ng/ml), or with control medium (control). Only PDGF-BB-treated cells show nuclear accumulation of FGF-2. Bar: 50 μM. These experiments were repeated at least three times with similar results.

PDGF-BB, as well as with hrFGF-2 (18 kDa), for 15 min or 6 h. PDGF-AA-treated cells showed ERK-1/2 activation at 15 min but not at 6 h; in contrast, FGF-2- or PDGF-BB induced activation of ERK-1/2 both at 15 min and 6 h (Fig. 1A). Similarly, both PDGF-AA and PDGF-BB induced rapid (15 min) activation of Akt but only PDGF-BB-induced activation was maintained up to 6 h. On the contrary, we could not detect Akt activation upon FGF-2 treatment.

Because HMW FGF-2 localizes predominantly to the cell nucleus and nucleolus, we analyzed the intracellular distribution of FGF-2 in RAOSMC treated with PDGF. Immunofluorescence analysis of PDGF-BB-treated cells showed nuclear and nucleolar accumulation of FGF-2 (Fig. 1C). In contrast, treatment of the cells with PDGF-AA or FGF-2 did not affect the intracellular localization of FGF-2. Thus, these results showed that PDGF-BB specifically upregulates RAOSMC expression of HMW FGF-2, and that this is accompanied by accumulation of FGF-2 in the cell nucleus and nucleolus.

To investigate the intracellular signaling mechanism(s) that mediate PDGF-BB induction of FGF-2 expression we tested the effect of synthetic inhibitors of the ERK-1/2 and PI3-K/Akt pathways. Treatment of RAOSMC with UO126, an inhibitor of MEK-1/2, showed that inhibition of ERK-1/2 activation completely suppressed PDGF-BB-induced expression of FGF-2 (Fig. 2A). In contrast, in hrFGF-2-treated cells UO126 did not significantly affect FGF-2 levels, particularly 18 kDa FGF-2, suggesting that the 18 kDa FGF-2 associated with the FGF-2-treated RAOSMC was indeed of exogenous origin. By immunofluorescence ERK-1/2 inhibition with UO126 blocked the accumulation of FGF-2 in the nucleus and nucleolus of PDGF-BB-treated cells (Fig. 2B).

Because PDGF-BB also induces Akt activation in RAOSMC, we tested the effect of wortmannin, a specific inhibitor of the PI-3K/Akt pathway. The results showed that wortmannin did not block the effect of PDGF-BB on FGF-2 expression although it completely inhibited Akt activation (Fig. 3). In contrast, treatment of the cells with actinomycin D, a transcription inhibitor, suppressed the PDGF-BB-induced upregulation of FGF-2 expression with an effect similar to that of UO126. However, it did not inhibit ERK-1/2 activation by PDGF-BB (Fig. 3).

Thus, these results showed that PDGF-BB upregulates RAOSMC expression and nuclear accumulation of HMW FGF-2 through sustained activation of the ERK-1/2 pathway.

DISCUSSION

The FGF-2 gene encodes different molecular-weight forms that are species-specific and have different intracellular distribution [Bikfalvi et al., 1997]. Several reports have described specific roles for cytoplasmic and nuclear FGF-2 [Bikfalvi et al., 1995; Delrieu, 2000; Claus et al., 2003]. 18 kDa FGF-2 is predominantly localized in the cytoplasm and controls migration and proliferation through autocrine and paracrine interaction with its cell membrane receptors. Conversely, HMW FGF-2 forms localize predominantly to the nucleus and may exert their biological activities, including cell transformation, with a mechanism(s) independent of membrane receptors [Bikfalvi et al., 1997]. A number of studies have identified conditions and/or agonists that control the expression of the specific FGF-2 forms (Table I). The data reported in this study show that PDGF-BB, but not PDGF-AA, specifically upregulates expression of HMW FGF-2, which is accompanied by accumulation of FGF-2 in the nucleus and nucleolus. This effect is mediated by sustained activation of the ERK-1/2 signaling pathway.

Our finding of FGF-2 immunoreactivity associated with the nucleolus of PDGF-BB-treated cells is consistent with an increase of HMW FGF-2 [Arese et al., 1999]. Indeed, although 18 kDa FGF-2 can also localize to the nucleus, it is not found associated with nucleoli [Arese et al., 1999; Pintucci, unpublished observation]. In our experiments treatment of RAOSMC with hrFGF-2 resulted in increased levels of 18 kDa FGF-2 and, to a minor extent, of HMW FGF-2. Inhibition of the ERK1/2 pathway reduced HMW FGF-2 expression but had no effect on the level of 18 kDa FGF-2. These findings indicate that, similarly to PDGF, 18 kDa FGF-2 also upregulates expression of its HMW isoforms with an ERK-1/2-dependent mechanism without affecting the expression of the 18 kDa isoform. The cell-associated 18 kDa FGF-2 detected in FGF-2-treated cells most likely reflects binding of the exogenous growth factor to the cell surface. This conclusion is consistent with our previous finding that treatment of cells genetically deficient in FGF-2 ($FGF\text{-}2^{-/-}$) with recombinant FGF-2 results in accumulation of the growth factor in extracts of the treated cells [Pintucci et al., 2002].

By immunofluorescence cells treated with hrFGF-2 did not show nuclear accumulation of FGF-2. In contrast, Western blotting analysis of these cells showed increased 18 kDa and HMW FGF-2 levels. A possible explanation for this discrepancy is that HMW FGF-2 and 18 kDa FGF-2 can form heterodimers [Pintucci et al.,

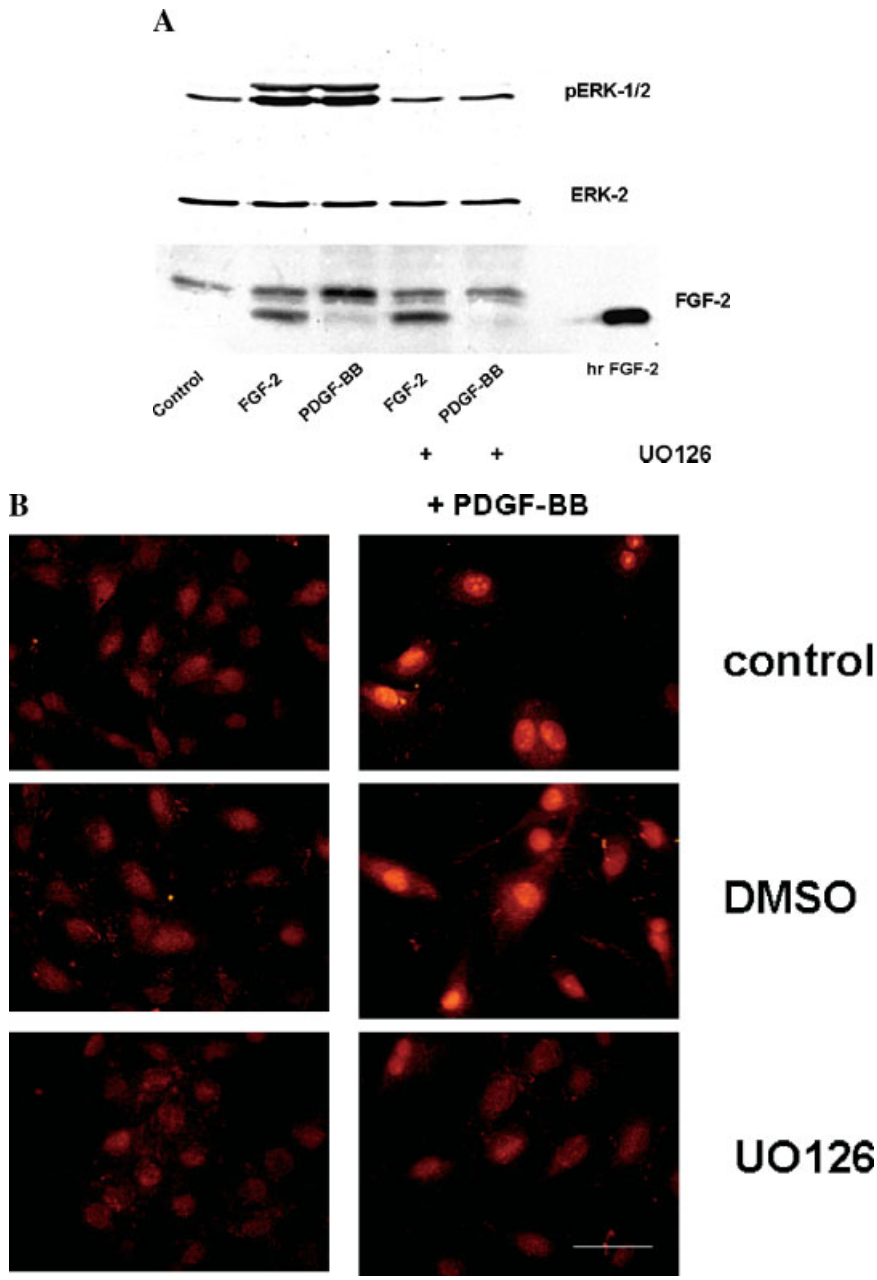

Fig. 2. Inhibition of ERK-1/2 activation blocks PDGF-BB-induced upregulation of FGF-2 expression. **A**: Western blotting analysis of ERK-1/2 activation and FGF-2 expression in RAOSMC treated for 6 h with the indicated growth factors in the presence (+) or absence of the MEK inhibitor UO126. Human recombinant 18 kDa FGF-2 (hr FGF-2) is shown in the rightmost lane as a control. **B**: Immunofluorescence analysis of FGF-2 expression in RAOSMC treated with either control medium (left column) or with PDGF-BB (20 ng/ml) in the absence (control) or presence of UO126 (10 μM) or 0.1% (v/v) DMSO (vehicle). PDGF-BB-treated cells show FGF-2 immunoreactivity in the nucleus, an effect blocked by UO126. Bar: 50 μM. Representative results of at least three experiments are shown.

unpublished results] through which two different isoforms could piggy-back one another into the nucleus or the cytoplasm depending on their relative concentrations. Based on this hypothesis, the nuclear accumulation of 18 kDa FGF-2 would result from increased HMW FGF-2 expression, which would hamper the 18 kDa FGF-2 release from the cell. Alternatively, relatively high concentrations of 18 kDa FGF-2, as those obtained when the growth factor is added exogenously to cultured cells, may prevent the nuclear accumulation of HMW FGF-2. This hypothesis, based on the relative balance of nuclear versus cytoplasmic isoforms of the same

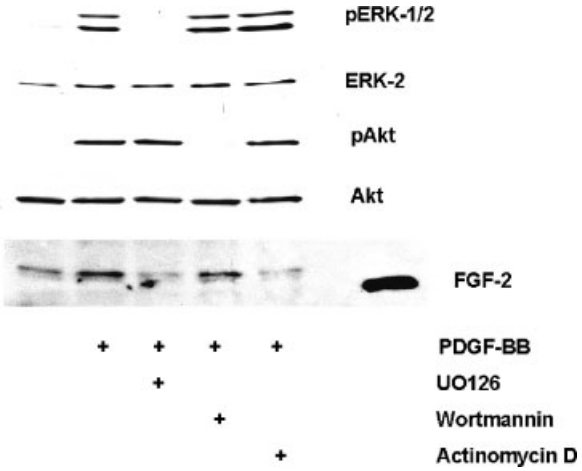

Fig. 3. Upregulation of FGF-2 expression by PDGF-BB is not controlled by the PI3-K/Akt pathway but requires mRNA synthesis. Western blotting analysis of ERK-1/2 and Akt activation and FGF-2 expression in RAOSMC treated for 6 h with control medium (leftmost lanes) or PDGF-BB (20 ng/ml) in the presence (+) or absence of either UO126 (10 μM) or wortmannin (1 μM) or actinomycin D (2 μg/ml). Human recombinant 18 kDa FGF-2 is shown in the rightmost lane as a control. FGF-2 expression is blocked by the MEK inhibitor UO126 and by the mRNA synthesis inhibitor actinomycin D but not by wortmannin, which inhibits Akt activation. These experiments were repeated at least three times with similar results.

gene product, has also been proposed for FGF-3, another member of the FGF family [Goldfarb, 2001]. Therefore, it is possible that the high levels of 18 kDa FGF-2 observed in FGF-2-treated RAOSMC prevent the nuclear accumulation of HMW FGF-2, which maintains a diffuse cytoplasmic distribution (Fig. 1C).

PDGF-BB induces increased expression and nuclear accumulation of HMW FGF-2 through activation of the ERK-1/2 pathway. However, the sustained activation of ERK-1/2 does not appear to be mediated by HMW FGF-2. Our finding that inhibition of transcription with actinomycin D suppresses FGF-2 expression but not ERK-1/2 activation in PDGF-treated cells supports this conclusion. However, the results obtained with actinomycin D do not rule out the possibility that PDGF-BB controls FGF-2 expression at the translational level.

Our data show that PDGF-BB and FGF-2, which induce sustained (6 h) activation of ERK-1/2, upregulate HMW FGF-2 expression. In contrast, PDGF-AA, which only activates ERK-1/2 transiently (15 min), has no effect on FGF-2 expression indicating that upregulation of FGF-2 expression requires sustained activation of ERK-1/2. These findings are consistent with our previous report on the requirement of sustained ERK-1/2 activation for the control of stromelysin-1 expression in endothelial cells [Pintucci et al., 2003]. Several lines of evidence have shown that the duration of MAPK activation determines specific cellular phenotypes, including cell differentiation or proliferation in different cell types [Cowley et al., 1994; Murphy et al., 2002].

Because nuclear HMW FGF-2 can exert its biological role with a mechanism independent of interactions with cell membrane receptors [Bikfalvi et al., 1995; Pasumarthi et al., 1996], our data indicate a novel mechanism through which PDGF can control vascular remodeling. The investigation of HMW FGF-2 and their role(s) in the biological activity of PDGFs should also take into account that in certain cell types HMW FGF-2 has been unequivocally linked to growth inhibition [Quarto et al., 1991b; Dono et al., 1998; Nindl et al., 2004]. Further studies will be needed to understand the role(s) of nuclear accumulation of HMW FGF-2 in smooth muscle cells in vivo, and their effect(s) on the SMC functions involved in the vessel wall remodeling that characterizes a variety of vascular pathologies. The findings that nuclear accumulation of FGF-2 correlates with certain tumors and their aggressiveness [Joy et al., 1997; el-Hariry et al., 1997; Fukui et al., 2002; Krejci et al., 2003; Polnaszek et al., 2003] also

TABLE I. Stimuli/Conditions and Proposed Mechanisms That Upregulate the Nuclear Forms (HMW) of FGF-2

Cell type	Stimulus/condition	Proposed mechanism	Reference
Bovine adrenal medullar cells	Carbachol, forskolin	cAMP activation	Stachowiak et al. [1994]
Human skin fibroblasts	Heat shock, oxidative stress	Translational control	Vagner et al. [1996]
SK-Hep-1	Cell transformation		
Human astrocytes, human glioma cells	Low cellular density	Release from contact inhibition	Joy et al. [1997]
Rat osteoblasts	Aging	Translational control	Cowan et al. [2003]
Rat microvascular endothelial cells	17β-estradiol	Translational control	Garmy-Susini et al. [2004]
Rat osteoblastic Py 1a line	Prostaglandin F2α	Increased ERK-2 expression	Sabbieti et al. [2005]
Rat aortic smooth muscle cells	PDGF-BB	Prolonged ERK-1/2 activation	This study

indicate that the relevance of FGF-2 isoform expression is not limited to the cardiovascular system, but may underlie novel pathophysiological roles for this pleiotropic growth factor.

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