

## Multiple Autocrine Growth Factors Modulate Vascular Smooth Muscle Cell Growth Response to Angiotensin II

Hiroshi Itoh, Masashi Mukoyama, Richard E. Pratt, Gary H. Gibbons, and Victor J. Dzau

Division of Cardiovascular Medicine, Falk Cardiovascular Research Center, Stanford University School of Medicine, Stanford, California 94305-5246

### Abstract

Angiotensin (Ang) II stimulates hypertrophic growth of vascular smooth muscle cells (VSMC). Accompanying this growth is the induction of the expression of growth-related protooncogenes (*c-fos*, *c-jun*, and *c-myc*), as well as the synthesis of the autocrine growth factors, such as PDGF-A and TGF- $\beta$ 1. In this study, we demonstrate further that Ang II also induces the synthesis of basic fibroblast growth factor (bFGF), a potent mitogen for VSMC. To examine how these factors interact to modulate the growth response of VSMC to Ang II, we used antisense oligomers to determine the relative contribution of these three factors. Treatment of confluent, quiescent smooth muscle cells with specific antisense oligomers complementary to bFGF, PDGF-A, and TGF- $\beta$ 1 efficiently inhibited the syntheses of these factors. Our results demonstrate that in these VSMC, TGF- $\beta$ 1 affects a key antiproliferative action, modulating the mitogenic properties of bFGF. Autocrine PDGF exerts only a minimal effect on DNA synthesis. An imbalance in these signals activated by Ang II may result in abnormal VSMC growth leading to the development of vascular disease. (*J. Clin. Invest.* 1993. 91:2268–2274.) Key words: angiotensin II • hypertrophy • hyperplasia • growth factors • antisense oligonucleotides

### Introduction

Abnormal growth of vascular smooth muscle cells (VSMC)<sup>1</sup> is central to the pathophysiology of atherosclerosis, hypertension, and restenosis after angioplasty. In atherosclerosis, VSMC replication is one of the important events in atheroma formation (1). In hypertension, medial hypertrophy occurs with endoreplication (polyploidy) in large conduit vessels and with true proliferation (hyperplasia) in the resistance vessels (2, 3). Abnormal VSMC proliferation is responsible for the restenosis that

develops at an alarming frequency after coronary and peripheral angioplasty, limiting the long-term efficacy of this procedure. The mechanisms responsible for this altered growth control are unknown, however, the expression of autocrine growth factors such as PDGF, basic fibroblast growth factor (bFGF), and TGF- $\beta$ 1 has been noted in vessels from hypertensive animals, atheromatous plaques from humans, and in injury-induced proliferation in rats (4–8).

Multiple lines of evidence suggest that angiotensin (Ang) II plays a role in the regulation of VSMC proliferation. In vivo, angiotensin converting enzyme inhibitors block the abnormal vascular growth in response to hypertension and vascular injury (9). Moreover, Ang II infusion induces vascular hypertrophy and proliferation in injured and uninjured vessels (10). Using cultured VSMC, we and others have demonstrated that Ang II induces increases in RNA and protein synthesis with little or no increase in DNA synthesis (11–15). Interestingly, Ang II exposure results in the increased expression of the protooncogenes, *c-fos*, *c-jun*, *jun-B*, and *c-myc* (16–18). Furthermore, we have previously reported that in VSMC, Ang II increases the mRNA for PDGF A chain (18) and TGF- $\beta$ 1 (19).

Our previous studies (19) suggest that Ang II induces in cultured VSMC both a proliferative and antiproliferative pathway and the interaction of these two pathways is responsible for the nonmitogenic growth response to Ang II. Immunologic blockade of TGF- $\beta$ 1 resulted in an increase in DNA synthesis in response to Ang II, suggesting that TGF- $\beta$ 1 mediated the antiproliferative pathway (19). However, the identity of the factor(s) mediating the proliferative pathway is still unclear. While a role for PDGF-AA was possible, it is unlikely since we have shown that these cells are not very responsive to exogenously added PDGF-AA (Itoh et al., manuscript submitted for publication). Accordingly, we examined whether other smooth muscle-derived growth factors may mediate the proliferative pathway. In this study, we show that Ang II increases the expression of bFGF. Moreover, using antisense technology, we demonstrate that the nonmitogenic growth response of VSMC to Ang II is primarily caused by the counterbalancing effects between the antiproliferative action of autocrine TGF- $\beta$ 1 and the proliferative action of autocrine bFGF but not that of PDGF-AA.

### Methods

*Growth of vascular smooth muscle cells.* Rat aortic smooth muscle cells (passage 5–10) isolated and cultured according to the method of Owens et al. (20), were plated into 24-well culture dishes at  $1 \times 10^4$  cells/well. At confluence, the cells were made quiescent by incubation for 48 h in a defined serum-free (DSF) medium containing insulin (5

Address correspondence to Victor J. Dzau, M.D., Falk Cardiovascular Research Center, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305-5246.

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1. Abbreviations used in this paper: Ang II, angiotensin II; bFGF, basic fibroblast growth factor; DSF, defined serum-free; VSMC, vascular smooth muscle cells.

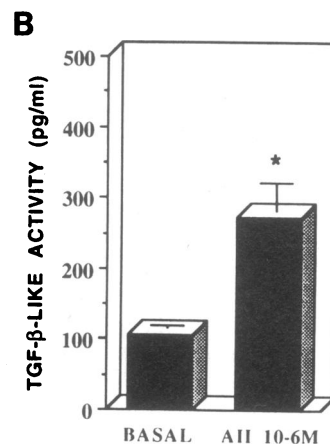
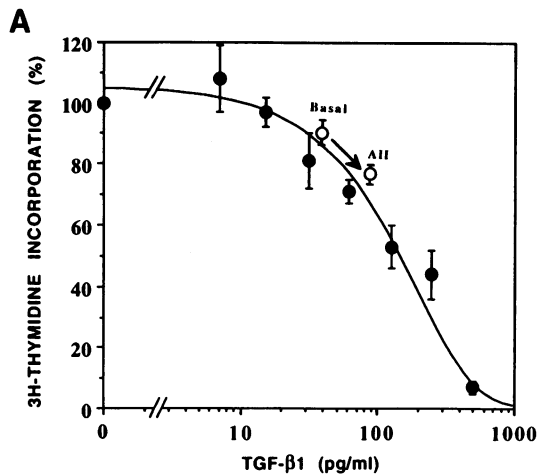
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**Figure 2.** Bioassay of TGF- $\beta$ 1 activity in VSMC conditioned media. Confluent, quiescent VSMC were exposed to vehicle (*Basal*) or Ang II ( $10^{-6}$  M), the media were collected and assayed for TGF- $\beta$ 1 activity using the mink lung epithelial cell bioassay. The mink lung cells respond to purified recombinant TGF- $\beta$ 1 with dose-dependent decrease in DNA synthesis (*A*, closed circles) expressed as percentage of basal [ $^3$ H]-thymidine incorporation (10,080 cpm). Conditioned media from basal or Ang II-treated VSMC were added to parallel cultures of mink lung cells and the effects on DNA synthesis examined (*A*, open circles). Comparison with the standard curve and multiplication by the dilution factor yield the concentration of TGF- $\beta$ 1 in the conditioned media from basal or Ang II-treated VSMC (*B*).  $n = 4$ ,  $*P < 0.05$ .

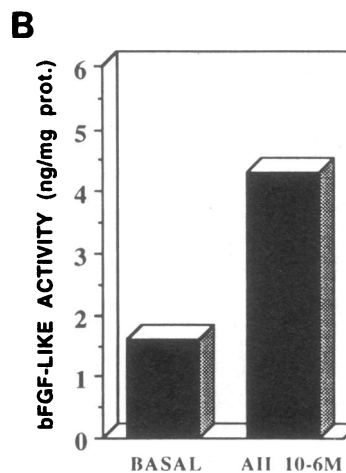
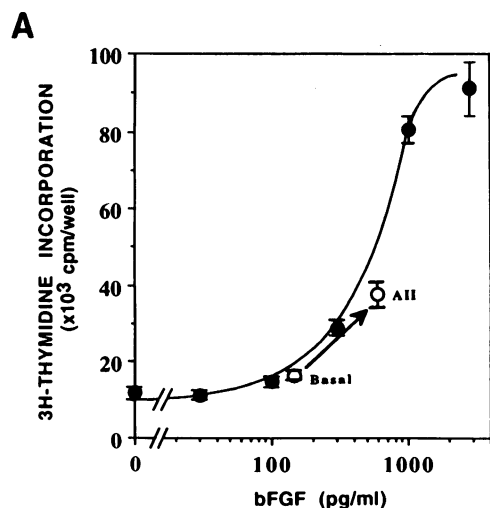
more, we have shown that cultured VSMC expressed TGF- $\beta$ 1 mRNA constitutively at a low level and that this expression was stimulated by Ang II (19). We assayed TGF- $\beta$ 1 activity using mink lung epithelial cells. In this assay, active TGF- $\beta$ 1 caused a dose-dependent inhibition of DNA synthesis of these cells ( $IC_{50} = 150$  pg/ml or  $6 \times 10^{-11}$  M) (Fig. 2). Media conditioned by Ang II or vehicle-treated VSMC were collected and added to cultured mink lung epithelial cells. Conditioned media from vehicle-treated VSMC inhibited mink lung epithelial cell DNA synthesis by 10%, while that from Ang II-treated cultures inhibited DNA synthesis by  $> 20\%$  (Fig. 2 *A*). Using the standard curve generated in Fig. 2 *A*, these levels of inhibition equated to  $105 \pm 5$  pg/ml of TGF- $\beta$ 1 activity in the conditioned media from vehicle-treated VSMC, which increased 2.5–3-fold after Ang II exposure ( $273 \pm 38$  pg/ml). The growth inhibitory activity in the conditioned media of the VSMC could be abolished by previous incubation of the conditioned media with a specific TGF- $\beta$ 1 neutralizing antibody, demonstrating the specificity of this assay for TGF- $\beta$ 1 (19).

VSMC synthesized bFGF basally and this production was stimulated by Ang II. We assayed bFGF activity in extracts of

VSMC using Swiss 3T3 cells as bioassay. Antibody-inhibitable bFGF activity in VSMC extracts, as detected by the Swiss 3T3 cells bioassay, was increased threefold (Fig. 3) by Ang II. The basal and Ang II-induced expressions of bFGF were confirmed by Northern blot analysis of bFGF mRNA (data not shown).

To examine the relative roles of these growth factors in Ang's growth effect, we synthesized antisense oligonucleotides (15 mer) complementary to human TGF- $\beta$ 1, bFGF, and PDGF A chain mRNAs (21–23) (Fig. 1). Control oligonucleotides either in the sense orientation or the reversed sequence, were also synthesized. The oligonucleotides were introduced into VSMC by cationic liposome-mediated transfection (lipofection), as described previously (24). The optimal in vitro concentration of cationic liposome and its ratio to DNA that minimize cell toxicity and optimize DNA uptake were determined to be 2–4  $\mu$ g/ml and 1:6 (wt/wt), respectively.

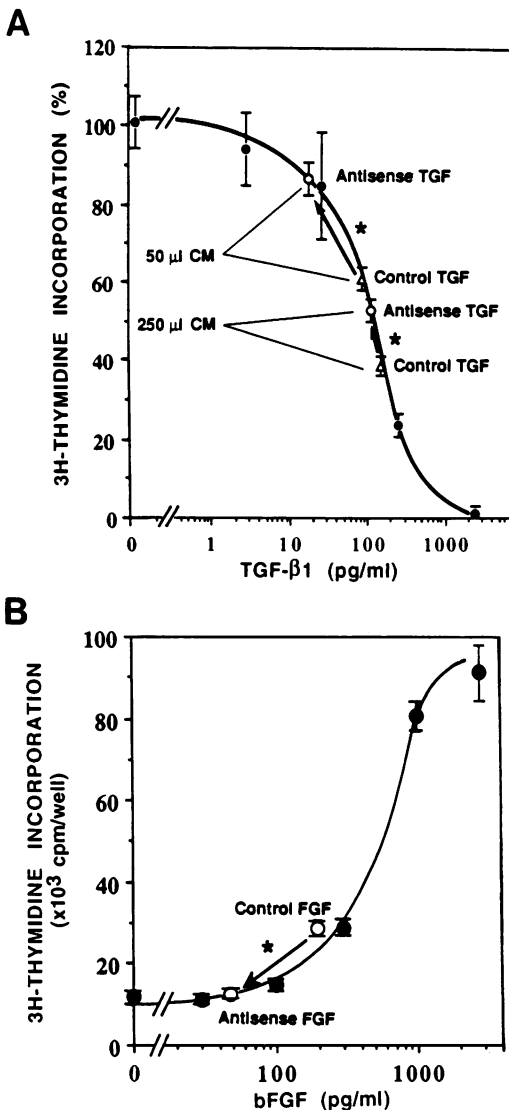
We next investigated the effect of the antisense oligomers on growth factor production. To examine the effectiveness of the blockade of TGF- $\beta$ 1 production by the antisense oligonucleotide against TGF- $\beta$ 1 mRNA, we measured the amount of TGF- $\beta$ 1 released by VSMC into the culture media again using



**Figure 3.** Bioassay of bFGF activity in VSMC extracts. Confluent quiescent VSMC were exposed to vehicle (*Basal*) or Ang II ( $10^{-6}$  M) and the cells were extracted and assayed for antibody-inhibitable bFGF activity using Swiss 3T3 fibroblasts as bioassay (*A*, open circles). The standard curve demonstrates the dose-dependent stimulation of DNA synthesis by bFGF (*A*, closed circles). Comparison with the standard curve and multiplication by the dilution factor yield the level of the VSMC extracts (*B*).

the mink lung epithelial cells bioassay (Fig. 4 A) (25). Compared to the control oligomer, the antisense oligomer decreased the TGF- $\beta$ 1 activity in the conditioned media by  $\sim 75\%$ .

The antisense oligomer directed against FGF was similarly efficient (Fig. 4 B) as determined using the Swiss 3T3 bioassay.



**Figure 4.** Effect of antisense oligonucleotides on the autocrine production of TGF- $\beta$ 1 and bFGF. (A) Confluent quiescent VSMC were treated with antisense or control oligonucleotides ( $5 \mu\text{M}$ ,  $25 \mu\text{g/ml}$ ) directed against TGF- $\beta$ 1. 50 and  $250 \mu\text{l}$  of conditioned media were collected after 20 h and assayed for TGF- $\beta$ 1 activity (A) using the mink lung epithelial cell bioassay as in Fig. 2. Closed circles represent the effects of purified TGF- $\beta$ 1 on DNA synthesis in the mink lung cells (expressed as a percentage of basal [ $^3\text{H}$ ]-thymidine incorporation; 11,848 cpm). Similarly, the open symbols represent the effects of the conditioned media from antisense or control oligomer-treated VSMC on DNA synthesis in parallel cultures of the mink lung cells. By comparison with the standard curve, antisense oligomer resulted in a 75% decrease in TGF- $\beta$ 1 activity. (B) bFGF content in extracts of control or antisense oligomer-treated VSMC was assayed using the Swiss 3T3 bioassay (B). Closed circles represent the effects of purified bFGF on the Swiss 3T3 cells, while the open circles represent the effects of extracts from antisense or control oligomer-treated VSMC on the Swiss 3T3 cells. The antisense oligomer inhibited bFGF production by 85%.  $n = 4$ ,  $*P < 0.05$ .

Extracts from quiescent VSMC contained 2.9 ng of bFGF per milligram of protein. This quantity was unaffected by incubation with control oligonucleotide but was decreased to below detectable levels ( $< 1 \text{ ng/mg}$  protein) when the cells were incubated with the  $5\text{-}\mu\text{M}$  antisense oligomer for 24 h. Moreover, in a related study, we demonstrated that the growth of cultured endothelial cells, which use bFGF as an autocrine growth factor (27), was also inhibited effectively with antisense oligomers directed against bFGF (28).

Fig. 5 demonstrates the effects of the antisense TGF- $\beta$ 1 oligonucleotide on DNA synthesis in basal and Ang II-stimulated VSMC. TGF- $\beta$ 1 antisense oligomer ( $5 \mu\text{M}$ ) potentiated DNA synthesis by 35% in basal state, but more significantly in Ang II-stimulated state (87%,  $P < 0.05$ ). In contrast, there was no change in the rate of DNA synthesis in Ang II-stimulated cells transfected with the control oligomer (Fig. 5 A). These results indicate that TGF- $\beta$ 1 exerts a tonic inhibitory action on VSMC proliferation and that in Ang II-stimulated state, it plays an even greater role in growth inhibition. These findings confirm our previous experiments using the anti-TGF- $\beta$ 1 neutralizing antibody, which resulted in a significant increase in cell number (50%) when these cells were stimulated with Ang II (19).

We next addressed the mediator of the proliferative effect of Ang II. Antisense oligomer ( $5 \mu\text{M}$ ) directed towards bFGF tended to suppress DNA synthesis in basal state, and inhibited this process significantly (by 30%) in Ang II-stimulated state (Fig. 5 B). Thus, bFGF can act as a promoter of VSMC proliferation especially in Ang II-stimulated state. In contrast, antisense oligomers directed against PDGF-A had no effect on basal or Ang II-stimulated [ $^3\text{H}$ ]thymidine incorporation (data not shown). These results, therefore, indicate that Ang II activates a growth-stimulatory pathway mediated primarily by bFGF.

We examined the effect of simultaneous blockade of bFGF and TGF- $\beta$ 1 production. The cotransfection of bFGF antisense oligomer with TGF- $\beta$ 1 antisense oligomer almost completely abolished Ang II-induced VSMC proliferation that was unmasked by the blockade of TGF- $\beta$ 1 production (Fig. 5 C). In contrast, transfection of the antisense directed against PDGF-A had no effect on the DNA synthesis uncovered by the antisense oligomer directed against TGF- $\beta$ 1 (data not shown). Moreover, the neutralization of PDGF-AA activity by anti-PDGF-AA antibody was also ineffective at inhibiting this proliferative response (Table I), suggesting that endogenous PDGF-AA plays an insignificant role in autocrine VSMC proliferation. This is also consistent with *in vitro* data that these cells express low levels of PDGF alpha receptors (29, 30).

To confirm further an interaction between TGF- $\beta$ 1 and bFGF, we added these factors to confluent, quiescent VSMC either alone or in combination at levels that approximated that detected in the cells or conditioned media in response to Ang II (i.e., 100–500 pg/ml and 5 ng/ml, respectively, see Figs. 2 B and 3 B). The choice of bFGF concentration is difficult since endogenously produced bFGF is not secreted but remains cell associated, exerting its effects intracellularly or intranuclearly. Moreover, the endogenously produced TGF- $\beta$ 1 may also act intracellularly since both the factor and its receptor are coexpressed in the same cells. Given these caveats, the results supported the overall hypothesis.

TGF- $\beta$ 1 dose dependently decreased while bFGF increased DNA synthesis. The bFGF-induced DNA synthesis could be

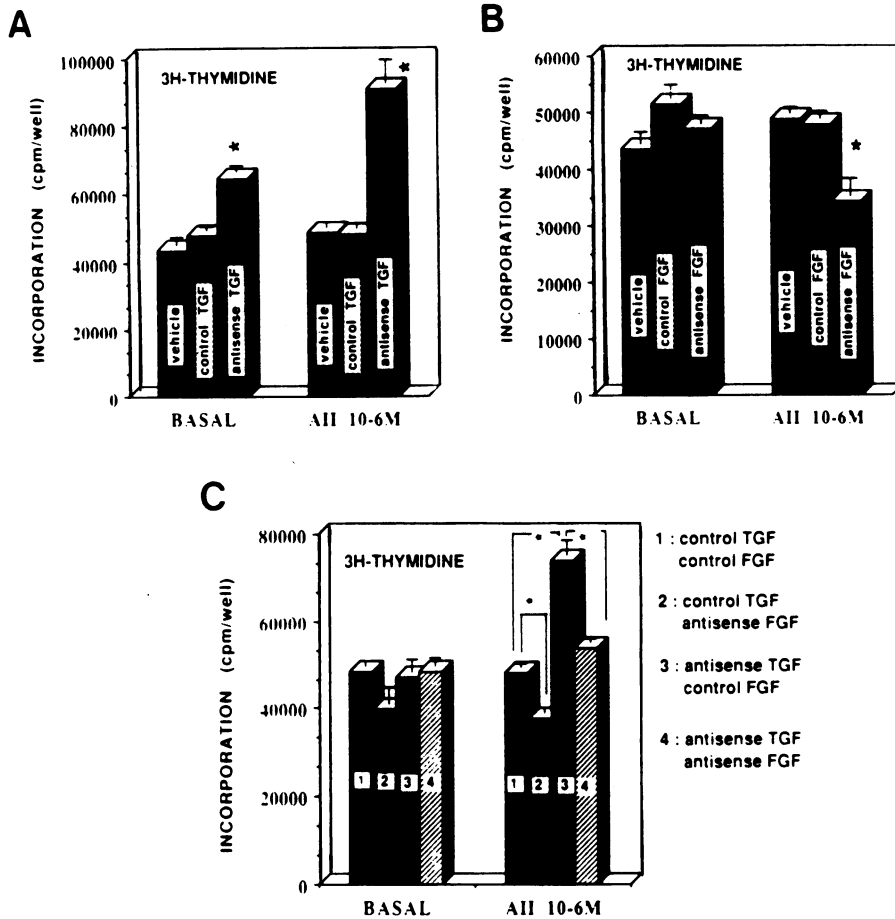


Figure 5. Effect of antisense TGF and/or antisense FGF oligonucleotide transfection on VSMC proliferation. Confluent quiescent VSMC were transfected with antisense or control oligonucleotides ( $5 \mu\text{M}$ ) 4 h before addition of vehicle or A II ( $10^{-6} \text{M}$ ). After 20 h, the cells were labeled with  $10 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine for 8 h. (A) Transfection with antisense or control (reverse) TGF oligonucleotide. (B) Transfection with antisense or control (reverse) FGF oligonucleotide. (C) Cotransfection with antisense or control TGF and FGF oligonucleotides.  $n = 6$ ;  $*P < 0.05$ .

blocked by the addition of TGF- $\beta$ 1 (Fig. 6 A). Thus, as has been demonstrated previously, TGF- $\beta$ 1 exerts a potent antiproliferative effect on VSMC (31, 32). Both TGF- $\beta$ 1 and bFGF increased RNA synthesis. Interestingly, the effects of

TGF- $\beta$ 1 and bFGF, when combined, appear additive (Fig. 6 B). Thus, taken together, these results demonstrate that TGF- $\beta$ 1 inhibits the proliferative effects of bFGF.

Table I. Effect of Anti-PDGF-AA Antibody on Ang II-Stimulated VSMC Proliferation Induced by TGF- $\beta$ 1 Antisense Oligonucleotide

Oligonucleotide	Antibody	[ $^3\text{H}$ ]thymidine incorporation
$5 \mu\text{M}$	$50 \mu\text{g/ml}$	cpm/well
Sense TGF	None	$33,600 \pm 5,960$
Antisense TGF	None	$57,000 \pm 4,700^*$
Antisense TGF	Control rabbit IgG from preimmune sera	$54,200 \pm 3,660^*$
Antisense TGF	Rabbit anti-PDGF-AA IgG	$52,200 \pm 6,000^*$

( $n = 6$ ,  $*P < 0.05$  significantly different from sense TGF oligomer-transfected group). Quiescent, confluent rat VSMC, treated with sense or antisense TGF oligonucleotides plus IgG ( $50 \mu\text{g/ml}$ ) from nonimmune sera or anti-PDGF AA antisera, were exposed to Ang II ( $10^{-6} \text{M}$ ). 20 h later,  $2 \mu\text{Ci/ml}$  of [ $^3\text{H}$ ]thymidine was added and the cells were incubated for 8 h.  $50 \mu\text{g/ml}$  of rabbit polyclonal anti-PDGF-AA IgG (Genzyme Corp.), inhibits the mitogenic activity of  $10 \text{ ng/ml}$  of recombinant PDGF-AA and blocks the mitogenic action of PDGF-AA released from VSMC by Ang II stimulation as assayed in the Swiss 3T3 cell bioassay.

## Discussion

The evidence herein demonstrates that autocrine bFGF and TGF- $\beta$ 1 counteract each other as a pro- and antimitogenic factors, respectively, within VSMC. Under control conditions, Ang II had little or no effect on DNA synthesis. However, after blockade of TGF- $\beta$ 1 synthesis with antisense oligonucleotides, Ang II significantly increased DNA synthesis. This is consistent with the observation that exogenous TGF- $\beta$ 1 decreased both basal and growth factor-induced DNA synthesis (31, 32). Therefore, TGF- $\beta$ 1 plays a major role as an antiproliferative factor in mediating the growth effects of Ang II. bFGF also plays a major role in this response. Exogenous bFGF increased DNA synthesis in VSMC. Blockade of the antiproliferative action of TGF- $\beta$ 1 also increased DNA synthesis to a similar extent. This increase was blocked with antisense oligonucleotides against bFGF demonstrating the proliferative role of autocrine bFGF. In addition to their effects on proliferation, TGF- $\beta$ 1 and bFGF also regulate RNA synthesis. We and others have shown that TGF- $\beta$ 1 induces hypertrophy of cultured VSMC (15, 31, 32). Exogenously added bFGF or TGF- $\beta$ 1 both increase RNA synthesis. Coadministration of bFGF and TGF- $\beta$ 1 increases RNA synthesis additively. In the absence of DNA synthesis, this increase in RNA synthesis is suggestive of a hypertrophic response. However, additional documentation of cellular hy-

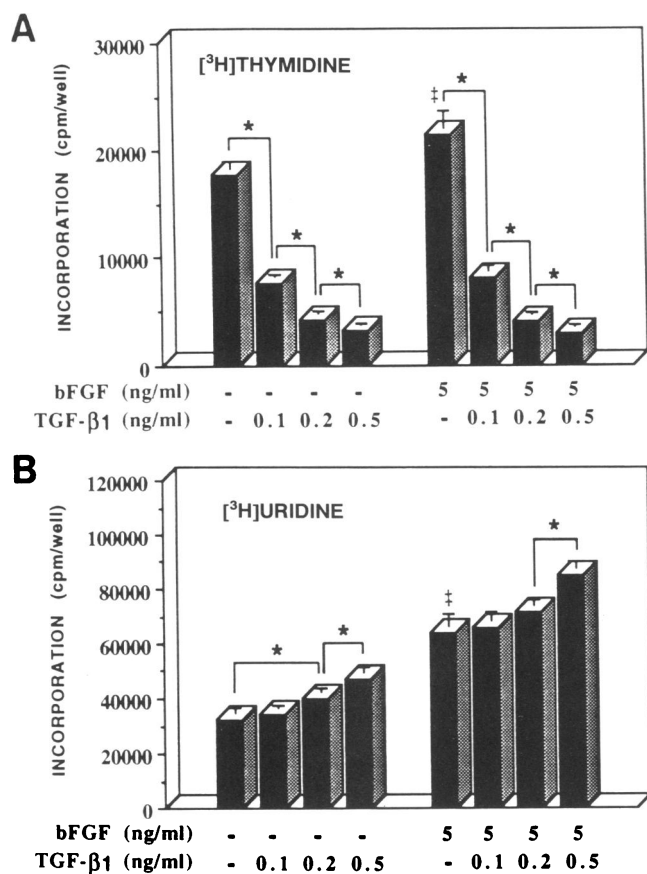


Figure 6. Effect of TGF- $\beta$ 1 and bFGF on DNA (A) and RNA (B) syntheses in VSMC. TGF- $\beta$ 1 with various concentrations (100–500 pg/ml) alone or in combination with bFGF (5 ng/ml) was administered to confluent, quiescent VSMC. After 16 h, these cells were pulse-labeled with [ $^3$ H]thymidine for 8 h (A) or [ $^3$ H]uridine for 4 h (B).  $n = 4$ . \* $P < 0.05$ ; † $P < 0.05$ , compared with the control (vehicle) group.

proliferation including an examination of cell volume and protein content will be required to fully establish this point. Thus, taken together, these results suggest that the combined action of bFGF and TGF- $\beta$ 1 mediated the growth effects of Ang II.

bFGF is unique among peptide growth factors in that this protein is not secreted but remains associated with the cell or extracellular matrix (33). Intracellularly, bFGF is associated with the nucleus, suggesting a nuclear site of action (34). However, cell surface receptors are also present. In endothelial cells, bFGF has been implicated as an autocrine growth factor (27, 28). Moreover, transfection of 3T3 cells with a bFGF expression vector results in a cell line with enhanced proliferation (35) and migration (36), indicating an autocrine or paracrine action of the expressed bFGF. However, the exact cellular site and mechanism of action are unclear.

Several reports have described the opposing effects of TGF- $\beta$ 1 and bFGF in cultured endothelial cells on proliferation, migration, and on urokinase and tissue type plasminogen activator production (37, 38). This study is the first demonstration that these two growth factors counteract each other within the same cell population in an autocrine and/or intracrine fashion. Since bFGF is known to promote the production of plasminogen activator, which is crucial for the activation of TGF-

$\beta$ 1 (21, 22), the interaction of these dual autocrine loops in the production and activation of these growth factors deserves further investigation. Parenthetically, we have demonstrated that in VSMC, bFGF enhanced TGF- $\beta$ 1 mRNA expression (39). Furthermore, in preliminary experiments, TGF- $\beta$ 1 ( $10^{-10}$  M) tended to reduce bFGF mRNA expression in VSMC. Therefore, these two growth factors, which are activated by Ang II, reciprocally modulate their expression within VSMC.

The results of this study illustrate the complex interaction of autocrine growth factor expressions and their actions in VSMC. A delicate balance between proliferative (bFGF) and antiproliferative (TGF- $\beta$ 1) factors determines the growth response of VSMC at basal state and in response to Ang II. With vascular injury such as that produced by coronary angioplasty, VSMC migrate and undergo rapid proliferation associated with autocrine expressions of these growth factors (6–8). This pathological proliferation may be the result of an imbalance of these growth factors. Angiotensin augments this process. Taken together, our data provide novel insight into the interaction of autocrine growth factors and demonstrate the usefulness of antisense methodology in the study of vascular biology. This technology may have further application in novel therapeutic strategies in vascular diseases such as restenosis.

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