Role of Transforming Growth Factor-β1 in the Cellular Growth Response to Angiotensin II

Yasushi Koibuchi, Wendy S. Lee, Gary H. Gibbons, and Richard E. Pratt

We have shown that angiotensin II (Ang II)-induced hypertrophy of vascular smooth muscle cells is dependent on the balance between proliferative and antiproliferative growth factors, specifically basic fibroblast growth factor and transforming growth factor-β1 (TGF-β1), respectively. We now present evidence, based on two phenotypically distinct cell cultures, that the ability to secrete the biologically active form of TGF-β1 is central to the growth response to Ang II. Two separate cultures were examined, one in which Ang II induces hypertrophy and the other in which Ang II induces hyperplasia. Ang II induces the expression of basic fibroblast growth factor twofold to fivefold in both cultures. Furthermore, both cultures express TGF-β1. In the culture that responds with hypertrophy, Ang II induces the expression of the active form of TGF-β1 twofold to threefold. However, in the culture that responds with hyperplasia, no active TGF-β1 was detected either at baseline or after Ang II exposure. Interestingly, all the TGF-β1 present was in the inactive, latent form. In the culture that responded with hyperplasia, Ang II induced a fourfold to fivefold increase in DNA synthesis. This increase could be abolished by the addition of active TGF-β1. Thus, in these two cultures the ability to activate TGF-β1 dictates the cellular response to Ang II. These results support our hypothesis that a balance of proliferative and antiproliferative autocrine signals mediates the growth control of vascular smooth muscle cells. (Hypertension 1993;21:1046–1050)

KEY WORDS • transforming growth factors • hyperplasia • hypertrophy • muscle, smooth, vascular • angiotensin II • fibroblast growth factor

Abnormal hypertrophic or hyperplastic growth of vascular smooth muscle cells (VSMC) is central to the pathophysiology of many vascular diseases. For example, hypertension results in vascular hypertrophy that in large conduit vessels, is due primarily to cellular hypertrophy, whereas in resistance arteries hyperplasia predominates. In atherosclerosis, vascular smooth muscle proliferation within the intima is a major cause of plaque formation. Moreover, the vascular response to injury after mechanical trauma such as balloon angioplasty also involves smooth muscle proliferation. The understanding of the mechanisms behind abnormal, uncontrolled proliferation is central to attempts to inhibit or control lesion formation.

Among the factors responsible for the regulation of vascular smooth muscle growth, vasoactive substances play a major role. Angiotensin II (Ang II) has been used as a paradigm for these studies. In vivo, angiotensin converting enzyme (ACE) inhibitors attenuate the development of hypertension-induced vascular hypertrophy to an extent greater than that predicted by its blood pressure-lowering effects. Moreover, infusions of Ang II result in vascular hypertrophy and DNA synthesis in vascular smooth muscle. These effects appear independent of the hemodynamic effects of Ang II. Furthermore, ACE inhibitors and Ang II receptor antagonists can attenuate the neointimal formation after balloon injury in the rat while ACE inhibitors have been shown to attenuate the development of atherosclerosis in rabbit and primates. Thus, in many models of abnormal vascular growth, Ang II may play a contributing role.

To understand the molecular mechanisms behind the induction of growth by Ang II, cell culture studies have been conducted. Interestingly, a controversy exists as to the cellular response to Ang II administration. Many investigators, including ourselves, have reported that Ang II induces primarily hypertrophy. Exposure to Ang II induces RNA and protein synthesis with little or no increase in DNA synthesis. This results in an increase in cell volume and protein content with no increase in cell number. On the other hand, others have reported that Ang II, either alone or synergistically with serum, induces a significant increase in DNA synthesis, increases in growth rates, and increases in cell number. To date, the reason for these conflicting reports is unclear.

The induction of growth by angiotensin is mediated via the induction of growth factors that act in an autocrine or paracrine fashion. Ang II induces the expression of transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF), and platelet-derived growth factor A chain (PDGF A) mRNA and...
protein. In these cells TGF-β1 exerts an antiprolifera
tive effect; exogenously added TGF-β1 will inhibit
growth factor–induced DNA synthesis while increasing
RNA and protein synthesis. Moreover, blockade of
derogogenous TGF-β1 synthesis or action with antisense
oligomers10 or antisera,9 respectively, will enhance DNA
synthesis. Thus, the hypertrophic response to Ang II is
due to the direct hypertrophic actions of TGF-β1 as well
as a modulating effect of TGF-β1 on the action of bFGF
and to a certain extent PDGF. We have hypothesized
that a decrease in the induction of TGF-β1 could result
in a proliferative response to Ang II.16

During the course of these studies, we came upon a
culture that responded to Ang II mitogenically. There-
therefore, in light of our hypothesis, we examined the levels
of growth factors induced by Ang II.

Methods

Growth of Vascular Smooth Muscle Cells

Rat aortic VSMC were isolated from 6-week-old or
3-month-old male rats (Wistar-Kyoto [WKY] and
Sprague-Dawley) from Charles River Breeding Labora-
tories, Wilmington, Mass.17 Animals were killed by
decapitation following Stanford University–approved
guidelines. These cell cultures were positive for
α-smooth muscle actin and grew in the typical hill and
valley pattern. Stock cultures were maintained in a 1:1
mixture of Dulbecco’s modified Eagle’s medium (DMEM)
and Ham’s F12 medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum (FCS). At confluence, the cells were made quiescent by
incubation for 48 hours in 2% FCS in DMEM/F12 followed by 48 hours in a defined serum-free medium (DSF) containing insulin (5×10⁻⁷ M), transferrin (5
µg/mL), and ascorbate (0.2 mM). This medium main-
tains smooth muscle cells in a quiescent noncatabolic
state and induces the expression of smooth muscle
cell–specific contractile proteins.18 Relative rates of
protein, RNA, and DNA syntheses were determined as
tritiated leucine, uridine, and thymidine incorporation,
respectively, into trichloroacetic acid precipitable ma-
terial as previously reported.9,10

Bioassay for Transforming Growth Factor-β1

CCL-64 mink lung epithelial cells18 were maintained
in MEM with 10% FCS and 0.1 mM nonessential amino
acids. Cells were plated at 4×10⁴ cells per well in
24-well plates 5–6 hours before the assay. The subcon-
fuent cells were washed once and fed with DSF contain-
ing vehicle or TGF-β1 (human TGF-β1, R & D Systems, Minneapolis, Minn.). Sixteen hours later, the
cells were pulse-labeled with 1 µCi/mL [³H]thymidine (1
µCi/mL). The incorporation of [³H]thymidine was
determined as described above and expressed as the
percentage of incorporation in the absence of TGF-β1.

The levels of TGF-β1, secreted during a 24-hour period
from quiescent or Ang II–treated VSMC, were similarly
assayed and calculated by comparison with the standard
curve.

Demonstration that the inhibitory effect of the con-
ditioned media was due to TGF-β1 (provided by Dr. M.
Sporn, National Institutes of Health). Fresh DSF me-
dia, human TGF-β1 (2 ng/mL), or the conditioned
media collected from VSMC were incubated at 37°C for
1 hour with either turkey preimmune serum or turkey
anti-human TGF-β1 antiserum19 (1/200 final dilution)
before the addition to the mink lung bioassay at a 0.5
dilution. This treatment completely abolished the
growth inhibitory action of the conditioned media.9

Bioassay for Basic Fibroblast Growth Factor

Confluent, quiescent VSMC, treated for 24 hours with
vehicle or Ang II, were harvested from monolayer
cultures by scraping, washed with phosphate-buffered
saline, and resuspended in 2 mL of 1 M NaCl/0.01 M
Tris-HCl, pH 7.5, containing leupeptin (1 µg/mL),
pepsin (4 µM), and phenylmethylsulfonyl fluoride (1
mM).20,21 After disruption by three cycles of freezing
and thawing and sonication for 1 minute, the homoge-
nate was centrifuged at 25,000g for 30 minutes, and the
supernatant was dialyzed overnight against 0.1 M NaCl/
0.01 M Tris-HCl, pH 7.5. All procedures were per-
formed at 4°C, and aliquots of cell extracts were stored
at −80°C until use. In this study, the bFGF activity is
expressed as bFGF antibody inhibitable mitogenic activity.
Therefore, for the measurement of bFGF activity,
human bFGF standards (0.03–3 ng/mL, Genzyme Cor-
poration, Boston, Mass.) or the extracted samples were
preincubated (2 hours at 37°C) with either anti-bFGF
immunoglobulin G (IgG) (R&D Systems, Minneapolis,
Minn.) or nonimmune IgG (both at 10 µg/mL). After
preincubation, the samples were incubated with quies-
cent Swiss 3T3 cells for 16 hours, after which the cells
were pulse-labeled with 1 µCi/mL [³H]thymidine for 8
hours. For quantitation of the mitogenic activity in the
cell extracts, the standard curve was plotted as counts
per minute incorporated versus nanograms of bFGF.

The antibody inhibitable mitogenic activity (counts per
minute) from the cell extracts was converted to nano-
grams of bFGF by comparison with the standard curve
and was expressed as nanograms FGF per milligram
protein extract or per T75 flask.

Controls were performed to validate the use of the
commercial antisera. Addition of nonimmune IgG had
no effect on basal or bFGF-stimulated thymidine incor-
poration into the 3T3 cells, nor did the administra-
tion of anti-bFGF IgG affect basal thymidine incor-
poration into the 3T3 cells. Anti-bFGF IgG (10 µg/mL)
completely abolished the mitogenic activity of 1 ng/mL
recombinant human bFGF, without affecting the mito-
genic activity of acidic FGF or PDGF. Serial dilution
curves of cell extracts were parallel to the standard
curve of bFGF.

Results

Consistent with previous reports,9–11 Ang II treat-
ment of CNC-3, a smooth muscle culture isolated from
6-week-old Sprague-Dawley rats, resulted in 40% in-
crease in protein synthesis (vehicle, 28,170±1,560; Ang
II, 39,850±4,700; cpm per well, p<0.05; n=8) and a
50% increase in RNA synthesis (vehicle, 93,150±8,030;
Ang II, 140,860±10,270; p<0.05; n=8). Similarly, Ang
II treatment of a second culture, Adu, cultured from
3-month-old WKY rats resulted in a 50% increase in
protein synthesis (vehicle, 15,880±550; Ang II,
24,750±1,030; p<0.05; n=8) and a 100% increase in
RNA synthesis (vehicle, 91,420±1,850; Ang II,
B

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FIGURE 1. Bar graphs show effects of angiotensin II (Ang II) on DNA synthesis in vascular smooth muscle cells. Confluent, quiescent vascular smooth muscle cells grown in 24-well (panels A and B) or 12-well (panel C) plates were treated with 2% or 10% fetal calf serum (FCS), 10^{-6} M Ang II, or vehicle. Panel A represents the response of CNC-3 cells; panels B and C represent the response of Adu cells. DNA synthesis was examined using [3H]thymidine by a 24-hour labeling commencing 12 hours after treatment with vehicle, Ang II, or FCS. Incorporation was assessed as acid precipitable counts per minute (CPM) per well and expressed as mean±SEM (n=8), p<0.05. All, angiotensin II; SER, fetal calf serum.

However, the response of these cells to Ang II with respect to DNA synthesis differed dramatically (Figure 1). The rates of DNA synthesis in the CNC-3 cells did not increase after Ang II exposure (Figure 1A). However, the response of the Adu cells to Ang II (Figure 1B) was similar to that seen with 10% FCS (Figure 1C). Thus, in the Adu cells, Ang II is a potent inducer of DNA synthesis.

We next examined the differential effects of Ang II on the relative cell size of the CNC-3 and Adu cells (data not shown). Hypertrophy is marked by an increase in cell size and protein content. CNC-3 cells treated with Ang II exhibit a larger cell size compared with cells grown in DSF media alone. On the other hand, Adu cells treated with Ang II have a smaller relative cell size than those grown under basal conditions, consistent with a hyperplastic response. Consistent with this, the number of cells per flask for the CNC cells (5×10^6 cells/T75) was identical between the control or Ang II-treated flasks. However, the Adu cells increased from 9×10^6 to 14×10^6 cells/T75 flask after Ang II treatment (n=2 per group).

We have hypothesized that the cellular response to Ang II is mediated by the simultaneous induction of a proliferative and an antiproliferative pathway mediated by bFGF and TGF-β1, respectively.^{9,10} Blockade of TGF-β1 activity with antibodies results in an increase in DNA synthesis and cell number in response to Ang II. Simultaneous blockade of both FGF and TGF-β will abolish this increase in DNA synthesis.^{10} Accordingly, we examined the expression of these factors basally and in response to Ang II. Both cultures express bFGF basally and at higher levels following Ang II exposure (Table 1). CNC-3 cells also express active TGF-β, and this expression is also increased after Ang II treatment. However, when the media from the Adu cells was examined, no detectable TGF-β1 activity could be observed in the media of either basal cultures or those treated with Ang II. TGF-β is synthesized as an inactive or latent precursor.\textsuperscript{23,24} CNC-3 cells have the ability to activate this latent form, either intracellularly or after secretion, such that both forms can be detected in conditioned media from these cells. To examine if the Adu cells fail to synthesize TGF-β or fail to activate the latent TGF-β1, the levels of latent TGF-β1 in media conditioned by the Adu cells were examined. Latent TGF-β1 was activated by incubation at 80°C for 10 minutes.\textsuperscript{25} In CNC-3 cells, heat activation results in a 10–20-fold increase in TGF-β1 activity yielding total concentrations of TGF-β1 of 600–1,200 pg/mL. Activation of conditioned media from the Adu cells resulted in the appearance of TGF-β1 activity to slightly higher levels (1,500–2,000 pg/mL). Thus, the lack of TGF-β1 activity in the Adu cells in response to Ang II is due to a lack of activation of the latent precursor.

The above data suggest that the lack of active TGF-β1 is responsible for the hyperplastic response of vascular smooth muscle cells to Ang II.

<p>| TABLE 1. Expression of Autocrine Growth Factors by Vascular Smooth Muscle Cells |
|-------------------------|-------------------------|-------------------------|-------------------------|</p>
<table>
<thead>
<tr>
<th>Cell culture</th>
<th>bFGF (ng/flask)</th>
<th>TGF-β1 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>18.5±2.2</td>
<td>0</td>
</tr>
<tr>
<td>Ang II</td>
<td>38.6±8.3*</td>
<td>160.1±12.8*</td>
</tr>
<tr>
<td>CNC-3</td>
<td>5.0±1.8</td>
<td>26.1±8.5</td>
</tr>
<tr>
<td>Ang II</td>
<td>60.3±14.4</td>
<td>160.1±12.8*</td>
</tr>
</tbody>
</table>

bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-β1; Ang II, angiotensin II.

Confluent, quiescent vascular smooth muscle cells were treated with vehicle or Ang II for 24 hours. For bFGF determination, cell extracts were assayed for antibody-inhibitable bFGF activity using a Swiss 3T3 bioassay and expressed as nanograms of antibody-inhibitable bFGF per T-75 flask (n=6 flasks).

For TGF-β1 assay, conditioned media from vehicle- or Ang II-treated cultures was assayed using a mink lung epithelial cell bioassay (n=6–8).

\*p<0.05 compared with vehicle-treated control.
the Adu cells to Ang II. To examine this, we added TGF-β1 to Ang II–stimulated Adu cells. As in Figure 1, Ang II induces a fourfold increase in DNA synthesis (Figure 2A). Addition of active recombinant human TGF-β1 to these cells decreases both basal as well as the Ang II–induced DNA synthesis. Further experiments demonstrated a dose-dependent inhibition of the Ang II–induced increase in DNA synthesis by TGF-β1 (ED₅₀ ∼200 pg/mL) (Figure 2B). These results further support the hypothesis that the hyperplastic response to Ang II is a result of the lack of active TGF-β1 production by these cells.

We next examined whether the cellular differences were due to age of the donor animal (3 months versus 6 weeks) or strain (WKY versus Sprague-Dawley rats). Multiple cultures from Sprague-Dawley rats aged 3 months or 6 weeks were examined. Two additional cultures isolated from 6-week-old rats exhibited a 1.08±0.15-fold increase in DNA synthesis after Ang II treatment (two separate cultures, assayed 2–3 times, n=6 wells per condition per assay, mean±SEM). On the other hand, three separate cultures isolated from 3-month-old rats exhibited a 3.72±0.6-fold increase (three separate cultures assayed 2–3 times, n=6 wells per condition per assay, mean±SEM). Thus, it appears that the cellular differences in response to Ang II are related to the age of the donor animal.

Discussion

This and previous reports support our hypothesis that the cellular response to Ang II is mediated by the paracrine or autocrine action of the growth factors induced by Ang II. Moreover, the data indicate that the synthesis of biologically active TGF-β1 plays a major role in determining whether the response is hypertrophic or hyperplastic.

TGF-β1 is initially synthesized as a protein of 390 amino acids consisting of a 29-amino acid signal sequence, a 249–amino acid latency protein, and 112–amino acid mature region. Both the mature growth factor and the latency protein exist as homodimers. In some cases, a modulator protein of 125–160 kd is disulfide-bonded to the latency protein. In vitro, TGF-β1 can be activated by acidification, alkalinization, heat, or exposure to proteases such as trypsin or plasmin. In vivo, however, the exact mechanism is unknown. Since plasminogen (present in the FCS) will bind extracellular matrix and Ang II can induce the synthesis of plasminogen activator in smooth muscle cells, we have hypothesized that plasmin is responsible for the activation in cell culture. Although this point requires further investigation, the existence of smooth muscle cultures that do not activate the latent TGF-β1 will be useful for these studies.

The identity of the growth factors responsible for the hyperplastic stimulus in these Adu cells is unclear. We have previously demonstrated that Ang II induces the expression of PDGF AA and bFGF. PDGF AA likely plays only a minor role, if any, since these cells are not particularly sensitive to exogenously added PDGF AA due to a low abundance of the PDGF α-receptor. These cells are, however, sensitive to bFGF. Moreover, antisense oligomers to bFGF inhibit both basal as well as Ang II–stimulated DNA synthesis in the CNC-3 cell line. Therefore, we speculate that Ang II induces proliferation of the Adu cells via the induction of FGF.

It is unclear why these two cell lines differ in their metabolism of TGF-β1. CNC-3 cells, similar to other cultures in our laboratory, were isolated from 6-week-old male Sprague-Dawley rats. On the other hand, the Adu cells were isolated from 3-month-old male WKY rats. Of the two readily apparent differences, the age difference is most probably related to the differences in ability to activate TGF-β1. Our data suggest that cells isolated from 3-month-old Sprague-Dawley rats respond in a manner similar to the Adu cells. Moreover, others have reported that vessels from older animals respond to vascular injury with a more robust neointimal formation and that cells from aged rats grow more rapidly in culture than those from younger animals. Therefore, age-related differences in the control of

![Figure 2](http://hyper.ahajournals.org/doi/abs/10.1161/01.ATP.104.12.1049?journalCode=hyp)
VSMC growth exists, and this may be due to a decrease in activation of the growth inhibitor TGF-β1.

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