Vascular Smooth Muscle Cell Hypertrophy vs. Hyperplasia

Autocrine Transforming Growth Factor-β_1 Expression Determines Growth Response to Angiotensin II

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Abstract

Recent observations in our laboratory suggest that angiotensin II (Ang II) is a bifunctional vascular smooth muscle cell (VSMC) growth modulator capable of inducing hypertrophy or inhibiting mitogen-stimulated DNA synthesis. Because transforming growth factor-β_1 (TGFβ_1) has similar bifunctional effects on VSMC growth, we hypothesized that autocrine production of TGFβ_1 may mediate the growth modulatory effects of Ang II. Indeed, this study demonstrates that Ang II induces a severalfold increase in TGFβ_1 mRNA levels within 4 h that is dependent on de novo protein synthesis and appears to be mediated by activation of protein kinase C (PKC). Ang II not only stimulates the synthesis of latent TGFβ_1, but also promotes its conversion to the biologically active form as measured by bioassay. The coincubation of VSMCs with Ang II and control IgG has no significant mitogenic effect. However, the co-administration of Ang II and the anti-TGFβ_1 antibody stimulates significantly DNA synthesis and cell proliferation. We conclude that: (*a*) Ang II induces increased TGFβ_1 gene expression via a PKC dependent pathway involving de novo protein synthesis; (*b*) Ang II promotes the conversion of latent TGFβ_1 to its biologically active form; (*c*) Ang II modulates VSMC growth by activating both proliferative and antiproliferative pathways; and (*d*) Autocrine active TGFβ_1 appears to be an important determinant of VSMC growth by hypertrophy or hyperplasia. (*J. Clin. Invest.* 1992. 90:456–461.) Key words: cell growth • growth factor • mitogenesis • protein kinase C • vasoconstrictor

Introduction

A growing body of evidence suggests that angiotensin II (Ang II)[1] can modulate vascular smooth muscle cell (VSMC) growth (1–3). Ang II induces VSMC hypertrophy, but not hyperplasia in confluent quiescent cells in a defined serum-free medium (1). Our laboratory has demonstrated further that the Ang II-induced hypertrophy is associated with increased mRNA levels of protooncogenes, c-fos, c-myc, and c-jun, and the increased expression and secretion of the autocrine growth factor platelet-derived growth factor (PDGF) AA homodimer (4, 5).

PDGF has been reported to be a potent mitogen and, therefore, the autocrine secretion of PDGF was expected to stimulate VSMC hyperplasia. However, recent experiments in our laboratory that utilize antibodies and antisense oligonucleotides directed against PDGF AA have demonstrated that autocrine PDGF AA is an important mediator of AngII-stimulated VSMC hypertrophy (6). This paradoxical response of hypertrophy but not mitogenesis led us to postulate that Ang II may be a bifunctional growth modulator of VSMCs by activating both proliferative and antiproliferative cellular events. Indeed, recent studies in our laboratory have demonstrated that prolonged exposure to Ang II inhibited basic fibroblast growth factor–induced DNA synthesis (7). Moreover, the Ang II inhibitory effect was mimicked by a protein kinase C (PKC) activator and blocked by down-regulation of the PKC pathway (7). These findings suggest that Ang II can activate an antiproliferative effect that is mediated by PKC.

Recently, transforming growth factor-β_1 (TGFβ_1) has been reported to exert a bifunctional effect on VSMC growth (8–12). Similar to observations with Ang II, administration of TGFβ_1 stimulates VSMC hypertrophy and inhibits mitogen-stimulated DNA synthesis (8). The autocrine production of biologically active TGFβ_1 in VSMCs has not been previously demonstrated. In the present study, we tested the hypothesis that the growth modulatory effects of Ang II may be mediated by the activation of autocrine production of TGFβ_1. Our results support the postulate that Ang II is a bifunctional growth factor that activates both proliferative and antiproliferative pathways. The autocrine production of biologically active TGFβ_1 is a major determinant of whether VSMCs grow by hypertrophy or hyperplasia. Inactivation of this antiproliferative autocrine growth factor unmasks the full mitogenic effect of Ang II. These findings may have implications for the role of Ang II as a determinant of vascular structure.

Methods

Cell culture. Primary cultures of VSMCs were initiated by enzymatic dissociation from the aortae of 7–8-wk-old male Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) by the method of Owens et al. (12). VSMCs were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DME/F12) (Gibco Laboratories, Grand Island, NY), 10% heat-inactivated fetal calf serum (FCS) (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), 25 mM Hepes buffer, glutamine, and glucose. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every two days and cells passaged by trypsin/EDTA with a split ratio of 1:10 in 75-cm^2 flasks (Costar, Cambridge, MA).

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1. *Abbreviations used in this paper:* Ang II, angiotensin II; PDGF, platelet-derived growth factor; PKC, protein kinase C; TGFβ_1, transforming growth factor-β_1; TGFAB, anti-TGFβ_1 neutralizing antibody; VSMC, vascular smooth muscle cell.

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Utilizing this technique, VSMCs exhibit typical, spindle-shaped morphology and a multilayered growth pattern, contain myofilaments by electron microscopy, and express α-actin as demonstrated by immunofluorescence studies with a smooth muscle specific anti-α-actin antibody (Sigma Chemical Co., St. Louis, MO). Studies were conducted on VSMCs (passages 7–12) that had achieved confluence for two days in 10% FCS/DME/F12. The cells were then incubated in a defined serum-free medium (DME/F12 plus insulin [5×10^{-7} M], transferrin [5 μg/ml], and ascorbic acid [0.2 mM]) (Sigma Chemical Co.) for 2 d. Libby and O'Brien (13) have shown that this medium maintains VSMCs in a quiescent, noncatabolic state for an extended period of time.

RNA analysis. Cells were harvested by lysis in guanidium isothiocyanate solution. RNA was isolated by standard techniques of centrifugation of the lysate through a cesium chloride gradient and ethanol precipitation. The RNA yield was quantitated by absorbance at 260 nm. For Northern blot analysis, RNA was electrophoresed in agarose (1.5%)/formaldehyde (2.2 M) gels, transferred to nylon membranes (Genescreen, Dupont-New England Nuclear, Boston, MA) by capillary action, and nucleic acids immobilized by UV irradiation (Stratagene, La Jolla, CA). The membranes were prehybridized and hybridized by standard techniques. The blots were washed in 0.1 × SSC/1% SDS at 55°C for 2 h and autoradiographed by standard techniques.

cDNA probes. The porcine TGFβ_1-specific cDNA 747-bp Pst fragment was employed for hybridization (kindly provided by Dr. Michael Sporn, National Cancer Institute, Bethesda, MD). DNA probes were labeled with 25 μCi of 32P deoxycytidine 5′-triphosphate (3,000 Ci/mmol, New England Nuclear, Boston, MA) using the random oligonucleotide method. The ^{32}P-labeled DNA was purified by Sephadex G-50 chromatography.

TGFβ_1 bioassay. Previous studies have established that mink lung epithelial cells can be utilized as a very sensitive bioassay for the growth inhibitory effects of active TGFβ_1 (14). Mink lung epithelial cells (CCL-64) (American Type Culture Collection, Rockville, MD) were grown in Eagle's minimal essential medium supplemented with nonessential amino acids and 10% FCS. Growth assays were conducted on freshly plated subconfluent cells exposed to serum-containing medium for 6 h, washed with defined serum-free medium, then incubated with TGFβ_1 or conditioned medium in the defined serum-free medium for 24 h. DNA synthesis in these cells was measured by pulsing with [^{3}H]-thymidine (2 μCi/ml) for a 6-h period beginning 18 h after agonist stimulation. A standard curve was constructed with increasing concentrations of purified human TGFβ_1 (R & D Systems, Inc., Minnesota, MN) in each experiment. Conditioned medium was obtained from VSMC 18 h after treatments with Ang II or vehicle and was administered to mink lung epithelial cells in a range of dilutions (1:10 to 1:40) to fall within the linear range of the standard curve. The specificity of the inhibitory response was confirmed by incubation with the anti-TGFβ_1 neutralizing antibody (TGFAB). Based upon the standard curve, the TGFβ_1 activity is expressed as picomolar concentration equivalents.

Antibody characteristics. Comparisons were made between control rabbit IgG (Sigma Chemical Co.) and a purified IgG fraction of rabbit polyclonal TGFAB (R & D Systems, Inc.). This TGFAB does not cross-react with either acidic or basic fibroblast growth factor, PDGF, or epidermal growth factor (manufacturer's data). The antibody recognizes the active, but not the latent form of TGFβ_1, inhibits specific TGFβ_1 binding to its surface receptor, and immunoprecipitates TGFβ_1 at a 1:10,000 dilution (15–17). Extensive experimental characterization has documented the ability of the antibody to block specifically the TGFβ_1-induced stimulation of anchorage-independent growth of rabbit kidney cells (15); inhibition of mink lung epithelium (16, 18), and VSMC growth (18); stimulation of fibroblast (19) and monocyte migration (20); and inhibition of endothelial cell migration (21). The capacity of the TGFAB to neutralize TGFβ_1-induced inhibition of mink lung epithelial cell growth was also determined specifically in this study. The effect of the TGFAB was assessed by preincubation of the antibody for 1 h with the ligand before exposure to the mink lung epithelial cells. As shown in Fig. 3 (*top*), the TGFAB significantly blocked the growth inhibitory effects of purified TGFβ_1 as compared to IgG. The TGFAB administered with vehicle had no significant growth stimulatory effect on mink lung epithelial cell DNA synthesis (data not shown).

Experimental protocols. The time course of the Ang II–stimulated increase in TGFβ_1 mRNA levels was assessed by incubating cells with Ang II (10^{-6}) and harvesting at 2, 4, 8, and 20 h after stimulation. The dose dependency of the response was studied by incubating the cells with Ang II at doses ranging from 10^{-12} to 10^{-6} M and harvesting cells at 8 h after stimulation.

The role of de novo protein synthesis in mediating TGFβ_1 expression was assessed by preincubating the cells with cycloheximide (25 μg/ml) for 45 min before stimulation with Ang II (10^{-6} M) and subsequently harvesting at 8 h after Ang II treatment.

The possible involvement of PKC activation as a mediator of the Ang II effect was examined by incubating VSMCs acutely with the PKC activator, phorbol myristate acetate (PMA) (10 ng/ml) and the effect of TGFβ_1 mRNA expression was examined. In addition, several investigators have established that prolonged PMA (100 ng/ml) pretreatment for 24 h blocks PKC activation in VSMC (21–25). We therefore employed this protocol to assess the effect of down-regulating the PKC pathway on the response to the acute administration of Ang II and PMA on TGFβ_1 gene expression.

Metabolic studies were conducted with radiolabeled precursors to characterize the growth response to Ang II. DNA synthesis was assessed by administering tritiated thymidine (2 μCi/ml) for a period of 24 h beginning 12 h after Ang II stimulation. Protein synthesis was measured by incubating cells with tritiated proline (10 μCi/ml) for a 4-h period beginning 15 h after Ang II stimulation. Preliminary experiments demonstrated that these incubation time periods correspond to periods of maximal incorporation of these radiolabeled precursors. At the end of the incubation period, the medium was removed, the cells washed twice with PBS, then washed once with 10% TCA, followed by incubation with 10% TCA for 30 min at 4°C. The TCA insoluble material was washed twice with ethanol, solubilized in 0.25 M NaOH, and then buffered to neutral pH. Tritiated thymidine and proline incorporation was determined by liquid scintillation spectrometry of a 50-μl aliquot.

We tested the hypothesis that TGFβ_1 modulates the Ang II–induced growth response by assessing the effect of the TGFAB (100 μg/ml) compared to an equal concentration of control IgG on the rate of protein and DNA synthesis and cell proliferation. Incubation with the TGFAB in the presence of vehicle induced a modest increase in thymidine incorporation compared to control IgG under basal conditions (see Results). In order to more closely examine the direct effect of Ang II stimulation, the data are also expressed as the difference between the Ang II–stimulated cells and vehicle-treated cells for each antibody group. Ang II's stimulatory effect on thymidine or proline uptake is expressed as the difference in counts per minute between Ang II–stimulated cells and the vehicle-treated control in the presence of either control IgG (100 μg/ml) or the TGFAB (100 μg/ml). To confirm that the potentiated growth response to co-incubation with Ang II and TGFAB was related to selective neutralization of active TGFβ_1, we preincubated the TGFAB (100 μg/ml) with TGFβ_1 (1 ng/ml) before administration into the culture medium. The effect of coincubation of Ang II with the TGFAB prebound to TGFβ_1 on DNA synthesis was compared to the response to coincubation of Ang II with TGFAB alone or control IgG. The data is expressed as the difference in counts per minute between Ang II–stimulated cells and the vehicle-treated control in the presence of control IgG, the TGFAB alone, or TGFβ_1 prebound to the TGFAB. Furthermore, to assess the specificity of the effect of co-incubation of with the TGFAB on DNA synthesis, we examined the growth response to submaximal concentrations of basic fibroblast growth factor (10 ng/ml) in the presence of control IgG (100 μg/ml) vs. TGFAB (100 μg/ml) (n = 16). To confirm that changes in thymidine incorporation were associated with mitogenesis (cell division with an increase in cell number), the effect of TGFAB on Ang II–induced cell prolifera-

tion was assessed by changes in cell number measured by hemocytometer 64 h after agonist stimulation. Ang II's stimulatory effect on cell density is expressed as the difference in cell number between the Ang II–stimulated cells and the vehicle-treated control (expressed as zero baseline) in the presence of either control IgG or TGFAB. Statistical analyses were performed with either ANOVA or Student's *t* test on the PC STAT statistical program.

Results

Confluent, quiescent adult rat aortic VSMCs exhibited low levels of constituitive TGFβ_1 mRNA expression. Incubation with Ang II resulted in a four- to eightfold increase in mRNA levels (Fig. 1, *top*). This response was detectable within 4 h of incubation and was sustained for at least 20 h. The response was dose dependent with a threshold of $\sim 10^{-9}$ M.

It is well established that PKC is an important mediator of cellular events stimulated by Ang II. Indeed, previous experiments have suggested that Ang II can produce an antiproliferative effect via a PKC-dependent pathway (7). We, therefore, examined the role of PKC as a mediator of Ang II–induced TGFβ_1 gene expression. Utilizing the protein kinase activator, PMA, we were able to demonstrate increases in TGFβ_1 mRNA levels similar to that achieved with Ang II. The inactive phorbol ester, 4-α phorbol didecanote had no effect on TGFβ_1 expression (Fig. 1, *bottom*). To further assess the role of PKC activation as a mediator of the Ang II effect on TGFβ_1 mRNA expression, we inactivated this pathway by a 24-h pretreatment with PMA. Down-regulation of the PKC pathway was confirmed by demonstrating that acute PMA incubation had no significant effect on TGFβ_1 mRNA levels. Indeed, 24-h PMA pretreatment blocked the stimulatory effect of Ang II on TGFβ_1 gene expression (Fig. 1, *bottom*).

In order to assess whether the Ang II effect on TGFβ_1 expression was dependent upon synthesis of a mediator protein,

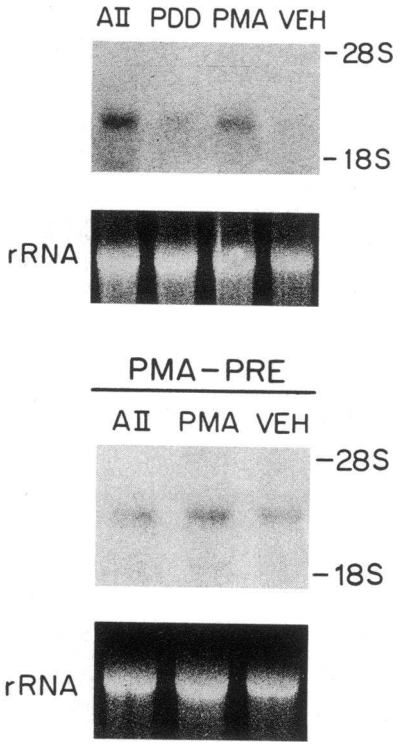

Figure 1. (*Top*) Ang II (*AII*) (10^{-6} M) and the PKC activator PMA (10 ng/ml) induce increased TGFβ_1 mRNA levels measured 8 h after stimulation compared to vehicle (*VEH*) or 4-α phorbol didecanote (*PDD*, 100 ng/ml). (*Bottom*) Effect of PKC pathway downregulation. PMA pretreatment for 24 h attenuates the induction of TGFβ_1 mRNA levels by Ang II or acute PMA treatment. 28S and 18S size markers are as shown. Lower panel on each part shows photograph of ethidium bromide–stained gel documenting integrity and comparable loading of total RNA (25 μg) in each lane.

cells were preincubated with the protein synthesis inhibitor cycloheximide. Inhibition of protein synthesis blocked the stimulatory effect of Ang II on TGFβ_1 mRNA levels. However, administration of cycloheximide 4 h after Ang II stimulation had no effect on the Ang II–induced increase in steady-state TGFβ_1 mRNA levels (Fig. 2). These data suggest that either a novel protein synthesized within the first few hours of agonist exposure or a short-lived constitutive protein mediates the increase in TGFβ_1 mRNA levels induced by Ang II. Similarly, induction of TGFβ_1 gene expression by Ang II required incubation for a period > 2 h, but < 4 h.

Previous studies have established that TGFβ_1 is normally secreted in a biologically inactive latent form (15, 16). To examine whether Ang II stimulates both the production of latent TGFβ_1 and conversion to its active form, we utilized the mink lung epithelial cell bioassay to assess the presence of active TGFβ_1. Conditioned medium from VSMC stimulated with Ang II significantly inhibited mink lung epithelial cell DNA synthesis compared to conditioned medium from vehicle-stimulated VSMC. Ang II induced a 10-fold increase in TGFβ_1 activity from a basal level of 6±4 to 62±8 pM ($P < 0.01$). This inhibitory activity was completely abolished by the TGFAB (Fig. 3, *top*).

In order to assess the role of autocrine TGFβ_1 as a modulator of Ang II–induced VSMC growth, we examined the effect of co-incubation with TGFAB. This antibody blocked the inhibitory effect of purified TGFβ_1 on mink lung epithelial cell growth (Fig. 3, *bottom*). In the presence of control IgG, Ang II induced a 100% increase in the rate of protein synthesis as measured by tritiated proline incorporation. Co-incubation with the TGFAB had no significant effect on the Ang II–induced increase in protein synthesis.

In the presence of control IgG, Ang II stimulated a significant increase in thymidine incorporation compared to vehicle (611±56 vs. 5,085±698 cpm; $P < 0.01$). However, co-incubation of the TGFAB with Ang II induced a marked enhancement of Ang II–stimulated DNA synthesis as measured by tritiated thymidine incorporation when compared to the IgG control (9,653±1,072 vs. 5,085±698 cpm; $P < 0.01$). The administration of TGFAB plus vehicle induced a modest increase in DNA synthesis under basal conditions (611±56 vs. 2,931±435 cpm; $P < 0.05$) without a significant change in cell number (data not shown). This effect on thymidine incorporation alone was not sufficient to account for the potentiated increase in Ang II–stimulated DNA synthesis (Table I). Indeed, during co-incubation with the TGFAB, Ang II further potentiated the increase in thymidine incorporation as compared to coincubation with IgG (6,722±999 vs. 4,474±657 cpm; $P < 0.01$).

In order to establish the specificity of the potentiation effect of TGFAB on Ang II–induced DNA synthesis, TGFAB was preincubated with the ligand TGFβ_1 to block its capacity to neutralize active TGFβ_1 synthesized by the VSMC. In this additional series of experiments, co-incubation with the TGFAB again potentiated the Ang II–stimulated increase in DNA synthesis above baseline by 50% as compared to co-incubation with IgG (15,325±865 vs. 9,225±415 cpm; $P < 0.01$). However, preincubation with the ligand abolished the capacity of the TGFAB to potentiate Ang II–stimulated DNA synthesis as compared to the IgG control (9,225±415 vs. 10,949±774 cpm; $P > 0.05$) (Fig. 4, *top*). To further confirm the specificity of the action of the TGFAB, we assessed its effect on basic fibroblast

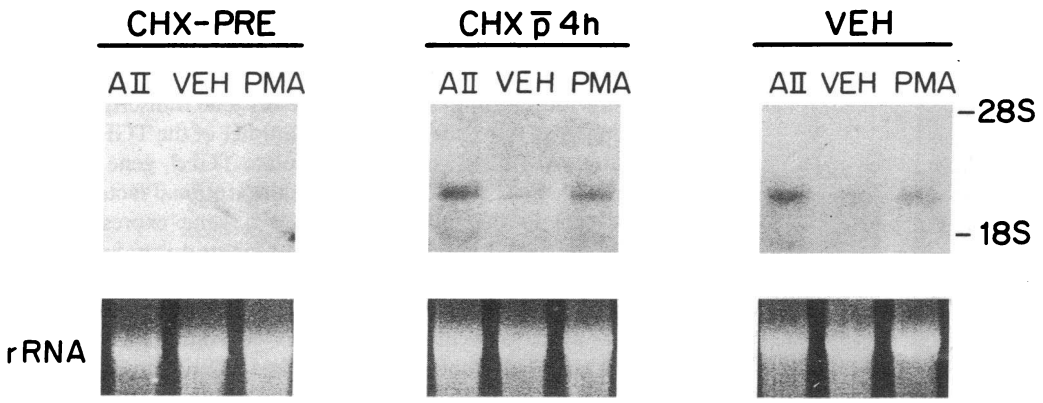

Figure 2. Role of de novo protein synthesis on induction of TGFβ_1 gene expression by Ang II (*AII*) (10^{-6} M) and PMA (10 ng/ml). Cells were either (*a*) pretreated with cycloheximide (*CHX*) (25 μg/ml), (*b*) treated with cycloheximide 4 h after agonist, or (*c*) treated with vehicle (*VEH*).

growth factor's mitogenic action. TGFAB had no effect on 10 ng/ml basic fibroblast growth factor–stimulated thymidine incorporation in VSMC as compared with coincubation of basic fibroblast growth factor with the IgG control (6,504±505 vs. 5,908±249 cpm; $P > 0.05$).

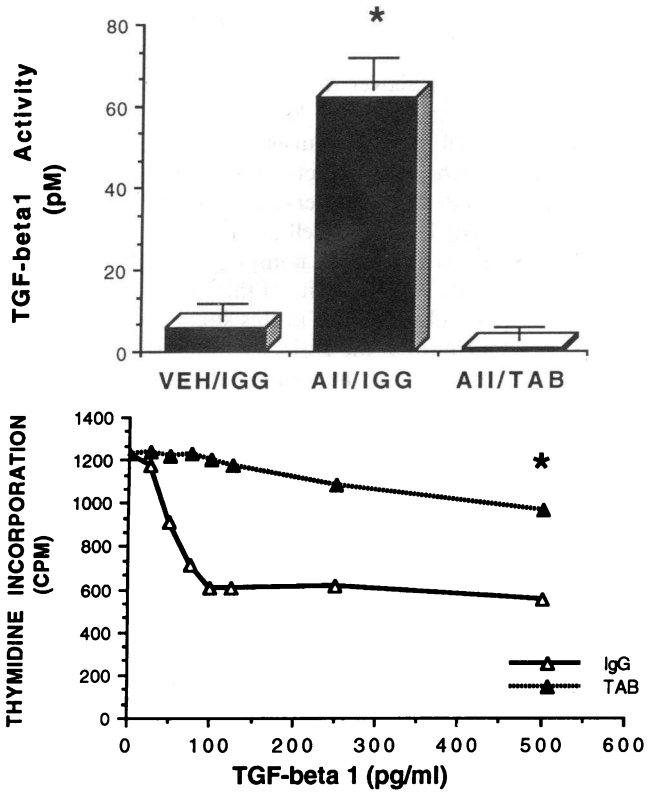

Figure 3. (*Top*) The production of active TGFβ_1 by Ang II–stimulated VSMC. Values are expressed as picomolar concentrations of TGFβ_1 activity as determined by the mink lung epithelial cell bioassay. The anti-TGFβ_1 antibody (*TAB*) abolished the TGFβ_1 activity in the conditioned medium from Ang II–stimulated VSMC. The statistical comparison shown was made between conditioned medium from Ang II–treated VSMC vs. vehicle-treated VSMC in the presence of control IgG as well as Ang II–treated VSMC conditioned medium preincubated with the anti-TGFβ_1 antibody ($n = 16$, $^*P < 0.01$). (*Bottom*) Effect of the anti-TGFβ_1 antibody on the growth inhibitory response of mink lung epithelial cells to purified TGFβ_1. Values are expressed as counts per minute. The statistical comparison shown was made between the standard curve with control IgG vs. anti-TGFβ_1 antibody ($n = 8$; $^*P < 0.01$).

In accordance with the thymidine incorporation data, Ang II induced a 50% increase in cell number in the presence of TGFAB ($P < 0.01$) (Fig. 4, *bottom*). In contrast, Ang II had no significant stimulatory effect on cell proliferation during coincubation with IgG as previously shown. Thus, blockade of autocrine TGFβ_1 activity unmasked Ang II–induced mitogenesis.

Discussion

Our laboratory has been intrigued by the observation that in the presence of serum, Ang II promotes hyperplasia (3), but in the absence of serum, Ang II induces only hypertrophy (1). We have shown that Ang II–induced hypertrophy is associated with increased expression of c-fos, c-myc, and c-jun and enhanced secretion of PDGF AA (4, 5, 25). Furthermore, we have recently shown that blockade of the Ang II–stimulated increase in autocrine PDGF AA expression attenuates the hypertrophic response to Ang II (6). Yet, despite activation of these growth promoting proteins, Ang II does not induce hyperplasia in serum-free media. We postulated that Ang II has a bifunctional effect on VSMC growth. Ang II may activate in VSMC a proliferative pathway (PDGF), as well as an antiproliferative pathway, such that the net effect is hypertrophy without hyperplasia.

Several studies have documented that the administration of TGFβ_1 to VSMC induces cellular hypertrophy and inhibits mitogen stimulated proliferation (8, 9, 11). We were intrigued by the observation that TGFβ_1 has bifunctional effects on VSMC growth similar to that induced by Ang II. Accordingly, we postulated that the bifunctional effect of Ang II on VSMC growth may be mediated by the autocrine production of TGFβ_1. How-

Table I. Effect of the Anti-TGFβ$_1$ Antibody on Ang II–stimulated VSMC DNA Synthesis

Treatment	Thymidine incorporation
	cpm
IgG + vehicle ($n = 41$)	611±56
IgG + Ang II ($n = 41$)	5,085±698
TAB + vehicle ($n = 31$)	2,931±435
TAB + Ang II ($n = 31$)	9,635±1,072

Values are expressed as the mean±SEM. IgG, co-incubation with control IgG (100 μg/ml). TAB, coincubation with the anti-TGFβ_1 antibody (100 μg/ml).

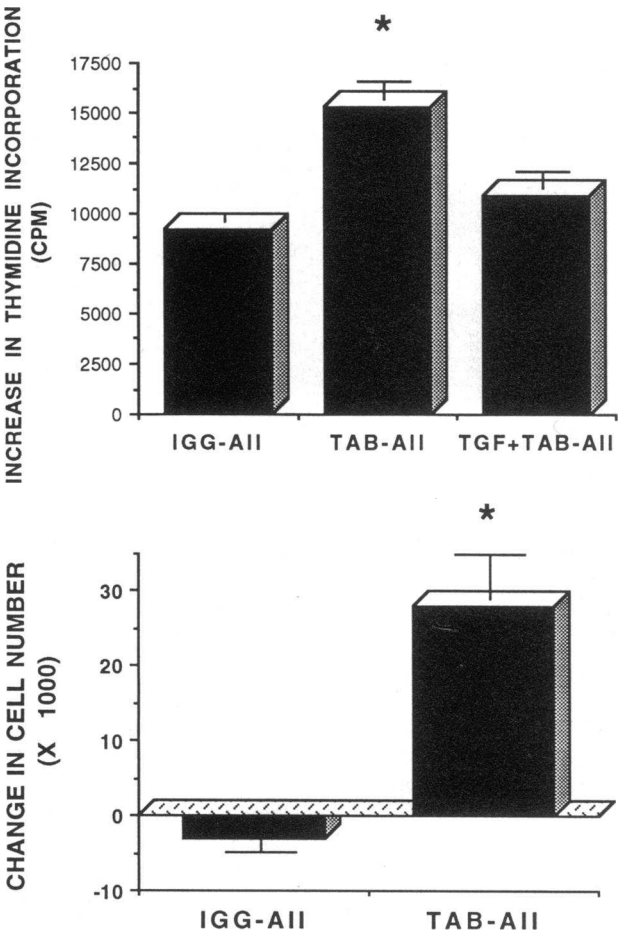

Figure 4. (*Top*) Coincubation with the anti-TGFβ_1 antibody (*TAB*) potentiates the Ang II (*AII*) (10^{-6} M) stimulated increase in DNA synthesis in VSMC above baseline as compared to control IgG or anti-TGFβ_1 prebound to TGFβ_1. Values are expressed as the increase in thymidine incorporation above the vehicle-treated baseline control±SEM. The statistical comparison shown was between TAB-AII vs. TGF + TAB-AII ($n = 16$; $P < 0.01$). IGG-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with control IgG (100 μg/ml). TAB-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with the anti-TGFβ_1 antibody (100 μg/ml). TGF + TAB-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with anti-TGFβ_1 antibody prebound to TGFβ_1 ($n = 16$; $*P < 0.01$). (*Bottom*) Effect of anti-TGFβ_1 antibody on Ang II (10^{-6} M)-stimulated cell proliferation compared to control IgG (*IGG-AII*) 64 h after stimulation. Values are expressed as the increase in the number of cells compared to the IGG-VEH control±SEM ($n = 22$; $*P < 0.01$). The baseline number of cells in the IgG control was 56,000±5,000.

ever, prior to our study, the autocrine production of biologically active TGFβ_1 by VSMCs in culture had not been reported.

In the present study we demonstrated that Ang II induces increased levels of TGFβ_1 mRNA. This process of induction is dependent upon a short-lived constituitive protein or the synthesis of a novel protein within the first few hours of stimulation. It remains to be determined whether Ang II's effect on steady-state TGFβ_1 mRNA levels is regulated at the transcriptional or post-transcriptional level. It is noteworthy that Ang II also induces the expression of the transcription factors c-fos and c-jun within the first few hours of exposure (4, 5). These proteins form a complex which activates gene transcription by binding to an AP-1 site (26). The promoter of the TGFβ_1 gene contains AP-1 sites which can modulate TGFβ_1 gene expression (27). We speculate that these transcriptional factors may participate in the induction of TGFβ_1 gene expression in VSMC by Ang II and phorbol esters. Our study confirms earlier reports that TGFβ_1 gene expression is induced by phorbol esters (27, 28). The Ang II–induced increase in gene expression appears to be mediated by PKC activation in that it is mimicked by phorbol esters and blocked by PKC down-regulation. It is noteworthy that Ang II–stimulated increase in TGFβ_1 mRNA levels was sustained for over 20 h whereas the induction by PMA was relatively transient. We speculate that this may reflect the ability of biologically active TGFβ_1 to autoinduce its gene expression via a PKC-dependent pathway (27). Blockade of the PKC pathway by prolonged exposure to PMA may have inactivated the autoinduction of TGFβ_1 expression resulting in a more transient increase in TGFβ_1 mRNA levels.

By utilizing the mink lung epithelial cell bioassay and the TGFAB, we demonstrated that the induction of TGFβ_1 gene expression by Ang II results in the production of the biologically active protein. Moreover, we observed that the TGFAB unmasked the mitogenic effect of Ang II. We did not observe any increase in cell number during incubation with the TGFAB alone, which probably reflects the low constituitive levels of active TGFβ_1 that we observed in this study. Thus the observation that Ang II induces cell proliferation in the presence of the antibody is not due to a nonspecific mitogenic effect of the antibody itself. The specificity of this antibody has been well characterized and confirmed in this study (14–21). Furthermore, preincubation of the antibody with purified TGFβ_1 abolished the potentiation of the Ang II–stimulated DNA synthesis resulting in levels of thymidine incorporation comparable to Ang II in the presence of control IgG. Hence sufficient ligand was available to effectively block the neutralizing capacity of the antibody without excess free ligand capable of directly attenuating the growth stimulatory effect of Ang II. These data further substantiate the conclusion that the potentiation response to the antibody reflects blockade of the antiproliferative effect of autocrine TGFβ_1 induced by Ang II. Indeed, recent preliminary studies suggest that a similar potentiation of Ang II–stimulated DNA synthesis is achieved in the presence of antisense oligonucleotides directed against the production of TGFβ_1 (29). Moreover, we observed that coincubation of basic fibroblast growth factor with the TGFAB failed to potentiate the mitogenic effect of this peptide. Preliminary data suggests that in contrast to Ang II, basic fibroblast growth factor fails to induce the production of biologically active TGFβ_1 (30). Hence, the potentiation of DNA synthesis by the TGFAB is due to specific antagonism of the effect of active TGFβ_1 generated by the VSMC. These data suggest that Ang II stimulates the production of biologically active TGFβ_1 which, in turn, exerts an antiproliferative influence on the growth stimulatory effects of Ang II. Consequently, the net growth response to Ang II is hypertrophy without hyperplasia. However, when the antiproliferative effect of TGFβ_1 is inactivated, the full mitogenic effect of Ang II becomes manifest. We postulate that this proliferative response is mediated by autocrine PDGF production induced by Ang II (4, 6).

Given that several cell types secrete a latent form of TGFβ_1 (17), it is interesting to note that Ang II can release a biologically active form of the protein from VSMC. The mechanism of activation of the TGFβ_1 precursor to the active form under these conditions is unknown at this time. The serine protease plasmin converts the latent TGFβ_1 to its active form in vitro (16). Recently, it has been demonstrated that the precursor of plasmin, plasminogen, binds to the extracellular matrix (31) and that Ang II stimulates plasminogen activator production by cultured vascular smooth muscle cells (32). We speculate that Ang II–induced generation of plasmin may result in the proteolytic activation of latent TGFβ_1.

In conclusion, we have established that Ang II has a bifunctional effect on VSMC growth (7). We have documented that Ang II activates a proliferative, as well as an antiproliferative pathway. Ang II's bifunctional properties result in the capacity to induce VSMC hypertrophy or hyperplasia, depending on the cellular milieu. The induction of biologically active TGFβ_1 is a major determinant of whether the cell undergoes hypertrophy or hyperplasia. It is interesting to speculate that under certain pathological conditions (e.g., endothelial injury and/or mitogen stimulation), this antiproliferative pathway may be inactivated. This postulate may explain the observations that Ang II may promote VSMC mitogenesis in the presence of serum in vitro (3, 18) or in response to vascular injury in vivo (2). Thus, the balance of autocrine proliferative and antiproliferative factors can modulate the growth response to vasoactive substances. These findings are consistent with the concept that vasoactive substances may participate in the process of structural remodeling within the vasculature in diseases such as hypertension and arteriosclerosis.

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