Matrix Metalloproteinase-2 Expression by Vascular Smooth Muscle Cells Is Mediated by Both Stimulatory and Inhibitory Signals in Response to Growth Factors

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In response to growth factors, vascular smooth muscle cells (VSMCs) undergo a phenotypic modulation from a contractile, non-proliferative state to an activated, migratory state. This transition is characterized by changes in their gene expression profile, particularly by a significant down-regulation of contractile proteins. Platelet-derived growth factor (PDGF)-BB has long been known to initiate VSMC de-differentiation and mitogenesis. Insulin-like growth factor (IGF)-I, on the other hand, has differing effects depending on the model studied. Here, we report that both IGF-I and PDGF-BB stimulated VSMC de-differentiation of rat heart-derived SMCs in culture, although only PDGF-BB was capable of inducing proliferation. Although both PDGF-BB and IGF-I stimulation resulted in decreased smooth muscle α-actin expression and increased matrix metalloproteinase (MMP)-2 expression, the response to IGF-I was significantly more rapid. The increased MMP-2 expression in response to both growth factors was due to increased transcription rates and was dependent on the action of phosphatidylinositol 3-kinase (PI3K) and its downstream effector, Akt. Both PDGF-BB and IGF-I activated PI3K/Akt to similar degrees; however, only PDGF-BB concomitantly stimulated an inhibitory signaling pathway that antagonized the effects of Akt but did not alter the extent or duration of Akt activation. Together, these findings suggest that changes in MMP-2 expression are part of the program of VSMC phenotypic modulation and that both PDGF-BB and IGF-I, despite their different abilities to induce proliferation in this model, are capable of inducing VSMC activation.

It is becoming increasingly clear that VSMCs, which are of fundamental importance in blood vessel structure, function, and dynamics, also play a critical role in the development of vascular lesions, particularly during athrogenesis. One reason VSMCs are able to participate in a variety of normal and disease processes is that they have the ability to alter their phenotype from a contractile, non-proliferative state to a migratory, non-contraction state, often accompanied by proliferation (1). During this phenotypic modulation, VSMCs down-regulate a set of contractile proteins, including SM α-actin, SM myosin heavy chain, calponin, and SM22α, and up-regulate genes involved in cell migration, extracellular matrix deposition, cell growth, and vessel remodeling (2, 3). This VSMC plasticity contributes to tissue remodeling as well as to serious vascular complications associated with disease (1), particularly in athrogenesis, during which VSMCs from the medial layer of vessels migrate to the intima, where they participate in the development of the fibrous plaque characteristic of the disease (5). VSMCs demonstrating the de-differentiated, or synthetic, phenotype in vivo have been found in arterial intimal lesions in atherosclerosis, as well as in most other vascular disease states (1).

Along with decreased contractile protein expression, VSMC de-differentiation involves the increased synthesis of extracellular matrix-degrading proteinases, which play an important role in VSMC proliferation and migration (6). MMP expression, in particular, is associated with the VSMC synthetic state (4). MMPs comprise a family of Zn2+-dependent extracellular enzymes that, along with their endogenous inhibitors, the TIMPs, regulate a wide variety of processes associated with vascular structure and remodeling (7). MMP-2 is known to play several important roles during changes in vascular structure associated with both normal and disease processes. The stimulation of VSMCs with PDGF leads to increased expression of MMP-2 (8), and this up-regulation was linked to increased cell migration (8). Cultured VSMCs, which often mimic the synthetic state, typically express MMP-2 (4, 9). Proliferating VSMCs express higher mRNA levels of MMP-2 and TIMP-2, which plays a dual role in MMP-2 activation and inhibition, and lower levels of TIMP-1, which inhibits MMP function (10). In vivo, MMP-2 expression by VSMCs has been linked to a variety of pathological situations, particularly in atherosclerotic plaques, which show significantly increased MMP-2 expression and activation most prominently in vulnerable regions (11, 12).

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MMP-2 Expression by Smooth Muscle Cells

Migration of VSMCs into the intima during atherogenesis is dependent on MMP activity, and VSMCs in atherosclerotic lesions show decreased contractile protein expression and increased MMP expression, indicative of the activated state (3). VSMCs derived from vessels of patients with aneurysms expressed significantly more MMP-2 than normal cells, with a concomitant increase in invasive capability (13). Importantly, MMP-2-deficient mice did not form aneurysms in an experimental model; this study also found that MMP-2 derived from the mesenchyme was the source for aneurysm formation (14). Consistent with the role of MMP-2 in vessel remodeling, blocking MMP activity can reverse or prevent the development of vascular pathologies. Inhibiting MMPs blocked proliferation of VSMCs derived from vessels of patients with aneurysms increased MMP expression, indicative of the activated state (3). Importantly, we show that PDGF-BB stimulates both processes. MMP-2 specifically inactivates adrenomedullin, a vasodilator, mediated by spectrophotometry and reverse-transcribed using PowerScript reverse transcriptase (BD Biosciences) and oligo(dT). RT-PCR, Semiquantitative PCR, and Real-time PCR—For wild-type and stable cells, RNA was harvested using the PureScript RNA Isolation kit (Genta Systems, Minneapolis, MN), and for nucleofected cells, RNA was harvested using an RNAqueous kit (Ambion, Austin, TX). Total RNA was quantified by spectrophotometry and reverse-transcribed using PowerScript reverse transcriptase (BD Biosciences) and oligo(dT).

EXPERIMENTAL PROCEDURES

Cell Culture—Wistar-Kyoto rat heart smooth muscle cells (WKY-HSMCs, kindly provided by Dr. Paula Grammas) were grown to post-confluence in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin. Cells were washed once with phosphate-buffered saline, and then placed on serum-free Dulbecco’s modified Eagle’s medium supplemented with 0.2% lactalbumin hydrolysate plus the antibiotic mixture. After 48 h of serum starvation, cells were treated with either the indicated concentrations of recombinant rat PDGF-BB, recombinant human IGF-I, or recombinant human M-CSF (all from R & D Systems, Minneapolis, MN). In experiments involving the PI3K inhibitor, LY294002, the JNK inhibitor SP600125 (both from Biomol, Plymouth Meeting, PA), and the MEK inhibitor, U0126 (Promega, Madison, WI), cells were pretreated with the indicated concentration of inhibitor for 15 min prior to growth factor stimulation. In some cases, cells were co-stimulated with 400 μM 1-mimosine (Biomol) plus PDGF-BB. Cell lysates were harvested with either boiling SDS lysis buffer (1% SDS, 10 mM Tris–Cl) or with cold radiioimmune precipitation assay lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate, 2 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 8, and 1 mM sodium vanadate) supplemented with 1X Protease Inhibitor Mixture (Sigma).

Immunocytochemistry—WKY-HSMCs were cultured on glass coverslips as above and treated with the indicated growth factors in serum-free medium. Following treatment, cells were fixed with 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) for 10 min. Fixed cells were stained with rhodamine phallolidin (Invitrogen) to visualize stress fibers. Coverslips were mounted in SlowFade® Gold anti-fade reagent (Invitrogen), and stress fibers were visualized using an Olympus AX70 fluorescence microscope (Melville, NY) with QCapture 2.68 software (IBM, Armonk, NY).

Stable Cell Lines and Nucleofection—WKY-HSMCs were transfected with Lipofectamine and PLUS Reagents (Invitrogen) and selected for 3 weeks with 10 μg/ml blasticidin (Invitrogen). After selection, cells were allowed to grow to confluence on maintenance (2.5 μg/ml) blasticidin and used to seed experiments. For transient expression of signaling pathway effectors, cells were nucleofected using the AMAXA Nucleofector (Amaxa, Inc., Gaithersburg, MD) with the Primary Smooth Muscle Cell supplement per manufacturer’s instructions.

RT-PCR, Semiquantitative PCR, and Real-time PCR—for wild-type and stable cells, RNA was harvested using the PureScript RNA Isolation kit (Genta Systems, Minneapolis, MN), and for nucleofected cells, RNA was harvested using an RNAqueous kit (Ambion, Austin, TX). Total RNA was quantified by spectrophotometry and reverse-transcribed using PowerScript reverse transcriptase (BD Biosciences) and oligo(dT). Semiquantitative PCR and real-time PCR were performed as described previously (26). Primers used for semiquantitative PCR: calponin (5‘-CACTTTAACCAGGTCTCTGCTAC-3‘ and 5‘-GCCAATGATGTTCTGCTCTCTCT-3‘); MMP-2 (5‘-TCAGATCCTGGTGTAGCTTCTCT-3‘ and 5‘-AAGTGTAAAGGAGAACAAAGAGG-3‘); MTI-MMP (5‘-GGACTGGAGAGGAGAGGAGAAGGACTGAGA-3‘ and 5‘-GGGGATGGAAGGAGGAGGAGAAGGACTGAGA-3‘); SM-α-actin (5‘-CCACTGCTGCTCTCTCTTTCTCTCCTCCCC-3‘ and 5‘-GAGGGTGGGGGCGTGACATCCACTGAGAAGGACTGAGA-3‘); SM-myosin heavy chain (5‘-GCACGAGAACAAAGAATCCAGGACTGAGAAGGACTGAGA-3‘ and 5‘-ATGGAAGGAGTCCTCGTTTCCCTCTCCTGAGAAGGACTGAGA-3‘); TIMP-1 (5‘- ACCACCTTATTACCAGGTGTAGAGAGGACTGAGAAGGACTGAGA-3‘ and 5‘-GAGGTACGTATCCGCTCCACAAGACA-3‘); TIMP-2 (5‘-CGTGGAGCAGGGAGGAGGACTGAGAAGGACTGAGA-3‘ and 5‘-GGGGATGGAAGGAGGAGGAGGAGGACTGAGA-3‘); TIMP-3 (5‘-GCCCTGCAAGTTGACACACTGAGAAGGACTGAGA-3‘ and 5‘-GGGGATGGAAGGAGGAGGAGGAGGACTGAGA-3‘). Real-time PCR was performed using the following primers: MMP-2 (5‘-CAAAAGAAGTGGTGCTGACCGCTC-3‘ and 5‘-CTGGAGAAGACTGAGAAGGACTGAGAAGGACTGAGA-3‘); TIMP-2 (5‘-AAAGAGATGGGCAAGATGCAC-3‘ and 5‘-GGAAGACTGAGAAGGACTGAGAAGGACTGAGA-3‘). Semiquantitative PCR images were captured and quantified using the ChemiImager 4400 and...
Western Blot Analysis—SDS cell lysates were sonicated prior to analysis, while radioimmune precipitation assay buffer cell lysates were centrifuged to remove insoluble material prior to BCA analysis (Pierce). Equal quantities of protein were resolved by SDS-PAGE, and gels were transferred to a 0.2-μm nitrocellulose membrane. Membranes were blocked with 3% bovine serum albumin and probed with the following primary antibodies: α-phospho-ERK, α-phospho-Akt (S473), α-Akt, and α-ERK (all antibodies from Cell Signaling, Beverly, MA). Proteins were visualized by chemiluminescence using secondary antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA). Blots were imaged using an Epi Chemi II Darkroom Imaging System with Lab Works 4.0 Software (UVP, Upland, CA).

Zymogram Analysis—Non-reduced conditioned media samples were resolved by SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, gels were incubated with rocking in 2.5% Triton X-100 for 30 min at room temperature, rinsed, and then incubated in 50 mM Tris-Cl, pH 8.0, 5 mM CaCl2, 0.02% NaN3 overnight at 37 °C. After incubation, gels were visualized with Coomassie R-250 stain and captured using the Chemi-Imager 4400.

Transcription Rate Analysis—Oligonucleotide primers were designed flanking the second intron of the MMP-2 gene and used to amplify that intron from Wistar-Kyoto rat genomic DNA using the following primers: 5′-AGATGTGGGCAACTAAACTGCTTTTC-3′ and 5′-TCATCCACTGCTCAGGG-TCCAGGTC-3′. This fragment was cloned and later nick-labeled with γ-[32P]dCTP for use as a probe in Southern blot analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) and DNase I-treated. RNA was then reverse-transcribed using random hexamers. The cDNA was then diluted 10-fold for use in semiquantitative PCR using the following primers to amplify a region within the second intron of the MMP-2 gene (5′-CATCCAGGGGCTTTGACATACAGAG-3′ and 5′-GTC-AAACAAAGGGCAGAGCTCTCA-3′). The PCR products were separated in 1.5% agarose gels, which were denatured and neutralized before transfer to a nylon membrane. Amplimers were detected using the MMP-2 intron 2 probe described above. Phosphorimages were made using an Imaging Screen K and the Molecular Imager® FX (Bio-Rad), quantified using Quantity One 1-D Analysis software (Bio-Rad), and normalized to TIMP-2 mRNA levels obtained by semiquantitative RT-PCR.

Proliferation Assay—Cells were plated into 96-well plates, grown to confluence, and serum-starved for 48 h. Cells were then stimulated in fresh serum-free medium plus the indicated concentrations of growth factor. After 48 h of growth factor stimulation, proliferation was measured using a Cell Titer 96® non-radioactive cell proliferation assay (Promega), per the manufacturer’s instructions.

Luciferase Assays—Cells were plated into various multiwell plates and transiently transfected overnight with Lipofectamine and PLUS reagents (Invitrogen). In the case of co-transfections of reporter constructs with signaling pathway effectors, lysates were collected and processed after 24 h of serum starvation per manufacturer’s instructions (Promega luciferase assay system). In transfections involving reporter constructs alone and co-transfections of reporter constructs with chimeric receptors, cells were treated with the indicated growth factor for 24 h prior to collection. Light production was measured using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

cDNA and Reporter Constructs—The constructs used are as follows: 1) pCI-Blast (pCIB), the blasticidin resistance gene and associated promoters were removed from pUB/Bsd (Invitrogen) and ligated into pCI-neo (Promega) from which the neomycin resistance gene and associated promoter had been removed. 2) pCI-Blast-mAkt and pIRES2-EGFP-mAkt, myristoylated bovine Akt was PCR-amplified from pCMV3(3my)-Akt-HA (a kind gift from Dr. Mark Coggeshall) and cloned into pBluescript II SK+ (Stratagene, La Jolla, CA). Myristoylated bovine Akt was then subcloned into pCI-Blast and pIRES-EGFP. 3) pCI-Blast-p110α-CA, constitutively active bovine p110α was PCR-amplified from pEF-p110α-CA (a kind gift from Dr. Mark Coggeshall) and directionally inserted into pCI-Blast. 4) Chimeric CSF-1R-PDGFRβ receptor, the extracellular domain of the human CSF-1R was PCR-amplified from Integrated Molecular Analysis of Genomes (IMAGE) clone 52844461 (Invitrogen) with XbaI- and MluI-containing 5′ and 3′ primers, respectively, which amplified 37 bases of the 5′-untranslated region and codons for amino acids 1 through 506. The trans- membrane and intracellular domain of the human PDGFRβ was PCR-amplified from Mammalian Gene Collection clone 34052 (ATCC) using MluI- and Sall-containing 5′ and 3′ primers, respectively, which amplified codons for amino acids 532 to the stop codon. Each receptor fragment was blunt-ligated into pl Bluescript II SK+. The CSF1R fragment was removed from pl Bluescript II SK+ with XbaI and MluI and cloned into pl Bluescript II SK+ containing PDGFRβ and cut with XbaI and MluI. The entire chimeric receptor was removed from pl Bluescript II SK+ with XbaI and Sall and ligated into pCI-Blast. 5) Chimeric CSF-1R-PDGFRβ receptor mutants, the Y740F,Y751F (amino acid numbering corresponds with the human PDGFRβ) mutant was generated through two rounds of mutagenic PCR performed on the pl Bluescript II SK+ PDGFRβ construct and inserted into a pCI-Blast construct containing only the extracellular domain of CSF-1R. 6) Rat MMP-2-promoter-pGL3B, the region from −1561 to −244 (+1 = A of translation start) of the rat MMP-2 promoter was cloned into pGL3Basic (27). Human MMP-2-promoter-pGL3B, the region −2.6 kb upstream of the translation start of MMP-2 was PCR-amplified from BAC clone RPC111-M17 (Children’s Hospital Oakland Research Institute, Oakland, CA). The region, from −2565 to −228 of the human MMP-2 promoter, was subsequently cloned into pGL3-Basic.

RESULTS

Phenotypic Modulation of VSMCs Occurs Concomitantly with Increased MMP-2 Expression but Is Delayed in Response to PDGF-BB When Compared with IGF-I—In response to several extracellular factors, VSMCs execute a program of de-differentiation that results in the down-regulation of contractile mark-
ers and the up-regulation of genes associated with extracellular matrix remodeling and cell migration (1–4). We have developed a culture model that mimics a number of aspects of this VSMC phenotypic modulation. This involves growing WKY-HSMCs isolated from rat heart microvasculature to post-confluence, followed by 2 days of serum deprivation. Previous studies have demonstrated that such treatment potentiates the maintenance of the contractile VSMC phenotype (28). Similar to other previously described cultured adult aortic SMCs, WKY-HSMCs assume a hill-and-valley monolayer when post-confluent and express the known markers for the differentiated SMC phenotype (29, 30). Importantly, they express SM myosin heavy chain, which distinguishes SMCs from other contractile cells such as myofibroblasts and is considered to be a key marker of differentiated SMCs (3).

To characterize our VSMC model, cells were grown on coverslips and subsequently fixed and stained to examine stress fiber organization. In the absence of de-differentiating factors, WKY-HSMCs displayed prominent stress fibers, consistent with the contractile phenotype, given that stress fiber formation is dependent on force generation (32). Stimulation with IGF-I or PDGF-BB induced a gradual loss of stress fibers 48 h post-stimulation, consistent with a migratory, non-contractile phenotype (Fig. 1).

An examination of gene expression revealed that, in response to IGF-I, WKY-HSMCs down-regulated their expression of several markers of the contractile/differentiated VSMC phenotype, including SM α-actin, SM myosin heavy chain, SM22α, and calponin (Fig. 2A). Several studies have demonstrated that IGF-I stimulates the migration of VSMCs in part by increasing the expression of proteolytic enzymes (1–4, 31). In agreement with this, we found that, concomitant with the loss of contractile markers, IGF-I stimulated an increased expression of MMP-2, both at the message level (Fig. 2A) and in protein accum-

**FIGURE 1.** PDGF-BB and IGF-1 induce changes in VSMC morphology. VSMCs were grown to post-confluence on coverslips, then serum-starved for 48 h. Culture medium was then replaced with fresh, serum-free medium (left panel) or serum-free medium plus 25 ng/ml IGF-I or PDGF-BB (middle and right panels, respectively). 48 h post-stimulation, cells were fixed and subsequently stained for stress fiber visualization using rhodamine phalloidin. Images magnified 750 × were acquired at similar exposure levels. Representative images are shown from three independent experiments.

**FIGURE 2.** Phenotypic modulation of VSMCs induced by PDGF-BB is delayed in comparison to IGF-I. VSMCs were grown to post-confluence, then serum-starved for 48 h. Culture medium was then replaced with fresh, serum-free medium plus the indicated growth factor. A, cells were stimulated with IGF-I (25 ng/ml) for the indicated times, and total RNA was isolated for analysis of MMPs, TIMPs, and contractile markers by RT-PCR. Note that the PCR images are inverted. B, cells were stimulated with IGF-I (25 ng/ml) or PDGF-BB (25 ng/ml) for the indicated times, and total RNA was analyzed for the presence of MMP-2, smooth muscle α-actin, or TIMP-2. C, cells were stimulated with several concentrations of IGF-I or PDGF-BB, and total RNA was isolated at the times indicated. Real-time PCR was used to evaluate the MMP-2/TIMP-2 ratio. D, medium conditioned by cells stimulated with 25 ng/ml IGF-I or PDGF-BB was analyzed by gelatin zymography to detect proMMP-2 and activated MMP-2 and by Western blot to detect TIMP-2.
MMP-2 Expression by Smooth Muscle Cells

Growth factor-mediated up-regulation of MMP-2 mRNA occurs through increased transcription. A, VSMCs were transfected with a luciferase reporter gene driven by the rat MMP-2 promoter, and cells were then sub-cultured into 48-well plates. After 2 days of serum starvation, cells were stimulated with 25 ng/ml IGF-I or PDGF-BB or with medium alone for an additional 24 h. Luciferase activity was measured in four individual wells per treatment for each of three experiments and normalized to no treatment control. B, total RNA was isolated from VSMCs that had been stimulated with IGF-I or PDGF-BB as described above, and heterogeneous nuclear RNA levels were measured using PCR primers specific for intron 2 in the MMP-2 gene. To control for the presence of genomic DNA, aliquots of each sample that were not reverse transcribed (No RT) were PCR-amplified. Southern blotting was used to detect PCR-amplified products. C, three separate experiments were used to quantify the relative transcription rate of MMP-2. D, aliquots of each sample from C were also evaluated for the relative levels of TIMP-2 mRNA by RT-PCR.

The Delay in PDGF-BB- versus IGF-I-induced MMP-2 Regulation Occurs at the Transcriptional Level—Previous studies have shown that decreased expression of SM α-actin during VSMC phenotypic modulation occurs at least in part due to decreased transcription. To determine if increased MMP-2 expression is similarly due to growth factor-mediated changes in MMP-2 transcription, we developed a luciferase reporter construct incorporating a fragment (\( \sim 1.3 \) kb) of the rat MMP-2 promoter and used this in transient transfection assays. Cells were transfected with the reporter construct, and then split into parallel culture wells for treatment with growth factors. Both PDGF-BB and IGF-I stimulated increased activity of the MMP-2 promoter (Fig. 3A), suggesting that both growth factors have the ability to regulate MMP-2 transcription; however, no delay could be detected in the case of PDGF-BB stimulation (data not shown). Titration of the reporter construct had no effect on PDGF-BB-mediated activation, suggesting that the transiently transfected plasmid did not mirror all aspects of the regulation of the endogenous MMP-2 gene. To examine the transcription rate of the endogenous MMP-2 gene in VSMCs stimulated with IGF-I and PDGF-BB, we isolated total RNA from cells, then evaluated the steady-state levels of MMP-2 heterogeneous nuclear RNA using primers specific for the second intron of the MMP-2 primary transcript. As illustrated in Fig. 3 (B and C), IGF-I-stimulated cells initiated increased MMP-2 transcription between 8 and 12 h post-stimulation, whereas PDGF-BB-stimulated cells showed a 12-h delay to reach comparable rates. The absence of signal in the no-RT control indicates that the signal being examined here was not simply due to genomic contamination. Thus, the delay in PDGF-BB- versus IGF-I-mediated regulation of MMP-2 occurred at the level of transcription.

Some reports have linked VSMC phenotypic modulation with a proliferative response, while others have suggested that this process occurs independent of mitogenesis (1). PDGF-BB is a potent mitogen for VSMCs and elicited cell proliferation in our VSMC model (Fig. 4A). IGF-I, on the other hand, was a poor mitogen (Fig. 4A), thus demonstrating that at least some aspects of VSMC phenotypic modulation can occur independently of proliferation. Based on these findings, we addressed the possibility that the delay in PDGF-BB-mediated MMP-2 up-regulation may be the result of a global transcriptional pause due to PDGF-BB-induced cycling. To test this idea, we stimulated VSMCs with PDGF-BB plus the S-phase inhibitor 1-mimosine and examined MMP-2 levels. The addition of 1-mimosine resulted in a somewhat more robust, but not accelerated, up-regulation of MMP-2 (Fig. 4B), suggesting that the delay in PDGF-BB-mediated MMP-2 up-regulation was not likely due to general transcriptional pausing.
PI3K Mediates the Increased Expression of MMP-2—Based on the significant differences in the ability of PDGF-BB and IGF-1 to induce a mitogenic response, we investigated the ability of these growth factors to stimulate the ERK and PI3K signaling pathways in our VSMC model, both of which have been implicated in VSMC phenotypic modulation. We found that PDGF-BB was able to stimulate prolonged ERK and Akt phosphorylation, whereas IGF-1 showed a very modest ERK activation, with PI3K activation comparable to that observed with PDGF stimulation (Fig. 5, A and B). Because IGF-1 does not stimulate robust ERK signaling in our VSMC model, we focused on the PI3K pathway as a candidate for MMP-2 up-regulation. It was recently demonstrated in a tumor cell model that IGF-I-mediated increases in MMP-2 expression was dependent on PI3K signaling (34). In our VSMC model, MMP-2 up-regulation induced by either IGF-I or PDGF-BB was similarly dependent on PI3K, based on the ability of the PI3K-specific inhibitor, LY294002, to inhibit this effect (Fig. 6, A–D). Note that the 15 μM dose of LY294002 was fully capable of inhibiting PI3K activity and actually resulted in increased ERK activation in response to both PDGF-BB and IGF-1 (Fig. 6C), as previously demonstrated (35). The positive role of the PI3K pathway was further demonstrated by the ability of a constitutively active form of the p110α subunit of PI3K, as well as active forms of the downstream kinase, Akt, to increase transcription of the rat MMP-2 promoter transiently co-transfected into cells (Fig. 6D and E). This was dose-dependent and was also observed when these constructs were co-transfected with the human MMP-2 promoter (Fig. 6F). Similarly, introducing these constructs into VSMCs by nucleofection resulted in the up-regulation of the endogenous MMP-2 gene (Fig. 6G and H). Transfection by the nucleofection technique typically resulted in 30% to 40% of the cells expressing the transgene of interest, so our results do not reflect the degree of up-regulation that would occur had all cells been transfected. Together, these results suggest that PI3K/Akt signaling is both necessary and sufficient for MMP-2 up-regulation.

To further examine the role of PI3Ks in MMP-2 up-regulation during VSMC phenotypic modulation, we constructed a chimeric receptor composed of the extracellular domain of CSF1-R and the trans-membrane and intracellular domains of the human PDGFRβ. The tyrosines at amino acid positions 740 and 751 (based on the position of the amino acids in the human PDGFRβ) were mutated to phenylalanines. The ability of the mutated chimeric receptor to stimulate growth, activate signaling pathways, and, finally, to regulate MMP-2 was examined in comparison with wild-type chimeras in stably transfected cells. The non-mutated chimeric receptor functioned similarly to the endogenous PDGFRβ, based on its ability to stimulate growth over varying concentrations of ligand (Fig. 7A), and to up-regulate the rat MMP-2 promoter construct when co-transfected into VSMCs (Fig. 7B). In all cases, PDGF-BB treatment was used to monitor the function of the endogenous PDGFRβ, whereas activation of the chimeric receptor involved stimulation with M-CSF. Mutation of the two tyrosine residues known to be involved in PI3K activation (Y740F,Y751F) reduced its ability to stimulate growth in response to M-CSF (Fig. 7A), and the PI3K mutant chimeric receptor was unable to stimulate MMP-2 promoter activity in the transient co-transfection assay (Fig. 7B). We next stably introduced these chimeric receptors into VSMCs to determine their effects on the endogenous MMP-2 promoter. Although the wild-type chimeric receptor was fully capable of activating both ERK and Akt, the mutated form was unable to stimulate Akt phosphorylation (Fig. 7C). In all cases, stimulating stably transfected pools of cells with PDGF-BB resulted in the activation of both ERK and Akt (Fig. 7C) and up-regulation of MMP-2, as expected, because the endogenous PDGFRβ is stimulated by this ligand (Fig. 7A), and the PI3K mutant chimeric receptor was unable to stimulate MMP-2 promoter activity in the transient co-transfection assay (Fig. 7B).
able to up-regulate MMP-2 expression, whereas the mutated receptor could not (Fig. 7, D and E). In fact, stimulation of the mutated receptor resulted in a significant decrease in MMP-2 expression, suggesting that an inhibitory signal is activated by PDGFR\(\beta\)-mediated signal transduction.

We next tested the potential contribution of the PDGFR\(\alpha\), which will also bind PDGF-BB. Although WKY-HSMCs express the PDGFR\(\alpha\) message, they did not proliferate in response to PDGF-AA (Fig. 8A), which is typical of differentiated adult VSMCs (29, 30). In addition, PDGF-AA induces some ERK activation but no detectible Akt phosphorylation (Fig. 8B). Finally, in addition to the inability of PDGF-AA to down-regulate SM\(\alpha\)-actin, it was unable to induce MMP-2 up-regulation (Fig. 8C), consistent with its inability to activate PI3K.

**PDGF-BB Induces Both Stimulatory and Inhibitory Signals That Influence MMP-2 Regulation**—In studies in which PI3K activity was blocked or unable to become activated, we noted an actual decrease in the level of MMP-2 message (Figs. 6 and 7, respectively). This implies that a negative signal, separate from the PI3K signal, may be involved in this down-regulation and may likely be responsible for the delay observed in PDGF-BB-versus IGF-I-mediated MMP-2 up-regulation. We reasoned that, if PDGF induces both stimulatory and inhibitory pathways with regard to MMP-2 regulation, then stimulating VSMCs with both PDGF-BB and IGF-I should result in a PDGF-BB-like delay. Indeed, co-stimulation resulted in a delay in MMP-2 up-regulation when both PDGF-BB and IGF-I were used to stimulate VSMCs (Fig. 9A). The activation of both Akt and ERK by co-stimulation with PDGF-BB and IGF-I resembled that induced by PDGF-BB alone (Fig. 9B), consistent with the idea that a separate, inhibitory signal is induced by PDGF-BB, and that the stimulatory, PI3K/Akt-mediated signal is blunted downstream of Akt activation. Based on our findings that PDGF-BB, but not IGF-I, stimulates a robust ERK activation, we investigated the possibility that one or more MAPK path-
ways are involved in repressing PDGF-BB-mediated MMP-2 up-regulation. The MEK inhibitor, U0126, was unable to accelerate MMP-2 up-regulation when added to cultures stimulated with PDGF-BB (Fig. 9C), whereas the JNK inhibitor, SP600125, was able to potentiate up-regulation (Fig. 9D). Although not definitive proof that JNK activation itself contributes to the suppression of VSMC phenotypic modulation, this result does support the idea that PDGF-BB stimulation leads to multiple independent signaling pathways that have antagonistic effects on MMP-2 expression.

**DISCUSSION**

We present evidence that the expression of MMP-2 is linked to the program of VSMC phenotypic modulation. We also demonstrate that both PDGF-BB and IGF-I stimulated MMP-2 transcription through the PI3K/Akt pathway and that this pathway is both necessary and sufficient for MMP-2 up-regulation in VSMCs. Finally, we show that PDGF-mediated phenotypic modulation involves both stimulatory and inhibitory signals that cause a delay in the up-regulation of MMP-2. Like PDGF-BB, IGF-I decreased contractile protein expression and increased MMP-2 levels, and its ability to do this at a significantly accelerated rate is due to its stimulation of primarily the potentiating pathways, not the inhibitory pathway(s). Interestingly, co-stimulation of VSMCs with both PDGF-BB and IGF-I resulted in a delayed response similar to that seen with PDGF-BB stimulation alone. PI3K signaling in co-stimulated cells was not impaired, suggesting that a separate, negative signal is generated that temporarily overwhelms the stimulatory signal generated by both PDGF-BB and IGF-I. This is supported
by the observation that VSMCs stimulated with PDGF-BB in the presence of a PI3K inhibitor demonstrate decreased MMP-2 mRNA levels and by studies in which a chimeric receptor unable to activate PI3K similarly resulted in MMP-2 down-regulation. Previous studies have suggested that PI3K activation causes the de-differentiation of VSMCs and stimulates VSMC migration, and this is consistent with the IGF-1-mediated MMP-2 up-regulation seen in our model (35–37). This pathway has been previously linked to increased MMP-2 expression in fibroblasts, gliomas, and mammary epithelial cells (38–40). Our findings support the idea that Akt activity alone is sufficient to up-regulate MMP-2 in VSMCs.

The role of PDGF-BB as a VSMC activator is well established; that of IGF-1 is less clear. On one hand, arterial injury is accompanied by increases in IGF-1 expression, and atherosclerotic lesions have elevated levels of IGF-1 (41, 42). Overexpression of IGF-1 in VSMCs in transgenic mice resulted in increased neointimal formation in response to injury (43). IGF is also associated with VSMC de-differentiation during disease processes (44) and has been reported to act in concert with PDGF in VSMC activation (45). On the other hand, IGF-1 has been shown to maintain the differentiated SMC phenotype, while factors such as PDGF induced de-differentiation (46, 47). This was most pronounced when cells were cultured on laminin and...
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was potentiated by the IGF-I-mediated activation of PI3K. In these cells, IGF-I induced the activation of PI3K, but not ERK, JNK, or p38 MAPK pathways, whereas PDGF-BB induced both PI3K and MAPK pathways, leading to de-differentiation. Interestingly, the visceral SMCs studied expressed higher levels of SM α-actin when de-differentiated, which is contrary to what occurs in VSMCs (48), suggesting that these cell types regulate gene expression quite differently. Other studies have shown that long term IGF-I stimulation of VSMCs results in increased contractility, whereas short term stimulation leads to reduced contractility (49). In other models, IGF-I induced VSMC proliferation and de-differentiation through the activation of MAPK and PI3K pathways (50, 51). However, in a number cases, IGF-I failed to activate ERK and was poorly mitogenic (52, 53). We observed that IGF-I stimulates increased MMP-2 expression independent of a mitogenic response which, in addition to its ability to alter the contractile state of VSMCs, supports its role in VSMC de-differentiation. However, IGF-I-mediated changes in MMP-2 expression may be highly cell type-dependent. For example, autocrine stimulation by IGF-I stimulated a decreased level of MMP-2 expression in mesangial cells isolated from non-obese diabetics rats, and this was linked to both PI3K and ERK activity (54). In contrast, overexpression of IGF-IR increased MMP-2 expression, whereas antisense-mediated down-regulation decreased MMP-2 expression, in tumor cells (55). Most primary SMCs, when exposed to culture conditions, show decreased SM α-actin and increased MMP-2 expression, concomitant with de-differentiation (9). This is consistent with our observations that show that VSMC de-differentiation in response to growth factor involves MMP-2 up-regulation.

The nature of the inhibitory pathways remains in question. We have found that the JNK inhibitor, SP600125, but not the ERK inhibitor, U0126, potentiates increased MMP-2 expression when used in cultures stimulated with PDGF-BB. However, given that these inhibitors, particularly SP600125, may inhibit multiple pathways (56), these results are not definitive. We have observed that VSMCs grown to sub-confluence showed an even greater delay in MMP-2 up-regulation in response to PDGF-BB when compared with post-confluent cells. Previous studies have shown that PDGF-BB will induce a decrease in VSMC markers in sub-confluent cells, but not in post-confluent cells, and that inhibiting phosphatase activity in post-confluent cells reverses this effect (57). High density cell cultures stimulate the activity of phosphatases that suppress epidermal growth factor and PDGF-mediated signaling in epithelial cells (58) and ERK-dependent signaling in endothelial cells (59). Interestingly, basic fibroblast growth factor stimulated the expression of MMP-2 and TIMP-2 in quiescent, but not proliferating, SMCs, comparable to our observations with PDGF-stimulated VSMCs (60). It has been demonstrated in a tumor cell model that, in response to increased concentrations of IGF-I, IGF-I-mediated increases in MMP-2 expression become significantly diminished due to increased activation of ERK (34). In that study, it was found that low to moderate levels of IGF-I caused the activation of PI3K/Akt pathways, which in turn inhibited the ERK pathway through Akt-mediated Raf phosphorylation. High levels of stimulating IGF-I overcame this inhibition and led to ERK induction. The PI3K/Akt pathway stimulated MMP-2 expression, whereas the Raf/MEK/ERK pathway was inhibitory. In our VSMC model, however, increased concentrations of IGF-I merely increased MMP-2 expression. Similarly, increasing amounts of PDGF-BB resulted in a more robust up-regulation of MMP-2, but not a more rapid increase. Although the signaling from the receptors differed in VSMCs, our findings are in agreement with the idea that growth factor stimulation results in both positive and negative signals that influence MMP-2 expression.

The role of cell proliferation in VSMC phenotypic modulation appears to be minimal, based on our observations and in agreement with others (1). We also present evidence against the possibility that delayed up-regulation of MMP-2 in response to PDGF-BB is due to nonspecific transcriptional delays associated with the cell cycle. Blocking cycling with mimosine did not accelerate MMP-2 up-regulation. In support of this, PDGF-BB-stimulated down-regulation of SM α-actin was delayed in comparison with IGF-I, suggesting that a global pause in transcription is not responsible for this delayed response. It is not clear why IGF-I fails to induce a mitogenic response or ERK activation in some SMC models but does so in others. Previous studies have shown that IGF-I, compared with PDGF-BB, is a weak mitogen for VSMCs and activates ERK only minimally (53). One possibility is that the balance between IGF-I receptor substrates, such as IRS or Shc proteins, varies in the different culture models. Most Grb2/SOS in insulin-stimulated cells is associated with Shc, suggesting that ERK activation is primarily mediated through Shc, not IRS-1 (61). It was recently demonstrated that de-differentiated VSMCs activate ERK in response to IGF-I, while, in differentiated VSMCs, this pathway is blocked by SHP-2-mediated dephosphorylation of IRS-1 (62). This is consistent with at least some of our observations, in that IGF-I was able to stimulate PI3K activation, but activated ERK very transiently. IGF-I signaling in our cell model resulted in a robust activation of PI3K, something that has previously been shown to antagonize ERK activation (35). Indeed, we noted that VSMCs treated with a pharmacological inhibitor of PI3K exhibited an enhanced IGF-I- and PDGF-BB-mediated activation of ERK. Thus, the stimulation of MMP-2 up-regulation appears to be highly dependent on environmental cues and the differentiation state of the cells.

We speculate that the inhibitory and the stimulatory signaling pathways stimulated by PDGF-BB exert their effects through separate transcriptional mechanisms. We base this on the observation that transiently transfected fragments of the MMP-2 promoter could be induced by both PDGF-BB and IGF-I, even in cells cultured under conditions in which PDGF-BB fails to up-regulate the endogenous MMP-2 gene. Several possibilities could explain our inability to detect a transcriptional delay with cells transiently transfected with reporter constructs. First, our promoter constructs may not have contained the sequence element responsible for the PDGF-BB-mediated delay. Examination of promoter constructs up to ~6.5 kb upstream of the start codon plus the entire first intron did

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3 G. M. Risinger, Jr., and E. W. Howard, unpublished observations.
not reveal a delayed response to PDGF-BB.\textsuperscript{3} Second, the transcription factor(s) responsible for the delay may not be as abundant as the factor(s) responsible for the up-regulation, allowing even a small amount of plasmid to titrate out such factors. Despite titration of the reporter construct to levels just above the limits of our detection capability, we were unable to mimic the PDGF-BB-mediated delay in MMP-2 expression using transiently transfected plasmids. Finally, a transfected plasmid does not necessarily reflect the context of the endogenous gene in its chromatin environment, and this may affect the precise regulation of the promoter construct being examined compared with the endogenous promoter.

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