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ARTICLES

Angiopoietin 1, PDGF-B, and TGF-β Gene Regulation in Endothelial Cell and Smooth Muscle Cell Interaction

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Abstract The vascular wall is mainly composed of endothelial cells (ECs) and smooth muscle cells (SMCs). The crosstalking between these two cell types is critical in the vascular maturation process. Genetic studies suggest that the Tie2/angiopoietin 1 (Ang1) pathway regulates vascular remodeling. However, the molecular mechanism is unclear. PDGF is a potent chemoattractant for SMCs, and TGF-β regulates SMC differentiation. Here, we examined gene regulation. PDGF-B stimulation upregulated Ang1 expression in SMCs through the PI3K and PKC pathways. PDGF-B stimulation also produced an acute induction of TGF-β expression in SMCs through the MAPK/ERK pathway. Interestingly, TGF-β negatively regulated Ang1 expression induced by the PDGF-B stimulation in SMCs. Reciprocally, we observed that stimulation of ECs with either Ang1 or TGF-β slightly downregulated PDGF expression. A combination of both TGF-β with Ang1 produced much stronger downregulation of PDGF. Our data showed complex gene regulations that include both positive and negative regulations between ECs and SMCs to maintain vascular homeostasis. J. Cell. Biochem. 91: 584–593, 2004.

Key words: Tie2; angiopoietin; PDGF; TGF-β; vascular maturation

The assembly of the blood vessel wall from its cellular components is a critical process in the development and maturation of the functional vasculature system. Blood vessels in general are composed of distinct cell layers with a single layer of endothelial cells (ECs) that form the lumen of the vessels with layers of mural cells, smooth muscle cells (SMCs) in large vessels, and pericytes in microvessels [Hirschi and D'Amore, 1996; Hungerford and Little, 1999]. Recent studies on vascular development have focused on the initial steps of the process, in which vascular ECs undergo cell proliferation, migration, and tubule formation. A series of signaling molecules that regulates these processes in normal and pathologic conditions have been identified [Hanahan, 1997; Yancopoulos et al., 2000]. While much has been learned regarding the formation of the endothelium, the mechanisms that regulate the subsequent recruitment and differentiation of vascular SMCs are not well defined. SMCs provide structural and functional support for the endothelium.

Tie2 is an endothelium-specific receptor tyrosine kinase. The ligands for Tie2 are angiopoietins (Ang). Genetic studies show that Tie2/Ang signaling mediates vascular maturation. Disruption of Tie2 or Ang1 function in knockout mice causes vascular defects with defective recruitment of SMCs [Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996]. On the other hand, a constitutive mutation of Tie2 causes venous malformation with abnormal SMCs [Vikkula

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et al., 1996]. Platelet-derived growth factor-B (PDGF-B) has been shown to regulate EC-induced recruitment and differentiation of SMCs in cell culture systems [Vikkula et al., 1996]. Inactivation of PDGF-B in transgenic mice display vascular defects with loss of pericytes [Lindahl et al., 1997]. Transforming growth factor-β (TGF-β) is produced by a variety of cell types including ECs and SMCs. TGF-β regulates SMCs differentiation [Hirschi et al., 1998, 1999]. Here, we studied gene regulation using cultured ECs and SMCs. Human umbilical vein ECs were used as an EC line. 10T1/2 cells are multipotent mouse embryonic cells and presumptive mural cell precursors. These cells have been used to study EC–SMC recruitment and SMC differentiation [Hirschi et al., 1998, 1999]. The data in this study showed that PDGF-B regulated Ang1 expression through the PKC and PI3 kinase pathways, and PDGF regulated TGF-β expression through the MAPK pathway in SMCs. TGF-β inhibited PDGF-induced Ang1 expression in SMCs. Reciprocally, we observed that Ang1 or TGF-β alone caused limited downregulation of PDGF-B expression in ECs. A combination of both PDGF-B and TGF-β produced a stronger inhibition of PDGF-B production in ECs. Our study provides molecular evidence indicating complicated gene regulations between ECs and SMCs are critical for vascular development.

MATERIALS AND METHODS

Materials

Recombinant human PDGF-BB and TGF-β were purchased from R & D Systems (Minneapolis, MN). Recombinant angiopoietin 1 was from Regeneron Pharmaceuticals (Yorktown, NY). Small chemical inhibitors, PD98059 (ERK inhibitor), BIM (PKC inhibitor), and LY294002 (PI3K inhibitor), were purchased from Calbiochem (San Diego, CA). The inhibitors were dissolved in DMSO. BIM, LY294002, or PD98059 was used at 10, 50, or 20 μM, respectively, in each experiment. DMSO was used as a vehicle control in the study. Anti-phospho-tyrosine kinase antibody (PY99) was purchased from Santa Cruz (Santa Cruz, CA). Anti-Tie2 antibody (33.1) was produced as described [Peters et al., 1998].

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). HUVECs were grown on 0.1% gelatin coated plates in endothelial growth medium (EGM, Clonetics) in a humidified incubator with 5% CO_2 at 37°C. HUVECs from passage 3 to 7 were used in this study. Prior to the treatment with each growth factor and inhibitor, the cells were cultured to 60% confluence, and then starved in endothelial basal medium (EBM, Clonetics) for 12 h. Undifferentiated SMCs, 10T1/2 cells, were purchased from ATCC cells (CCL 226; ATCC, Rockville, MD). The cells were maintained in DMEM plus 10% FCS. The cells were passaged before they reached 75% confluency to prevent cell differentiation based on the manufacture's recommendation.

Northern Blot

Both HUVECs and 10T1/2 cells were grown to 60% confluency, followed by serum starvation with EBM or DMEM, respectively, for 24 h. The cells were then stimulated with each growth factor in the presence or absence of each inhibitor. Cells were harvested at various time points and total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). Ten micrograms of total RNA was electrophoresed in 1% agarose gel, transferred to hybond-nitrocellulose membrane. The membrane was stained with either ethidium bromide or methylene blue as a loading control. The membrane was then probed with ^{32}P-labeled probes. The full length of murine and human Ang1 coding sequences, full length of TGF-β sequence, full length of human PAI-I, and a partial human PDGF-B sequence corresponding 3–641 nt were used as probes. The probes were labeled with ^{32}P-dATP (NEN, Boston, Massachusetts) using a Prime-it II probe label kit (Stratagene, Cedar Creek, TX) and labeled probe was purified using a Probe Quant Sephadex G-50 column. The membrane was hybridized with each labeled probe and this was followed by exposure to X-ray film.

Quantification of mRNA Levels

Northern blot films were scanned. The intensity of each mRNA band was measured by NIH image 1.12 software.

Western Blot

HUVECs were stimulated with Ang1* followed by cell lysis in the RIPA buffer plus proteinase inhibitors and vanadate [Lin et al.,

1998]. Cellular proteins were collected and the protein content was measured using a BCA protein assay kit (Bio-Rad, Hercules, CA). Proteins (20 μg per sample) were separated on a SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed with an anti-phospho-tyrosine antibody (PY99) for 1 h at room temperature. The membrane was washed and incubated with horseradish peroxidease conjugated goat anti-rabbit IgG. The membrane was developed using ECL Western blotting detection reagents. The same membranes were striped and reblotted with an anti-Tie2 antibody (33.1).

Statistics

Results are reported as mean ± SD for mRNA levels for each group. A two-tailed Student's *t*-test was used to analyze statistical differences between control treated group and treated group. Differences were considered statistically significant at $P < 0.05$.

RESULTS

PDGF-BB Upregulated Ang1 Expression in SMCs/Pericytes via the PI3 Kinase and PKC Pathway

Genetic studies indicate that Tie2/Ang1 plays a role in the recruitment of SMCs during the vascular maturation and remodeling process [Sato et al., 1995; Suri et al., 1996]. PDGF-B is a potent chemoattractant factor for SMCs and it has been shown to recruit SMCs [Hirschi et al., 1998, 1999]. Disruption of PDGF-B expression in knockout mice leads to embryonic lethality with vascular defects characterized with defects in vascular maturation [Lindahl et al., 1997]. Since Ang1 is mainly expressed in SMCs, we examined its production upon PDGF-B stimulation. We used a multipotent cell line, 10T1/2, and presumptive mural cell precursors in the study. The cell line has been shown to response to PDGF stimulation in the cell migration assay [Hirschi et al., 1998]. Cultured 10T1/2 cells were serum-starved for 24 h, followed by stimulation with recombinant PDGF-B at 3 ng/ml. The cells were harvested at various time points and total RNAs were isolated and subjected to Northern blot. Ang1 mRNA was detected with a murine Ang1 probe labeled with ^{32}P-CTP. PDGF stimulation induced a bi-phastic Ang1 expression in a time dependent manner. The mRNA levels of Ang1 were decreased at 2 h post-stimulation, but gradually and dramatically increased afterwards with a peak time around 6 h post-stimulation (Fig. 1A). NIH image analysis was used to quantitate the mRNA levels in each sample as described in Materials and Methods (Fig. 1B).

Next, we examined the molecular mechanisms of the gene induction. We used specific inhibitors for PI3 kinase (LY294002), PKC (BIM), and MAPK/ERK (PD98059) in the study. 10T1/2 cells were pretreated with LY294002 at 20 μM, PD98059 at 20 μM, or BIM at 10 μM, respectively, for 30 min prior to PDGF-B stimulation. The control was treated with DMSO. The cells were harvested at 6 h post-PDGF stimulation and total RNAs were analyzed by Northern blot and probed with Ang1 probe. Again, stimulation with PDGF-B upregulated Ang1 expression in 10T1/2 cells (Fig. 1C,D). Blocking PKC or PI3 kinase activity using specific inhibitors inhibited endogenous Ang1 expression as well as PDGF-induced Ang1 expression (Fig. 1C,D). However, blocking MAPK/ERK activity failed to inhibit PDGF-induced Ang1 expression in 10T1/2 cells (Fig. 1C,D). The data showed that PDGF-B upregulated Ang1 expression in SMCs, and PDGF-induced Ang1 expression was mediated through the PI3 kinase and PKC pathways, but not the MAPK pathway.

PDGF-B Upregulated TGF-β Expression in SMC/Pericytes Through the MAPK Pathway

TGF-β has been shown to regulate SMCs differentiation and cell shape [Hirschi et al., 1998, 1999]. Therefore, we examined the effects of PDGF-B stimulation of SMCs on TGF-β expression. 10T1/2 cells were serum-starved, followed by stimulation with PDGF-B at 3 ng/ml for up to 24 h. Cells were harvested at various time points and total RNAs were analyzed by Northern blot. The filter was probed with a ^{32}P-labeled TGF-β cDNA probe. PDGF-B stimulation induced an acute and robust induction of TGF-β expression in SMCs. TGF-β mRNA peaked at 1-h post-stimulation, and it gradually decreased and reached base line in 24 h (Fig. 2A,B). Then, we examined the signaling pathways that regulated TGF-β expression by using specific pathway inhibitors. The cells were pretreated with LY294002 at 20 μM, PD98059 at 20 μM, or BIM at 10 μM, respectively, for 30 min prior to PDGF stimulation. The control was treated with DMSO. Cells were harvested at 2 h after

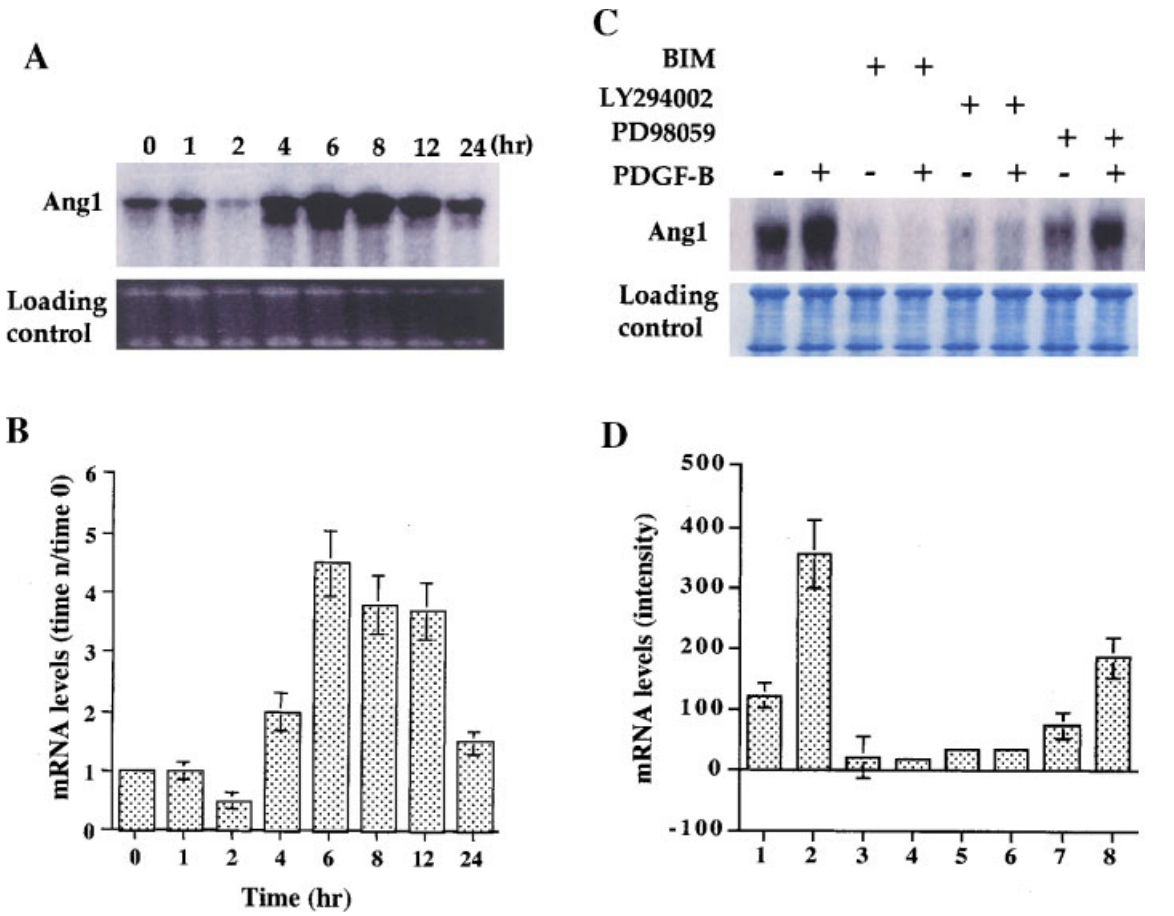

Fig. 1. PDGF-B regulated Ang1 expression in smooth muscle cells (SMCs) through the PKC and PI3 kinase. 10T 1/2 cells were grown to 50% confluency. Cells were serum starved for 24 h and then stimulated with PDGF-B at 3 ng/ml. Total RNAs (10 μg/sample) were analyzed by Northern blot. The nitrocellulose membrane with RNA samples was stained with ethidium bromide as a RNA loading control (**bottom panel** of **A**). The nitrocellulose membrane was then probed with a ^{32}P-labeled Ang1 cDNA probe (**upper panel** of A). The mRNA levels of Ang1 from (A) were quantitated by using NIH image 1.12 program (**B**). To study the gene regulation mechanisms, we used specific inhibitors, LY294002 at 20 μM, BIM at 10 μM, or PD98059 at 20 μM to block PI3 kinase, PKC, or MAPK/MEK activity, respectively. 10T1/2 cells were pretreated with each inhibitor for 30 min followed by PDGF-B stimulation at 3 ng/ml. Total cellular RNAs were analyzed by Northern blot for Ang1 expression (**upper panel** of **C**). The nitrocellulose membrane with RNA samples was stained with methylene blue as a RNA loading control (**bottom panel** of C). The mRNA levels of Ang1 from (C) were quantitated by using NIH image 1.12 program (**D**). The experiments were repeated twice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PDGF stimulation followed by Northern blot analysis for TGF-β expression. Interestingly, blocking either PKC or PI3 kinase activity did not affect TGF-β expression, but blocking MAPK/ERK activity significantly inhibited PDGF-induced TGF-β expression (Fig. 2C,D). Collectively, the data demonstrate that PDGF-B regulates both Ang1 and TGF-β expression in SMCs, but PDGF-B utilizes different signaling pathways in regulating different gene expression. It induces Ang1 expression through the PI3 kinase and PKC pathways (Fig. 1C,D), and it induced TGF-β expression through the MAPK pathway (Fig. 2C,B).

TGF-β Downregulated Ang1 Expression and TGF-β Blocked Ang1 Expression Induced by PDGF-B Stimulation in SMCs

TGF-β, PDGF, and Ang1 collectively regulate SMC differentiation and SMC recruitment in vascular remodeling. Therefore, we examined the effects of TGF-β on Ang1 expression in SMCs. Cultured 10T1/2 cells were serum-starved for 24 h followed by stimulation with either recombinant TGF-β protein at 6 ng/ml or TGF-β plus PDGF-B at 3 ng/ml. DMSO was used as a vehicle control. Cells were harvested at 2, 4, and 8 h after stimulation. Total RNAs

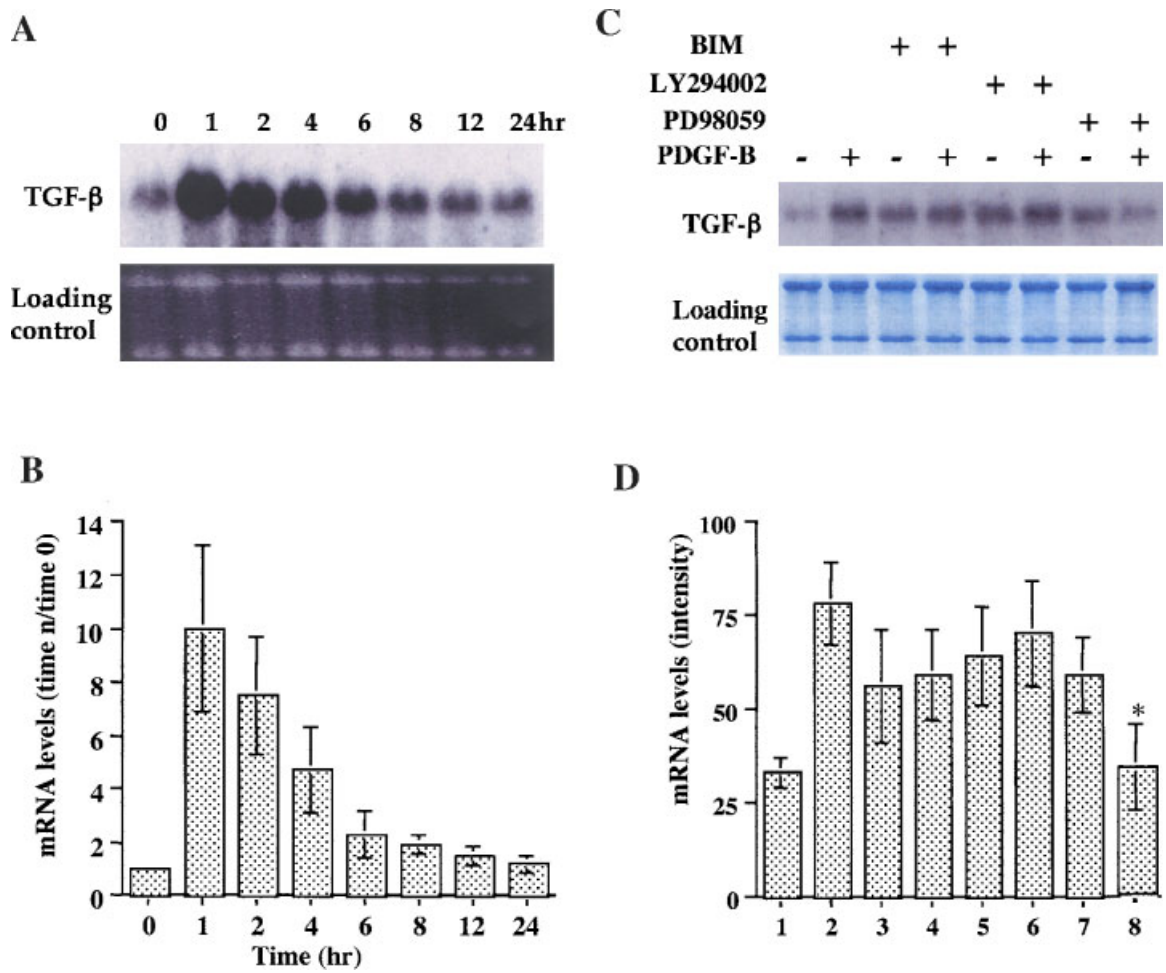

Fig. 2. PDGF-B stimulated TGF-β expression through the MAPK/ERK pathway in SMCs. 10T1/2 cells were stimulated with PDGF-B at 3 ng/ml in the presence of absence of different inhibitors. Cells were harvested at various time points and total cellular RNAs were analyzed by Northern blot for TGF-β expression. The nitrocellulose membrane with RNA samples were stained with ethidium bromide (**bottom panel** of **A**) or methylene blue (**bottom panel** of **C**), respectively, to ensure equal sample loading. PDGF-B stimulation produced an acute and robust induction of TGF-β expression in 10T1/2 cells (**upper panel** of A). The mRNA levels of TGF-β from (A) were quantitated by using NIH image 1.12 program (**B**). To study the gene regulation mechanism, 10T1/2 cells were pretreated with LY294002 at 20 μM, PD98059 at 20 μM, or BIM at 10 μM, respectively, for 30 min prior to PDGF stimulation. The control was treated with DMSO. Cells were harvested at 2 h after PDGF stimulation followed by Northern blot analysis for TGF-β expression (**upper panel** of C), which was confirmed by the quantification data of mRNA levels (**D**). Differences in mRNA levels are significant with $P < 0.05$ as indicated as a asterisk. The experiments were repeated twice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were analyzed by Northern blot and probed with a ^{32}P-labeled Ang1 probe. Endogenous Ang1 levels partially decreased 2 h after TGF-β treatment, and Ang1 mRNA was completely diminished 4 and 8 h after the treatment (Fig. 3A). Quantification data of mRNA levels confirm that TGF-β significantly inhibited Ang1 expression in SMCs (Fig. 3B, $P < 0.05$). In addition, TGF-β completely blocked Ang1 expression in response to PDGF stimulation (Fig. 3A,B, $P < 0.05$). The data showed that TGF-β negatively regulated PDGF-induced Ang1 expression in SMCs. It suggests that the levels of Ang1 may be critical in regulating vascular growth and maturation.

Ang1 and TGF-β Alone Marginally Downregulated PDGF-B Expression, and a Combination of Both Ang1 and TGF-β Produced a Profound Effect on PDGF-B Expression in ECs

Vascular walls are mainly composed of ECs and SMCs. During vascular maturation, the endothelium sends signals to SMCs and recruits SMCs towards the endothelium. Cell–cell crosstalking between the endothelium and

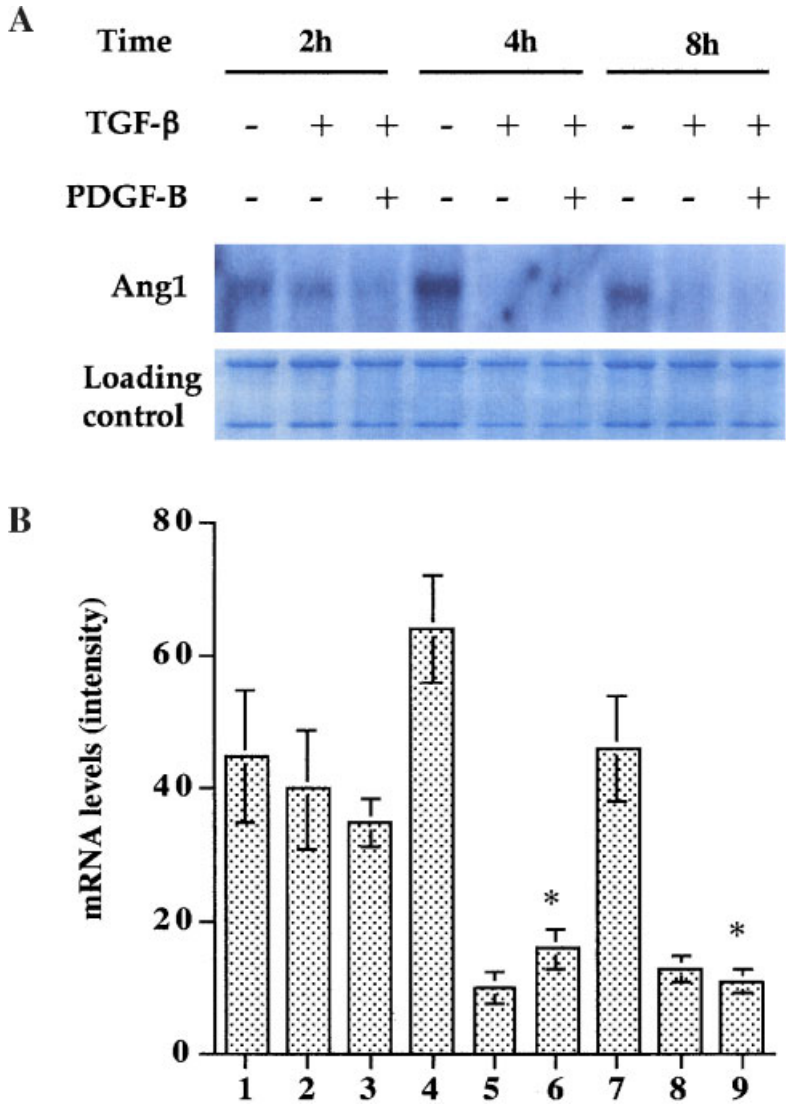

Fig. 3. TGF-β negatively regulated Ang1 expression, and TGF-β blocked PDGF-B-induced Ang1 expression in SMCs. 10T1/2 cells were serum-starved for 24 h, followed by stimulation with TGF-β at 6 ng/ml in the absence or presence of PDGF-B at 3 ng/ml for up to 8 h. Total cellular RNAs were harvested and analyzed by Northern blot for Ang1 expression (**upper panel** of **A**). The nitrocellulose membrane was stained with methylene blue to ensure equal sample loading (**bottom panel** of A). The mRNA levels of Ang1 from (A) were quantitated by using NIH image 1.12 program (**B**). Differences in mRNA levels are significant with $P < 0.05$ as indicated as a asterisk. The experiments were repeated twice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

SMCs plays a pivotal role in this process. We showed that PDGF-B regulated Ang1 and TGF-β expression in SMCs (Figs. 1–3). Here, we examined the effects of Ang1 and TGF-β on PDGF-B expression in ECs. Cultured HUVECs were serum starved for 24 h, followed by stimulation with either a recombinant Ang1* protein at 200 ng/ml, or TGF-β at 6 ng/ml, or a combination of both Ang1* and TGF-β, respectively. The cells were harvested at different time points. Total cellular RNAs were analyzed by Northern blot and probed with a ^{32}P-labeled PDGF-B cDNA probe. Treatment of HUVECs with Ang1* produced a marginal response in PDGF-B expression. Ang1* stimulation slightly downregulated PDGF-B expression in HUVECs (Fig. 4A). To make sure that Tie2 signaling was indeed activated by the Ang1 stimulation, we harvested the cells 10 min after stimulation. Cells lysates were processed for Western blotting and probed with an anti-phospho-tyrosine antibody or a Tie2 antibody, respectively. We observed Tie2 activation upon Ang1 stimulation in HUVECs (Fig. 4B). This data supported the

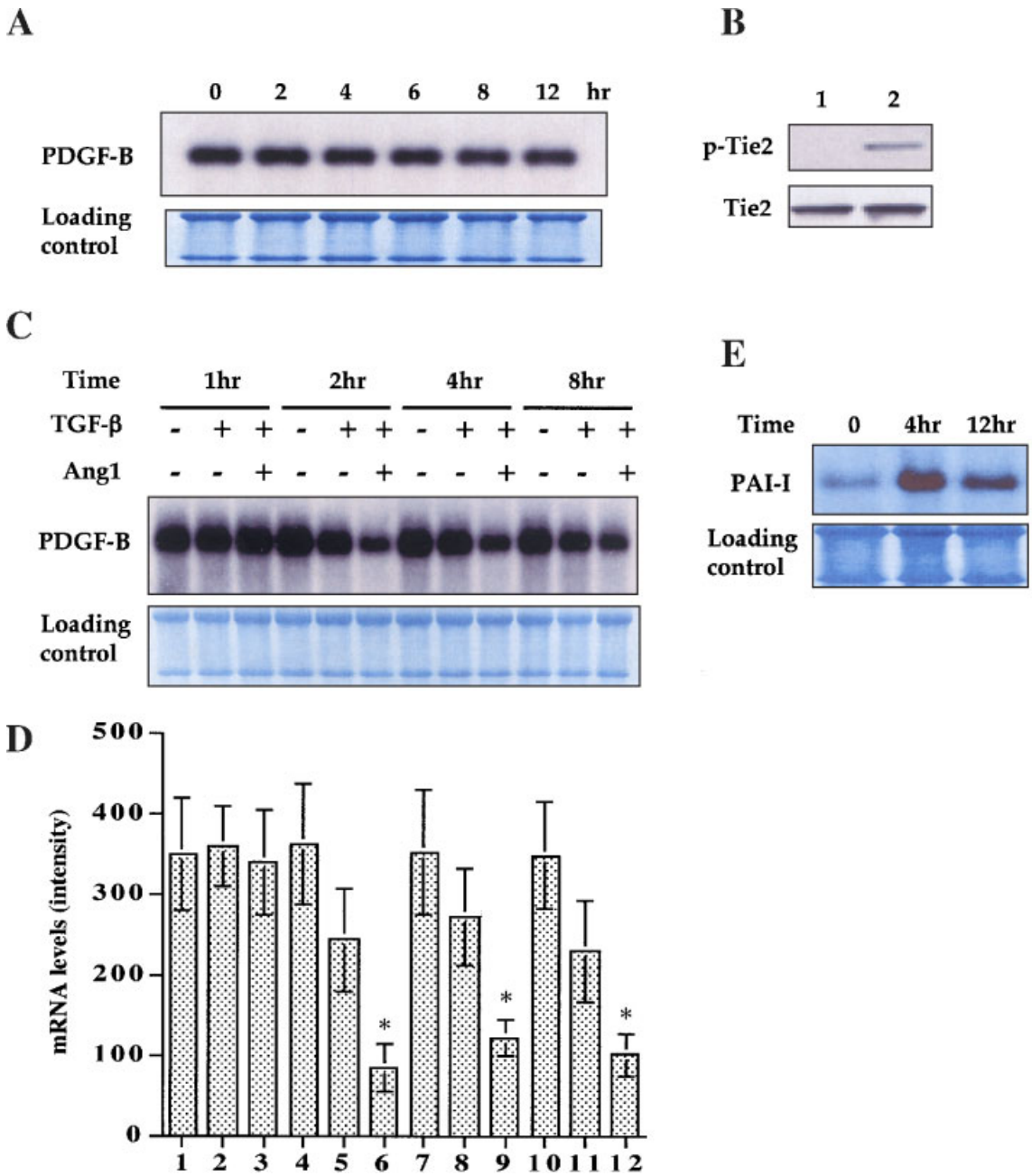

Fig. 4. Ang1 in combination with TGF-β negatively regulate PDGF-B expression in endothelial cells (ECs). HUVECs were serum-starved for 24 h in EBM. The cells were then stimulated with either Ang1* at 200 ng/ml, or TGF-β at 6 ng/ml, or a combination of both Ang1* and TGF-β, respectively, for various periods of time as indicated. The nitrocellulose membrane with RNA samples was stained with methylene blue as a RNA loading control (**bottom panels** of **A**, **C**, and **D**). The membranes were then incubated with a ^{32}P-labeled PDGF-B specific cDNA probe. Ang1 stimulation of HUVECs produced a very marginal inhibition of PDGF-B expression in a time dependent manner (**upper panel** of A). To demonstrate the Ang1 activity, HUVECs were stimulated with a vehicle control (**lane 1** of **B**) or Ang1* at 200 ng/ml (**lane 2** of B) for 10 min. Cell lysates were analyzed by Western blot and probed with an anti-phospho-tyrosine antibody (PY99) for Tie2 activation (**upper panel** of B). The same membrane was reprobed with an anti-Tie2 antibody (33.1) for the detection of Tie2 protein (B). Again, the treatment of HUVECs with TGF-β yielded slight decrease of PDGF expression (**upper panel** of C). A combination of both Ang1* and TGF-β produced a strong downregulation of PDGF-B expression in HUVECs (upper panel of C). The quantification data of mRNA levels confirmed that a combination of Ang1 and TGF-β significantly downregulated PDGF-B expression in ECs (D). Differences in mRNA levels are significant with $P < 0.05$ as indicated as a asterisk. To demonstrate TGF-β activity, HUVECs were stimulated with TGF-β at 6 ng/ml. Cells were harvested at 0, 4, and 12 h post-stimulation. Total cellular RNAs were analyzed by Northern blot and probed with a ^{32}P-labeled PAI-I cDNA probe (**E**). The experiments were repeated twice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

conclusion that Ang1 alone had little effects on PDGF-B expression in HUVECs.

Then we examined the effects of TGF-β on PDGF-B expression in ECs. Once again, stimulation of HUVECs with TGF-β yielded a limited downregulation of PDGF-B (Fig. 4C). Interestingly, a combination of both TGF-β and Ang1 produced a much stronger downregulation of PDGF-B expression in HUVECs (Fig. 4C). Quantification data of the mRNA levels confirm that a combination of TGF-β and Ang1 significantly downregulated PDGF-B expression in ECs (Fig. 4D). Since we observed no induction or limited downregulation of PDGF-B by TGF-β stimulation in HUVECs, we tried to be certain that the TGF-β stimulation was effective. We performed a Northern blot analysis and probed with a PAI-I probe. PAI-I has been used as a positive control for TGF-β mediated gene regulation. Indeed, we observed a strong PAI-I induction that peaked at 4 h after TGF-β stimulation in HUVECs (Fig. 4E). In summary, our data showed that either Ang1 or TGF-β alone had limited effects on PDGF-B expression, but Ang1 and TGF-β collectively regulated PDGF-B expression in ECs.

DISCUSSION

Crosstalking between ECs and SMCs is critical in vascular development. Recent studies have identified several important signaling pathways that regulate the endothelium formation process [Hanahan, 1997; Yancopoulos et al., 2000]. However, the molecular mechanisms of vascular maturation are less clear. PDGF-B and TGF-β have been shown to mediate EC-induced recruitment and differentiation of SMCs in cell culture systems [Hirschi et al., 1998, 1999]. Genetic evidence suggests that a new class of receptor tyrosine kinase, Tie2/angiopoietin, may regulate the recruitment of SMCs in vascular maturation [Sato et al., 1995; Suri et al., 1996]. Here, we studied gene regulation in ECs and SMCs. Our data showed that PDGF-B regulates Ang1 expression in SMCs in a time-dependent manner. We observed a bi-phastic gene regulation of Ang1. PDGF downregulated Ang1 expression at 2 h post-stimulation, but upregulated Ang1 expression afterwards. We showed that PDGF-B regulated Ang1 expression through the PI3 kinase and PKC pathways, but not the MAPK pathway (Fig. 1C,D). In addition, PDGF-B also produced an acute and robust induction of TGF-β in SMCs. TGF-β levels peaked at 1-h post-stimulation and gradually returned to the base line in 24 h (Fig. 2A,B). Interestingly, it was the MAPK/ERK pathway that regulated TGF-β expression induced by the PDGF-B stimulation (Fig. 2C,D), but not PI3 kinase or PKC that has been shown to regulate Ang1 expression in SMCs (Fig. 1C,D). In addition, we observed that TGF-β negatively regulated Ang1 expression in SMCs, and TGF-β also blocked PDGF-B induced Ang1 expression in the cells (Fig. 3), indicating a delicate balance of Ang1 expression might be important in vascular homeostasis. After examining PDGF-B expression in cultured ECs, we observed that either Ang1 or TGF-β alone marginally downregulated PDGF-B expression in ECs. A combination of Ang1 and TGF-β, however, produced a much stronger downregulation of PDGF-B expression in ECs (Fig. 4). Our study demonstrated complex gene induction mechanisms involving these important mediators in SMC recruitment and differentiation. It provides molecular evidence suggesting a fine balance of these important genes is required for normal vascular development.

Developmental studies indicate that Tie2/Ang1 signaling mediates recruitment of SMCs during vascular maturation. In embryos lacking a functional Tie2 or Ang1, there were profound defects in the morphogenesis of microvessels in both embryonic and extraembryonic tissues including a reduction in the complexity of vascular branching and a defect in recruitment of pericytes and/or SMC [Sato et al., 1995; Suri et al., 1996]. In addition, an activating mutation of Tie2 was found to be responsible for causing an inherited form of venous malformation [Vikkula et al., 1996]. The lesions in this syndrome consist of enlarged, ecstatic venous channels surrounded by highly variable numbers of SMCs. Since the lesions in the venous malformation syndrome occur sporadically, these data, when considered together, suggest that tight regulation of Tie2 activity is required for maintenance of the appropriate, mature vasculature phenotype. The expression data by in situ hybridization and Northern blot analysis suggest that Ang1 is expressed by cells adjacent to the endothelium such as SMCs/pericytes. In this study, we examined Ang1 expression in a multipotent, presumptive mural cell precursor cell line in the study. We observed

that PDGF-B, a potent chemoattractant mediator of SMCs [Hirschi et al., 1998, 1999], regulated Ang1 expression in a bi-phastic manner. PDGF downregulated Ang1 expression at an early time point, but upregulated the gene expression afterwards. Current efforts are directed toward understanding the mechanisms of the bi-phastic gene regulation. An earlier report shows that PDGF downregulates Ang1 expression in human fibroblast cells [Enholm et al., 1997]. The difference between our study and the published report may arise from either the cell lines or the dose of PDGF. We used an undifferentiated SMC cell line, 10T1/2, in the study. This cell line responses to PDGF-B mediated SMC recruitment and TGF-β-induced the 10T1/2 differentiation toward SMCs [Hirschi et al., 1998, 1999]. In contrast, the published data used a fibroblast cell line. As for differences in PDGF dose levels, we used 3 ng/ml of PDGF-B to stimulate 10T1/2 cells, and the published report used 100 ng/ml of PDGF-B in their study. We believe that the cell line and the dose of PDGF-B used in our study are more physiologically relevant to the recruitment of SMCs in vascular maturation. In addition, we observed a downregulation of Ang1 expression at an earlier time point after PDGF stimulation, suggesting that the action of PDGF on Ang1 production in SMCs is time-dependent.

TGF-β regulates SMC differentiation [Hirschi et al., 1998, 1999]. Loss of endoglin, a TGF-β binding protein expressed on the endothelium surface, in transgenic mice leads to poor vascular smooth muscle development and arrested vascular remodeling [Li et al., 1999]. We examined the effects of PDGF-B on TGF-β expression in SMCs. We observed that PDGF-B induced a robust and acute induction of TGF-β expression. Interestingly, the PDGF-B-induced TGF-β expression in SMCs was mediated through the MAPK/MEK pathway, but not the PI3 kinase and PKC pathways, which mediated Ang1 expression in SMCs, induced by PDGF-B. The data show that PDGF-B utilizes different signaling mediators in regulating gene expression in SMCs. Furthermore, we observed that TGF-β negatively regulated Ang1 expression in SMCs. The data is in agreement with published data in which TGF-β downregulated Ang1 expression in a human fibroblast line [Enholm et al., 1997]. In addition, our data showed that TGF-β also blocked PDGF-B-induced Ang1 expression in SMCs.

Tie2 is an endothelium-specific receptor and Ang1 is predominantly expressed in SMCs surrounding the endothelium. We postulate that crosstalking between ECs and SMCs regulates vascular remodeling with SMCs producing Ang1 that affects the endothelium and the endothelium in return produces PDGF-B that regulates SMCs behavior. After examining the expression of PDGF expression in ECs, we observed that Ang1 slightly downregulated PDGF-B expression. TGF-β exhibited no effect or slightly downregulated PDGF-B expression in HUVECs (Fig. 4B). Interestingly, a combination of both Ang1 and TGF-β produced a more profound downregulated of PDGF-B expression in ECs. TGF-β has been shown to upregulate PDGF-B expression in ECs [Daniel et al., 1987; Daniel and Fen, 1988; Taylor and Khachigian, 2000]. However, we did not observe such a response, although we demonstrated that TGF-β stimulation of HUVECs indeed activated TGF-β signaling in our system as shown with the induction of PAI-I expression (Fig. 4E), a widely used positive control for TGF-β activation in gene regulation. We do not know what caused the difference between our data and published results at this moment. One potential explanation could be the differences of ECs used in each study. There is mounting evidence demonstrating that the endothelium is very heterogeneous [Arap et al., 2002].

In summary, we studied gene regulation in the vascular maturation process. Our data showed that PDGF-B regulated Ang1 and TGF-β expression in SMCs. TGF-β blocked PDGF-B-induced Ang1 expression in SMCs, revealing a negative feedback regulation mechanism of Ang1 production in SMCs. The data also showed that Ang1 and TGF-β collaborately regulated PDGF-B expression in ECs. It presents complex and highly regulated mechanisms that govern the expression of these important mediators. It suggests a tight regulation of Tie2 activity, PDGF receptor activity, and TGF-β receptor activity are required for maintenance of the appropriate, mature vasculature phenotype.

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