Angiotensin II–Induced Mitogenesis of Spontaneously Hypertensive Rat–Derived Cultured Smooth Muscle Cells Is Dependent on Autocrine Production of Transforming Growth Factor-β

George A. Stouffer and Gary K. Owens

Angiotensin II (Ang II) has been implicated in the regulation of smooth muscle cell proliferation after vascular injury, but the molecular mechanisms of this effect remain obscure. The aims of the present study were 1) to determine if Ang II was mitogenic (in a defined serum-free medium) for aortic smooth muscle cells derived from spontaneously hypertensive rats, either alone or in combination with epidermal growth factor, basic fibroblast growth factor, or platelet-derived growth factor-BB; and 2) to determine if the Ang II effects were mediated by autocrine production of transforming growth factor-β (TGF-β). Results demonstrated that Ang II increased the proliferative response of smooth muscle cells to epidermal growth factor or platelet-derived growth factor-BB. Ang II alone and in combination with basic fibroblast growth factor induced a small delayed increase (48–72 hours after treatment) in DNA synthesis and [3H]thymidine labeling indexes without an increase in cell number. Ang II effects were at least partially mediated by autocrine production of active TGF-β in that 1) treatment with Ang II increased TGF-β activity in conditioned media and 2) TGF-β neutralizing antibody inhibited Ang II–induced increases in DNA synthesis. However, treatment with exogenous TGF-β at concentrations induced by Ang II failed to elicit a mitogenic response, thus implicating other autocrine factors in mediation of Ang II effects. Results suggest a potential mechanism whereby Ang II might regulate smooth muscle cell mitogenesis after vascular injury. (Circulation Research 1992;70:820–828)

Key Words • angiotensin II • smooth muscle cells • transforming growth factor-β • epidermal growth factor • fibroblast growth factor • platelet-derived growth factor

Excessive smooth muscle cell (SMC) proliferation leading to clinically important restenosis occurs in approximately 30% of atherosclerotic arteries diluted by percutaneous transluminal coronary angioplasty.1 The rate of restenosis has not been reduced by the introduction of newer technologies (e.g., directional atherectomy and excimer laser)2 and remains the major limitation of percutaneous coronary revascularization. Despite a major worldwide research effort, the molecular mechanisms underlying excessive SMC proliferation after vascular injury remain obscure. This lack of understanding has impeded patient risk stratification and the development of effective therapeutic interventions.

Angiotensin II (Ang II) has been implicated in the regulation of smooth muscle proliferation after balloon catheter injury in the rat. Powell et al3 have shown that treatment with angiotensin converting enzyme inhibitors decreased neointimal formation by 80% in a carotid injury model. The effect was similar with angiotensin converting enzyme inhibitors from two distinct chemical classes (cilazapril and captopril) but was not produced by verapamil, a calcium channel antagonist, despite a similar reduction in blood pressure. Subsequently, this group has also shown that treatment with the nonpeptide Ang II antagonist DuP 753 reduced neointimal formation, that continuous infusion of Ang II significantly increased neointimal formation, and that Ang II infusion abolished the effect of angiotensin converting enzyme inhibitors but not DuP 753.4 Daemen et al,5 using a similar model, found that chronic Ang II infusion after injury increased the neointimal cross-sectional area by 60–100% and increased [3H]thymidine SMC labeling indexes by more than 10-fold in the injured vessel.

The molecular mechanisms whereby Ang II might promote SMC proliferation in vivo have not been elucidated. Our understanding in this area is confounded by observations that Ang II treatment induces cellular hypertrophy and not proliferation in cultured SMCs derived from Sprague-Dawley (SD) rats6,7 but promotes

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proliferation in SMCs derived from spontaneously hypertensive rats (SHR). Thus, the SMC response to Ang II varies as a function of the target cell examined. Although the reasons for the differential Ang II–induced growth response in SHR-derived versus SD-derived cultured SMCs are not known, studies of SHR-derived SMCs may provide insight regarding the mechanisms whereby Ang II promotes SMC proliferation in vivo. The objectives of the present study were 1) to determine whether Ang II stimulated proliferation in SMCs derived from SHR in a defined serum-free medium; 2) to determine whether Ang II increased the proliferative response of SMCs to other growth factors present at the site of vascular injury in vivo including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor-BB (PDGF-BB); and 3) to determine whether Ang II–induced proliferation in SHR-derived SMCs was mediated by autocrine production of transforming growth factor-β (TGF-β), a multifunctional growth factor whose expression is increased in the intima of rats after vascular injury11 and in cultured SMCs treated with Ang II.12,13

Materials and Methods

Cell Culture

SMCs were isolated from SHR aortas by enzymatic digestion, as previously described.6 The cells were cultured in medium containing a 1:1 formulation of Dulbecco’s modified Eagle’s medium ( Gibco Laboratories, Grand Island, N.Y.) and Ham’s F12 medium (GIBCO) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 1-glutamine (0.68 mM, Sigma Chemical Co., St. Louis, Mo.), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were harvested for passaging at subconfluence with a trypsin-EDTA (0.05% trypsin and 0.02% EDTA, Gibco) solution. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2–95% air. SMC identity and the purity of cultures were verified by immunocytochemical analysis using antibodies specific for smooth muscle α-actin.14

SMCs between passages 8 and 20 were plated at 3×10⁴ cells/cm² in serum-containing media. They were grown to confluence and then growth arrested in a defined serum-free medium containing a 1:1 formulation of Dulbecco’s modified Eagle’s medium and Ham’s F12 supplemented with transferrin (5 μg/ml, Sigma), insulin (5×10⁻⁷ M, Sigma), ascorbate (0.2 mM, Sigma), glutamine, and penicillin/streptomycin. This serum-free medium has been shown to maintain SMCs in a quiescent, noncatabolic state and to promote expression of SMC-specific contractile proteins.15

[^H]Thymidine Incorporation

Relative rates of DNA synthesis were assessed by determination of [^H]thymidine incorporation into trichloroacetic acid-precipitable material. Cells were pulsed for 24 hours with [^H]thymidine (New England Nuclear, Boston, Mass.) and then washed with a calcium- and magnesium-free phosphate-buffered saline solution (mM: NaCl 137, Na₂HPO₄ 8.1, KCl 2.7, and KH₂PO₄ 1.5, pH 7.4). This was followed by 10-minute washes with 10% trichloroacetic acid, first at 4°C and then at room temperature. Cells were then dissolved in 1N NaOH and placed in Ready-Safe scintillation fluid (Beckman Instruments, Inc., Palo Alto, Calif.). Counting was done with a Wallac LKB scintillation counter.

[^H]Thymidine Autoradiography

 Cultures were pulse labeled for 24 hours (48–72 hours after treatment) with [^H]thymidine (10 μCi/ml, 6.7 Ci/mmol, New England Nuclear) added to regular culture medium. Cells were then washed twice with phosphate-buffered saline, fixed in 2% glutaraldehyde for 5 minutes, dehydrated, and coated with NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted 1:1 with distilled water. Dishes were exposed for 7 days at 4°C and then developed in D19 (Eastman Kodak), fixed with Rapid-Fix (Eastman Kodak), and stained with hematoxylin (Sigma). The percentage of cells synthesizing DNA was determined by counting the number of labeled cells in a random sample of at least 500 cells from each dish. Triplicate dishes were analyzed for each group.

Growth Curves

 Cultures were washed with phosphate-buffered saline, harvested with trypsin-EDTA, diluted with 0.9% NaCl (Columbia Diagnostics Inc., Springfield, Va.), and counted using an Electrozone Celsoscope (Particle Data, Inc., Elmhurst, Ill.) with orifice size of 95 μm and sample volume of 500 μl.

Transforming Growth Factor-β Bioassay

Mink lung epithelial cells (MV 1 Lu, American Type Culture Collection, Rockville, Md.) were cultured in minimum essential medium (GIBCO) supplemented with 5% fetal bovine serum. Harvesting for passaging was at subconfluence by using a trypsin-EDTA solution. Bioassays were performed as described by Cheifetz et al.16 Briefly, MV 1 Lu cells were plated at 1×10⁵ cells/cm² in minimum essential medium supplemented with 0.5% fetal bovine serum. Twenty-four hours later, SMC conditioned medium (CM) was added at a 1:6 dilution. The MV 1 Lu were pulsed with [^H]thymidine at 44 or 47 hours and then harvested at 48 hours.[^H]Thymidine incorporation was determined as described above. CM was obtained from SHR-derived SMCs that were grown to confluence and growth arrested as described above. Ang II (10⁻⁶ M) was added to the treatment group for various time periods before removal of the CM. Untreated SMCs growth arrested under similar conditions provided control CM. A TGF-β dose–response was run in parallel with the CM experiments as a positive control for TGF-β effects. The proportion of inhibitory activity attributable to TGF-β was ascertained by addition of a rabbit anti-human TGF-β neutralizing antibody (R&D Systems, Minneapolis, Minn.).

Reagents

Ang II (human) was obtained from Peninsula Laboratories, Belmont, Calif. TGF-β was isolated from human platelets by R&D Systems, Minneapolis, Minn. Recombinant human EGF, bFGF, and PDGF-BB were obtained from Upstate Biotechnology Inc., Lake Placid, N.Y. TGF-β neutralizing antibody was obtained from R&D Systems and was prepared in rabbits.
by injection of porcine platelet TGF-β. The antibody neutralizes both TGF-β1 and TGF-β2 and does not exhibit any cross-reactivity with bFGF, EGF, or PDGF (R&D Systems' data).

Statistical Analysis

Results are presented as mean±SD unless otherwise stated. Student's t test for unpaired data was used for statistical analysis when comparing two data points (e.g., autoradiography results). One-way analysis of variance followed by the Newman-Keuls multiple range test was used to analyze the TGF-β concentration curve data and the results obtained from the mink lung epithelial cell bioassay. Two-way analysis of variance followed by Tukey's multiple range test was used to analyze the remainder of the data. A value of p≤0.05 was used to determine statistical significance. Triplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of three times.

Results

Treatment With Angiotensin II Induced a Delayed Increase in DNA Synthesis in Cultured Smooth Muscle Cells Derived from Spontaneously Hypertensive Rats

The initial aim of these studies was to determine whether Ang II induced an increase in DNA synthesis in confluent SHR-derived SMCs made quiescent in a defined serum-free medium. Results demonstrated that no increase in DNA synthesis was observed between 0 and 48 hours after stimulation with Ang II (Figure 1). A delayed increase (range, twofold to eightfold; mean, 3.6) was observed beginning 48 hours after treatment. The time course of induction of DNA synthesis in Ang II–treated SMCs was thus different from that observed with mitogens such as EGF, bFGF, or PDGF-BB where [³H]thymidine incorporation was increased within 24 hours of treatment (authors’ unpublished observations). The delayed Ang II–induced increase in DNA synthesis was concentration dependent (Figure 2), demonstrating an ED₅₀ of approximately 1.0×10⁻⁹ M. However, the actual Ang II concentration in the CM was presumably less between 48 and 72 hours since Ang II is known to be degraded under the conditions of these experiments (A.A. Geiterfer and G.K. Owens, unpublished observations).

Angiotensin II–Induced Increases in DNA Synthesis Were Inhibited by Treatment With Transforming Growth Factor-β Neutralizing Antibody

The delayed increase in Ang II–induced DNA synthesis suggested that this effect was mediated via production of a secondary growth factor. Because TGF-β mRNA has been reported to be increased in Ang II–treated SMCs, subsequent studies examined whether the Ang II–induced increases in DNA synthesis could be inhibited by a neutralizing antibody to TGF-β. Results demonstrated that administration of a TGF-β neutralizing antibody at the time of addition of Ang II completely inhibited the delayed increase in DNA synthesis (Figure 3).

Angiotensin II Treatment Was Associated With an Increase in Concentration of Active Transforming Growth Factor-β in Conditioned Media

Results of experiments with anti–TGF-β antibodies indicated that Ang II–induced increases in DNA synthesis were at least partially mediated by autocrine production of TGF-β. To determine whether Ang II treatment increased the concentration of active TGF-β
in CM, active TGF-β levels were assayed using a mink lung epithelial cell bioassay described by Cheifetz et al.16 Because of the expense and limited supply of TGF-β neutralizing antibody, a two-step approach was used. Initial studies examined the temporal sequence of the appearance of growth inhibitory activity in CM from Ang II-treated SMCs. Results of these studies demonstrated that CM from SMCs treated for 48 hours with Ang II induced maximum inhibition (Figure 4, top panel). Subsequent studies, using this selected time point, then demonstrated that Ang II–treated SMC CM growth inhibitory activity could be abolished by treatment with TGF-β neutralizing antibody (Figure 4, middle panel). Ang II or anti–TGF-β antibody alone had no direct effect on [3H]thymidine incorporation in mink lung epithelial cells (data not shown).

Based on analysis of the effects of various concentrations of purified human TGF-β on DNA synthesis in mink lung epithelial cells (Figure 4, bottom panel), the concentration of active TGF-β in CM from Ang II–treated SMCs was estimated to be between 0.5×10^{-12} and 5×10^{-12} M, whereas the concentration of TGF-β in CM from quiescent SMCs was below the threshold detectable with this bioassay (10^{-15} M). Actual concentrations of TGF-β in CM from Ang II–treated SMCs may differ from this estimate because of the interactive effects of additional undefined Ang II–induced components that could influence mink lung epithelial cell DNA synthesis. Nevertheless, these data provide compelling evidence that Ang II induced a qualitative increase in active TGF-β in CM as compared with control CM.

**Figure 3.** Bar graph showing effect of transforming growth factor-β neutralizing antibody (TGF-β Ab) on angiotensin II (Ang-II)–induced increase in DNA synthesis. Smooth muscle cells derived from spontaneously hypertensive rats were grown to confluence and growth arrested as described in “Materials and Methods.” Four days later the serum-free medium was changed, and then Ang-II (10^{-6} M) alone or with an affinity-purified anti–TGF-β antibody (10 μg/ml) or an affinity-purified nonimmune rabbit immunoglobulin was added. The smooth muscle cells were pulsed with [3H]thymidine at 48 hours. [3H]Thymidine incorporation was assayed 24 hours later. Results are expressed as mean±SEM. Cnt, control.

**Treatment With Exogenous Transforming Growth Factor-β Alone Did Not Increase DNA Synthesis in the Initial 24 Hours After Treatment**

Taken together, data from TGF-β neutralizing antibody and mink lung epithelial bioassay experiments implicated TGF-β in mediation of Ang II effects. Subsequent studies addressed whether TGF-β was sufficient to induce SMC mitogenesis or whether Ang II–induced effects were mediated via interactions of TGF-β and other growth factors. Results of these studies (Figure 5) demonstrated that exogenous purified human TGF-β failed to elicit a mitogenic response within 24 hours of treatment, a time point compatible with concentrations of active TGF-β being increased 48 hours after Ang II treatment (Figure 4, top panel) and Ang II–induced increases in DNA synthesis occurring between 48 and 72 hours after treatment (Figure 1). Additional studies in this laboratory have demonstrated that TGF-β elicits a delayed (48–72 hours after treatment) mitogenic response (authors’ unpublished observations) in these cells; however, the concentrations of TGF-β necessary for this response were several log orders greater than concentrations induced by Ang II (Figure 4, middle and bottom panels). Data indicate that whereas Ang II–induced responses are dependent on TGF-β, TGF-β alone is not sufficient to mimic the response, suggesting that other SMC autocrine factors (e.g., PDGF-AA or endothelin) are involved in Ang II–induced effects.

**Angiotensin II Increased DNA Synthesis in Epidermal Growth Factor–, Basic Fibroblast Growth Factor–, or Platelet-Derived Growth Factor-BB–Stimulated Smooth Muscle Cells and These Effects Were Markedly Inhibited by Treatment With Transforming Growth Factor-β Neutralizing Antibody**

Because Ang II actions after vascular injury in vivo most likely occur in the presence of other growth factors, additional studies addressed whether Ang II enhanced EGF-, bFGF-, or PDGF-BB–induced growth responses. Available evidence suggests that these three growth factors are present at the site of vascular injury.17,18 Results of in vitro experiments demonstrated that cotreatment with Ang II increased DNA synthesis approximately twofold between 48 and 72 hours after treatment in SMCs stimulated with EGF, bFGF, or PDGF-BB (Figure 6).

To determine whether the Ang II–induced increases in DNA synthesis in EGF-, bFGF-, or PDGF-BB–stimulated SMCs were mediated via TGF-β, the effect of TGF-β neutralizing antibody on these responses was measured. Addition of TGF-β neutralizing antibody completely inhibited the Ang II–induced increases in DNA synthesis in EGF- or bFGF-treated SMCs and markedly reduced the Ang II–induced increase in DNA synthesis in PDGF-BB–treated SMCs between 48 and 72 hours after treatment (Figure 6). Consistent with this result, separate studies have shown that exogenous TGF-β increased the proliferative response of SHR-derived SMCs treated with EGF, bFGF, or PDGF-BB19 (authors’ unpublished observations). Additionally, the
concentration of TGF-β that increased [3H]thymidine incorporation in EGF- or PDGF-BB–treated SMCs (approximately $10^{-12}$ M) was an order of magnitude less than the TGF-β concentration required to increase [3H]thymidine incorporation in bFGF-treated SMCs.

Angiotensin II Increased the Proliferative Response to Epidermal Growth Factor or Platelet-Derived Growth Factor-BB

Cotreatment with Ang II increased the proliferative response to EGF or PDGF-BB, eliciting an 11% and 30% increase in cell number, respectively, 5 days after a single treatment (Figure 7). No significant increases in cell number were observed in SMCs treated with Ang II alone or in combination with bFGF. The failure to observe a change in cell number after Ang II treatment despite an increase in [3H]thymidine incorporation was explored in further studies. Potential explanations for this apparent discrepancy included the following: 1) the increase in [3H]thymidine incorporation represented a pool effect and not a true increase in DNA synthesis; 2) the absolute increase in the fraction of replicating SMCs was insufficient to induce significant increases in cell number; and/or 3) Ang II treatment induced an increased fraction of cells with tetraploid DNA similar to the effect seen by Geisterfer et al. in SD-derived SMCs after Ang II treatment. To address each of these possibilities, autoradiographic and flow cytometric studies were performed. Results of autoradiographic studies demonstrated that the fraction of labeled cells was $2.9\pm0.4\%$ in controls versus $4.9\pm0.2\%$ in Ang II–treated SMCs ($p<0.01$) between 48 and 72 hours after treatment. Consistent with these findings, [3H]thymidine incorporation was increased by Ang II treatment in CM from Ang-II–treated SMCs (Figure 4, Top panel).
The cells described in "Materials and Methods." Four days later the serum-free medium was changed, and then various concentrations of TGF-β or vehicle (veh) were added. Approximately 0.5 hour later, the smooth muscle cells were pulsed with [3H]thymidine. [3H]Thymidine incorporation was assayed 24 hours later.

Discussion

In vivo studies demonstrating that angiotensin converting enzyme inhibitors as well as nonpeptide Ang II antagonists markedly inhibit neointimal lesion formation after balloon injury strongly implicate a role for Ang II in regulation of SMC proliferation after vascular injury. The cellular and molecular mechanisms whereby Ang II might promote proliferation of SMCs in vivo are not known, and our understanding in this area is confounded by studies in cultured SMCs demonstrating variable Ang II–induced growth responses in different laboratories (reviewed by Owens and Schelling et al). For example, results of studies in our laboratory, later confirmed by others, demonstrated that Ang II treatment increased protein synthesis and content (i.e., cellular hypertrophy) in cultured rat aortic SMCs derived from SD rats but did not stimulate SMC proliferation when administered in serum-free medium, in the presence of serum, or in combination with other purified growth factors such as PDGF. In contrast, a number of laboratories have reported Ang II–induced proliferative responses, albeit small effects, in cultured...
SMCs under similar conditions\textsuperscript{8,9,23–25} with the most convincing effects being observed in cultured SMCs derived from SHR.\textsuperscript{8,9,25,26} The cells were trypsinized and counted at 5 days as described in “Materials and Methods.”

Results of the present studies, carried out in aortic SMCs derived from SHR, demonstrated that Ang II stimulated modest increases in [\textsuperscript{3}H]thymidine incorporation and labeling indexes but not in SMC number when administered alone in serum-free medium (Figures 1 and 7). However, in contrast to our previous findings in SD-derived SMCs\textsuperscript{8} (A.A. Geisterfer and G.K. Owens, unpublished data), Ang II increased the proliferative response of SMCs treated with PDGF-BB or EGF (Figure 7). Taken together, these results indicate that Ang II-induced growth responses vary as a function of the target SMC culture used. These variations may be attributed to differences in methods of cell isolation and culture (i.e., enzyme dissociation or explant cultures, passing protocols, serum lots, etc.) and/or genetic differences between SMCs derived from different strains of rats.

In the present studies, SMCs were derived and cultured from SHR aortas by using procedures and reagents similar to those used in our previously reported studies using aortic SMCs derived from SD rats.\textsuperscript{6} Although it is thus tempting to speculate that differences in Ang II responsiveness in SD- versus SHR-derived SMCs may be attributed solely to genetic differences, this is unlikely to be the case. Cultured SMCs are known to undergo extensive phenotypic modulation in vitro, which is characterized by diminished expression of SMC-specific contractile proteins and accelerated growth responsiveness.\textsuperscript{27,28} Moreover, studies by Rosen et al\textsuperscript{29} demonstrating that Wistar-Kyoto but not SHR SMCs revert to a polyploid state in vitro when grown under identical culture conditions (the opposite of what has been found in vivo\textsuperscript{30}) provide clear evidence that SMCs from different rat strains undergo differential phenotypic modulation in culture. Thus differences in Ang II–induced growth responses in SD- and SHR-derived SMCs may also be attributed to differential phenotypic modulation in vitro. Although definitive data are lacking, there is evidence supporting the hypothesis that the phenotypic state of the SMC is a critical determinant of Ang II growth responsiveness. For example, Daemen et al\textsuperscript{31} demonstrated that intimal SMCs, which are phenotypically modulated,\textsuperscript{27,28} show an enhanced proliferative response as compared with medial SMCs after Ang II infusion. However, the specific nature of phenotypic changes that influence Ang II growth responsiveness of SMCs, as well as the specific mechanisms and factors that control these phenotypic changes, remains to be determined.

Results of the present study demonstrating that Ang II potentiated the mitogenic response to PDGF-BB and EGF (Figures 6 and 7) may provide some insight regarding the mechanisms whereby angiotensin converting enzyme inhibitors and Ang II receptor antagonists inhibit neointimal lesion formation in vivo. There is evidence suggesting that the vessel wall contains a complete renin–Ang II system\textsuperscript{32,33} and that peptide growth factors are present at sites of vascular injury.\textsuperscript{10,11,17,18,31–33} In a recent study, Majesky et al\textsuperscript{34} demonstrated that TGF-β mRNA expression in the vessel wall was increased after vascular injury. They further demonstrated that neointimal SMCs stained positive for TGF-β in a much higher percentage than medial SMCs from either injured or normal vessels and that the pattern of staining of the neointimal SMCs was suggestive of intracellular synthesis. Additionally, infusion of TGF-β after vascular injury increased the labeling index of intimal SMCs. Thus, the available in vivo data, though very limited, are consistent with the hypothesis that Ang II regulates neointimal SMC proliferation by inducing autocrine production of TGF-β. The relevance of the results presented here to in vivo events remains speculative, however, as it remains to be determined whether the phenotype exhibited by SHR-derived SMCs in culture mimics a phenotype exhibited by SMCs in neointimal lesions. Additionally, Bell and Madri\textsuperscript{24} have presented evidence based on culture studies suggesting that the role of Ang II in lesion development may be related to effects on endothelial cell and SMC migration rather than to inhibition of SMC proliferation.

Production of active TGF-β is necessary but not sufficient for Ang II–induced increases in DNA synthesis in the present studies. Although TGF-β neutralizing antibody markedly inhibited Ang II–induced effects and Ang II treatment was associated with increased concentrations of active TGF-β in CM, exogenous TGF-β was not mitogenic. Interpretation of these data suggests that other Ang II–induced growth factors are required for Ang II–induced mitogenesis. Growth factors that may be involved include PDGF-AA or endothelin, which have been shown to be increased by Ang II treatment in cultured SMCs.\textsuperscript{12,35} However, the SHR-derived SMCs used in the present study fail to respond mitogenically to PDGF-AA (authors’ unpublished observations), indicating that involvement of this growth factor would require a concurrent change in PDGF-AA responsiveness. Further studies are needed to test the possible involvement of autocrine factors in mediation of Ang II responses.

**Figure 7.** Bar graph showing proliferative response of spontaneously hypertensive rat–derived smooth muscle cells treated with angiotensin II (Ang-II) and epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or platelet-derived growth factor (PDGF-BB). Smooth muscle cells from spontaneously hypertensive rats were grown to confluence and growth arrested as described in “Materials and Methods.” Four days later the serum-free medium (SFM) was changed, and then Ang-II (10⁻⁶ M) and EGF (1.7 nM), bFGF (1.25 nM), or PDGF-BB (0.7 nM) was added. The cells were trypsinized and counted at 5 days as described in “Materials and Methods.”
A number of observations in the present studies support the hypothesis that Ang II-induced growth responses were mediated by autocrine production of TGF-β. These observations include the following: 1) Ang II–induced increases in [3H]thymidine incorporation were delayed and could be blocked with a neutralizing antibody to TGF-β; 2) anti–TGF-β antibody inhibited Ang II–enhanced growth responses to EGF, PDGF, and bFGF; and 3) Ang II treatment was associated with increased concentration of active TGF-β in CM. The mechanism by which TGF-β regulates proliferation in SHR-derived SMCs, however, is unknown. Majack et al. found that TGF-β inhibited PDGF- and serum-induced proliferation of subconfluent SMCs but potentiated cell growth in confluent cells. Our laboratory demonstrated that TGF-β was a potent inhibitor of serum-induced proliferation in subconfluent SMCs derived from SD rats. This inhibition was associated with increased cell cycle transit time and development of cellular hypertrophy. Battegay et al. reported that TGF-β induced a proliferative response in both sparse and dense human SMCs, which varied depending on the concentration of TGF-β available per cell. Studies in our laboratory (unpublished observations) with SHR-derived SMCs indicate that TGF-β mitogenic synergism with EGF and PDGF-BB is concentration dependent. Low concentrations induced a transient mitogenic effect, while higher concentrations initially delayed S phase entry but then increased mitogenesis at later time points. The mechanisms responsible for differential responses of SMCs to TGF-β are unclear but may be a function of receptor subtype expressed by the cell, altered postreceptor coupling mechanisms, or different responses to secondary mediators such as PDGF-AA, PDGF-BB.

Results of the present studies demonstrated that Ang II treatment was associated with an increase in the level of active TGF-β in CM. A number of studies have reported that Ang II treatment increases TGF-β mRNA levels, suggesting that at least part of the effect in the present studies may be due to increased TGF-β production. TGF-β, however, is secreted in a latent form and thus additional mechanisms must be invoked to explain the increase in active TGF-β. TGF-β can be activated ex vivo by transient acidification, proteolysis, the use of chaotrophic agents, or plasmin, but the mechanism of activation of TGF-β by cultured SMCs has not been determined. Sato et al. studying TGF-β activation in endothelial cell/SMC co-cultures, have proposed that TGF-β formation is a self-regulating system encompassing an unidentified protease, possibly plasminogen activator, which activates TGFB-β, which then induces secretion of plasminogen activator inhibitor, which inhibits the protease. Additional studies have shown that SMCs secrete plasminogen activator both in culture and in vivo after vascular injury. It is not known whether plasminogen activator can activate TGF-β directly or whether there are low levels of plasminogen in cultured SMC CM that would enable the generation of plasmin. Similarly, the effects of Ang II treatment of SMC on plasminogen activator or plasmin levels are unknown. Further studies are needed to clarify the time course of activation and production of TGF-β after Ang II treatment. Additionally, the effects of Ang II on production of TGF-β activating proteases (when identified), plasminogen activator inhibitor, and/or essential cofactors (if identified) need to be explored.

In summary, Ang II has been implicated in regulation of SMC proliferation after vascular injury in the rat. Ang II is at best a weak mitogen for quiescent SMCs, and the growth response varies depending on the target SMCs. In the present study, Ang II increased the mitogenic response of SHR-derived SMCs to EGF and PDGF-BB, and this effect was at least partially mediated via autocrine secretion of TGF-β. Thus, these results suggest a potential mechanism whereby Ang II might regulate SMC mitogenesis after vascular injury.

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