J Korean Med Sci 1999; 14: 487-96
ISSN 1011-8934

Angiotensin II Stimulates Proliferation of Adventitial Fibroblasts Cultured from Rat Aortic Explants

It has been proposed that the local renin-angiotensin system is activated in the adventitia after vascular injury. However, the physiological role of Angiotensin II (Ang II) in the adventitia has not been studied at a cellular level. This study was designed to assess the role of Ang II in the growth response of cultured adventitial fibroblasts (AFs). Adventitial explants of the rat thoracic aorta showed outgrowth of AFs within 5-7 days. Ang II caused hyperplastic response of AF cultures. The Ang II-induced mitogenic response of AFs was mediated primarily by the AT_1 receptor. Ang II caused a rapid induction of immediate early genes (*c-fos*, *c-myc* and *jun B*). Induction of *c-fos* expression was fully blocked by an AT_1 receptor antagonist but not by an AT_2 receptor antagonist. Epidermal growth factor (EGF), platelet-derived growth factor-BB (PDGF-BB) and basic fibroblast growth factor (bFGF) induced DNA synthesis in AFs. Co-stimulation of AFs with the growth factors and Ang II potentiated the incorporation of ^{3}H-thymidine into DNA. Results from this study indicate that Ang II causes mitogenesis of AFs via AT_1 receptor stimulation and potentiates the responses to other mitogens. These data suggest that the Ang II may play an important role in regulating AF function during vascular remodeling following arterial injury.

Key Words: *Fibroblasts adventitial; Angiotensin II; Arteries; Wounds and injuries; Rats*

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Received: 12 April 1999
Accepted: 18 May 1999

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*This work was supported by grants from the Science and Technology Policy Institute (98-N1-02-02-A-07), the Samsung Biomedical Research Institute (C-95-003-2) and the Samsung Medical Center.

INTRODUCTION

Angioplasty induces lesions in the intima, media and adventitia, resulting in a luminal narrowing, known as a restenosis. Data from previous studies have suggested that angioplasty results in a restenosis due to a neointimal hyperplasia, comprising proliferation and migration of smooth muscle cells towards the intima and secretion of an extracellular matrix. However, recent findings that the cicatricial process, which induces restenosis, is due to constrictive remodeling rather than neointimal hyperplasia caused us to consider another mechanism of restenosis (1-4).

The vessel is composed of an endothelial layer, intima, media and adventitia. The adventitia was presumed inert and therefore little attention has been paid to its regulation. Contrary to this notion, recent evidence has raised the possibility that the adventitia may play an important role in remodeling after vascular injury (1, 5-7). The adventitial damage caused by the stretching and tears that occur in angioplasty leads to cellular proliferation and extracellular matrix production of AFs, which result in the formation of a neoadventitia and constrictive shrinkage. In addition, recent findings of the phenotypic modulation of AFs to adventitial myofibroblasts (AMFs) and the migration and presence of AMFs in neointima after vascular injury suggest that AFs may be involved in the process of neointimal formation (6). AFs and AMFs contribute to the problem of post-angioplasty restenosis by proliferating, forming a fibrotic scar that surrounds the injury site, and migrating into the neointima.

Angiotensin II (Ang II) is a potent vasoconstrictor peptide that plays an important role in blood pressure and fluid homeostasis through the systemic renin-angiotensin system. Ang II also promotes vascular and cardiac hypertrophy (8). More recently, evidence has accumulated demonstrating the existence of a tissue renin-angiotensin system (9). Ang II, acting through the local renin-angiotensin system, may be involved in the regulation of vascular growth associated with hypertension, atherosclerosis and vascular injury. The presence of angiotensinogen mRNA (10-12), and the induction of angiotensin con-

verting enzyme (ACE) (13, 14) in the adventitia of injured vessels, have been reported. Rakugi et al. (12) have shown that in an injury model of the rat abdominal aorta, angiotensinogen mRNA was detected clearly in the adventitia as well as in the media of the control and injured rat aortae. Fishel et al. (13) have reported the prominent induction of ACE in the adventitia of injured vessels in pig iliac arteries and rat carotids after balloon injury. Using quantitative in vitro autoradiography, it has been demonstrated that the adventitia of large vessels of humans, rats, rabbits, and dogs has a high concentration of ACE (15-17). ACE is more abundant in the adventitia than in the endothelium (17). These results suggest that the local renin-angiotensin system is activated in the adventitia after vascular injury. The physiological role of the renin-angiotensin system in the adventitia is currently unknown. Among the potential roles of the adventitial renin-angiotensin system is the formation of tissue Ang II, which activates AFs and affects the growth of AFs. Ang II has been shown both in vivo and in vitro to be capable of producing vascular smooth muscle cell (VSMC) hypertrophy or hyperplasia according to the experimental conditions (8). Ang II has also been found to be mitogenic for neonatal rat cardiac fibroblasts in culture (18). Therefore, Ang II produced locally in the adventitia might exert an effect on the proliferation of AFs, the main component of the cell types in the adventitia, and contribute to the adventitial thickening which leads to restenosis after angioplasty.

The role of Ang II on the proliferation of AFs has not been investigated previously at a cellular level. In this study, we have established an explant culture technique of AFs and investigated the effect of Ang II on the proliferative response of AFs. We also characterized the subtype of AT receptor that mediates the action of Ang II in AFs. The interactions between Ang II and other growth factors were also analyzed.

METHODS

Materials

Culture reagents were purchased from Gibco BRL, Gaithersburg, MD, U.S.A. All radiochemicals were obtained from Du Pont-New England Nuclear, Boston, MA, U.S.A. All other chemicals were from Sigma Chemical Co, St Louis, MO, U.S.A.

Cell isolation and culture

AFs were isolated from male Sprague-Dawley rats (4-6 weeks old, Charles River, Japan) (Fig. 1). The thoracic aorta was removed and stripped of loose perivascular

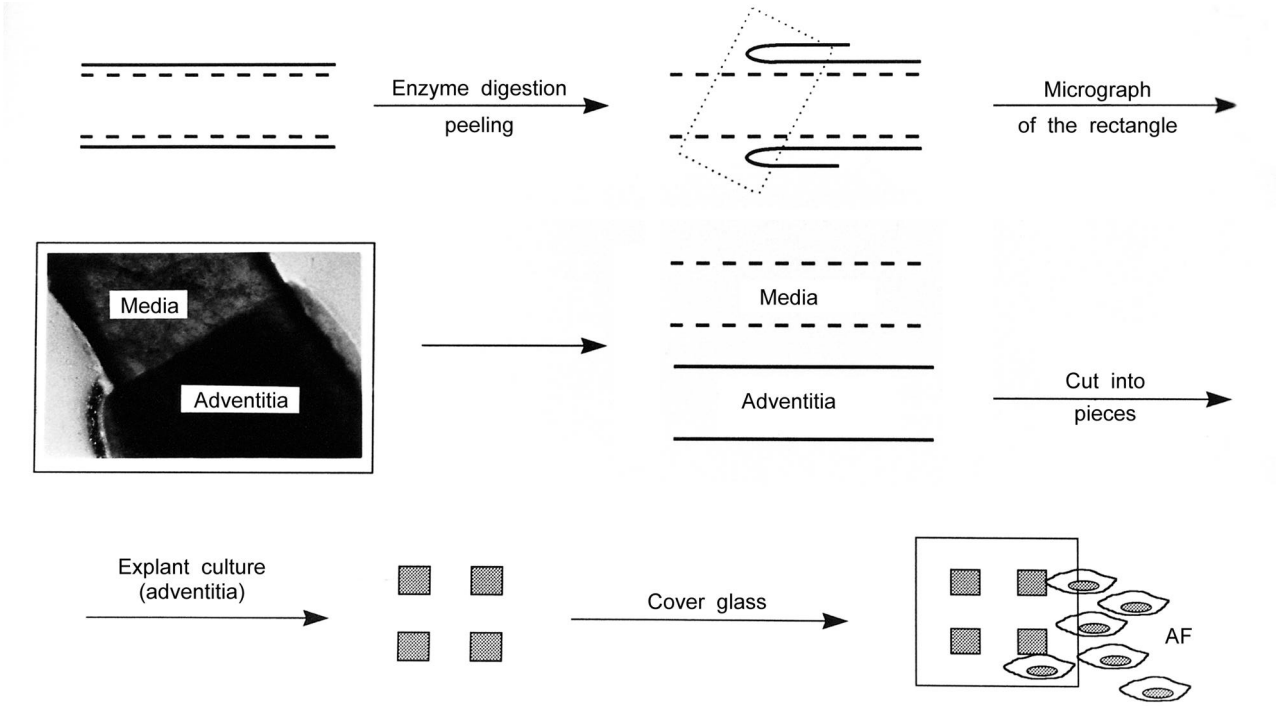

Fig. 1. Schematic illustration of the explant culture of AFs. After brief enzymatic digestion of the aorta, the adventitia was peeled from the underlying media. The micrograph shows the separation of the adventitia from the media. Dissected adventitia from the rat aorta was placed under the cover glass.

connective tissue and placed in an enzyme dissociation mixture consisting of DMEM/F12 medium; 1 mg/mL type 2 collagenase, 0.25 mg/mL elastase, 1 mg/mL soybean trypsin inhibitor (each from Worthington Biochemical Co., Freehold, NJ, U.S.A.); and 2 mg/mL bovine serum albumin (BSA) (fraction V) at 37℃ for 10 min. This procedure facilitated dissection of the adventitia from the underlying media and removed the endothelial cells. Under a dissecting microscope, the adventitia was dissected using fine forceps. The dissected adventitia was cut into small pieces and placed in 6 well plates (4 pieces per well) under a cover glass. The culture medium was added cautiously to cover the cover glass. After 2 days, an additional 3 mL of the medium was added and then routinely cultured. The explants were cultivated until large colonies of cells formed surrounding the explants. The cover glass and explants were then removed. When about 1/3 of a plate was covered by cells, the cells were trypsinized and seeded on a 25 cm^2 tissue culture flask. The culture medium was DMEM/F12 supplemented with 10% FBS, 100 mg/mL penicillin, and 0.1 mg/mL streptomycin. The cells were incubated at 37℃ in a humidified 5% CO_2/95% air atmosphere. The culture medium was changed after 18-24 hr and thereafter routinely changed at intervals of 48-72 hr. Cells were routinely passaged at a 1:3 to 1:5 ratio and studied between passages 5 and 15.

Measurement of DNA and RNA synthesis

At confluence, the cells were grown in serum-free conditions for 48-72 hr by incubating them in a defined serum-free (DSF) medium containing 0.2% bovine serum albumin. DNA synthesis was assessed by administering ^{3}H-thymidine (5 μCi/mL) for 4 hr, beginning 12 hr after Ang II or growth factor stimulation. RNA synthesis was measured by incubating the cells with ^{3}H-uridine (5 μCi/mL) for 4 hr, beginning 12 hr after Ang II or growth factor stimulation. At the end of the incubation period, the medium was removed, the cells were washed twice with phosphate-buffered saline (PBS), then washed once with 10% trichloroacetic acid (TCA), followed by incubation with 10% TCA for 30 min at 4℃. The TCA-insoluble material was washed twice with ethanol, and solubilized in 0.25 M NaOH. Incorporation of ^{3}H-thymidine and uridine was determined by a liquid scintillation counter (Beckman LS6500). Experimental values for the treated groups were normalized to the untreated control values.

Cell count determinations

AFs plated into 24-well culture dishes at 10^4 cells/well were cultured in the growth medium for 3 days, and switched to the serum-free medium for 2 days. After 24 hr of Ang II stimulation, cells were harvested by gentle trypsinization, and then cell number was counted using a hemocytometer.

RNA isolation and Northern blot analysis

Total cellular RNA was extracted from adherent cells using a modification of the method of Chomczynski and Sacchi (19). Twenty micrograms of total RNA was electrophoresed on 1% denaturing formaldehyde agarose gel, and blotted onto a Hybond-N nylon membrane (Amersham International). The blotted filters were hybridized with ^{32}P-labeled probes of *c-fos*, *c-myc* and *jun B* [1×10^6 counts per min (cpm)/mL], which were generated by random priming using a multiprime DNA labeling system (Amersham International). A 612-bp fragment of the human *c-fos* cDNA probe and a 478-bp fragment of the human *c-myc* cDNA probe were generated from human genomic DNA by a polymerase chain reaction (PCR) using commercial primer sets (Human *c-fos*, *c-myc* amplifier set, Clontech, CA, U.S.A.). The probe *jun B* was a 1.8-kb *Eco*RI fragment of the mouse *jun B* cDNA (20). Hybridization was carried out at 42℃ for 12-18 hr. Filters were washed sequentially and autoradiography was performed for 1-3 days using Kodak XAR-5 film with intensifying screens at -70℃. After autoradiography, the filters were stripped and were hybridized again with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The GAPDH cDNA probe was the 1272-bp *Pst*I segment from rat GAPDH cDNA.

Statistical analysis

Data are expressed as mean±SEM. The difference between the two groups was tested by an unpaired Student's t test. Differences among more than two groups were tested by ANOVA for multiple-sample comparison. Significance was accepted at $p<0.05$.

RESULTS

Explant culture of AFs

First, we established the culture of AFs. AFs were growing outwards from the explants after 2 or 3 days and formed a colony after 5-7 days (Fig. 2A, 2B). At confluence, cultured AFs showed contact growth inhibition and remained in a monolayer, a typical pattern of fibroblast (Fig. 2C).

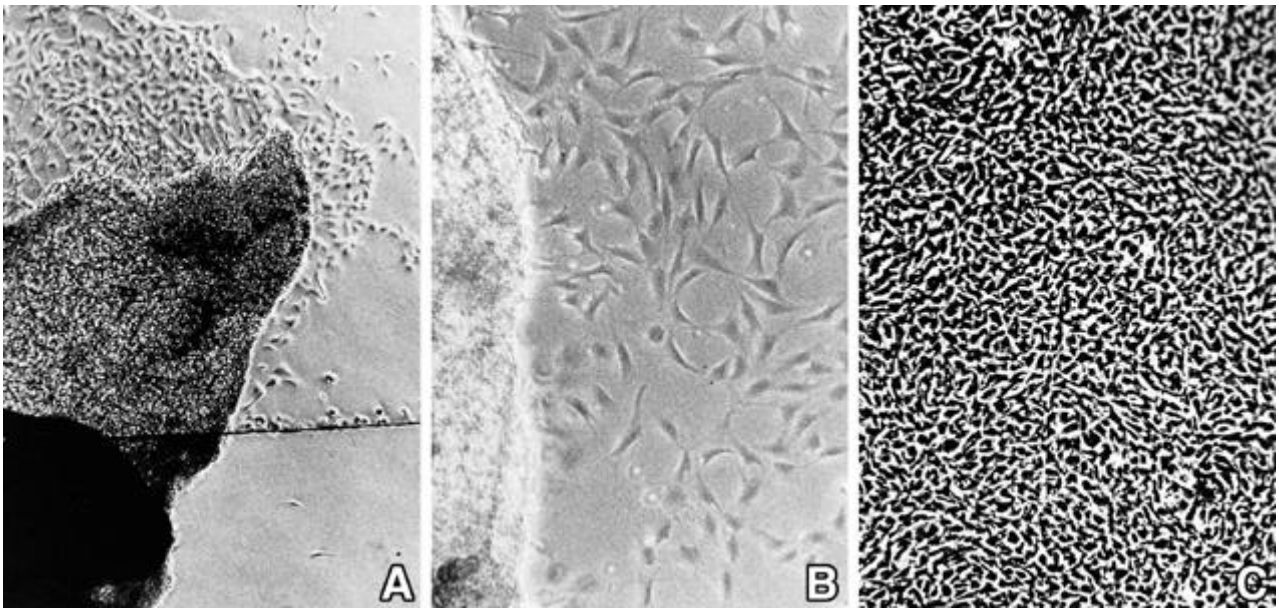

Fig. 2. AFs grew outwards from the tissue under the cover glass (A) (×40). The outgrowing cells showed the morphology of typical fibroblasts, which are polygonal and spindle-shaped (B) (×100). At confluence, the AFs were in a homogenous monolayer (C) (×100).

Ang II causes hyperplasia of AFs

Next, we examined the effects of Ang II on the DNA and RNA synthesis of cultured rat AFs. Ang II (10^{-6} M) caused a significant increase in the ^{3}H-thymidine uptake of AFs over 4 hr, and also caused a significant increase in ^{3}H-uridine incorporation in AFs over 4 hr (Fig. 3A). The increase in DNA synthesis caused by Ang II was 292% of that caused by 10% FBS. To test whether the Ang II-induced DNA synthesis was due to mitosis, cell number was counted at 24 hr following Ang II stimulation. Cell number increased significantly over the test period (Fig. 3B), which demonstrated that Ang II induces a hyperplastic response in AFs. The ED_{50} of Ang II on the proliferation of AFs was approximately 10^{-8} M (Fig. 4).

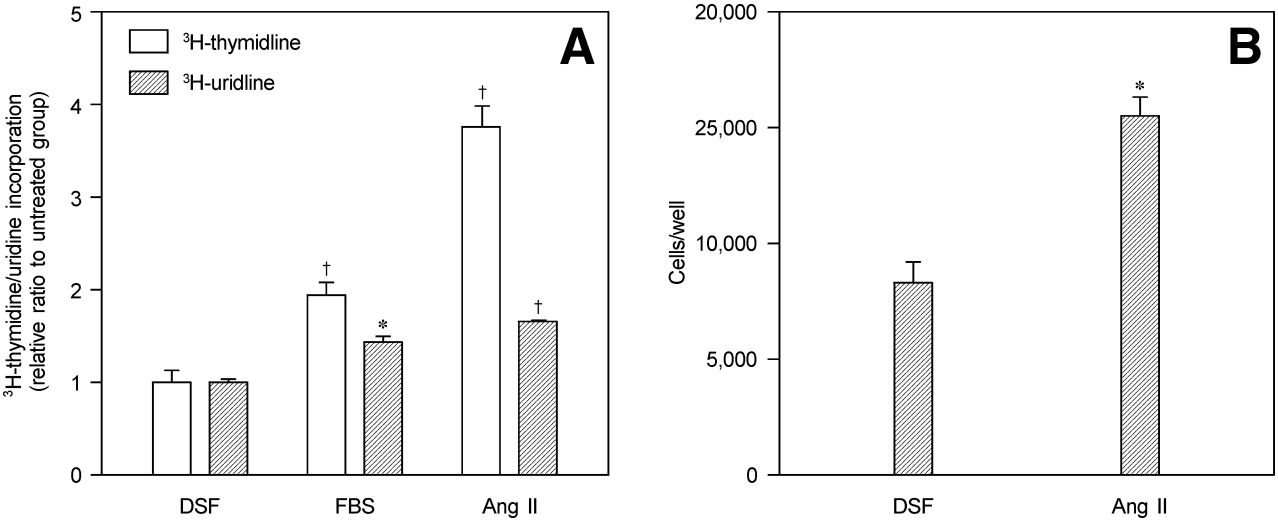

Fig. 3. Bar graphs show the effect of Ang II on ^{3}H-thymidine incorporation and ^{3}H-uridine incorporation in AFs (A). After confluence, the cells were grown in a defined serum-free medium (DSF) for 48-72 hr. ^{3}H-thymidine or ^{3}H-uridine was administered for 4 hr, beginning 12 hr after Ang II stimulation (10^{-6} M). Compared to basal thymidine incorporation in cells grown in DSF, Ang II increased both thymidine and uridine incorporation. FBS means 10% fetal bovine serum. Cell counts were performed at 24 hr after Ang II stimulation (10^{-6} M) (B). The cell number was increased significantly over the time period. Data are expressed as mean±SEM obtained from four samples in each group. *$p<0.05$, †$p<0.01$ vs DSF.

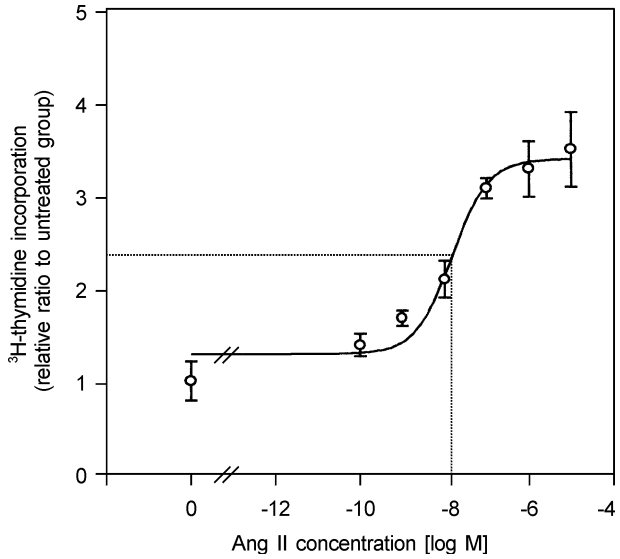

Fig. 4. Graph shows the concentration dependence of the Ang II-induced increase in DNA synthesis. The AFs were pulsed with ^{3}H-thymidine at 12 hr and assayed 4 hr later. Data are expressed as mean±SEM obtained from three samples in each group.

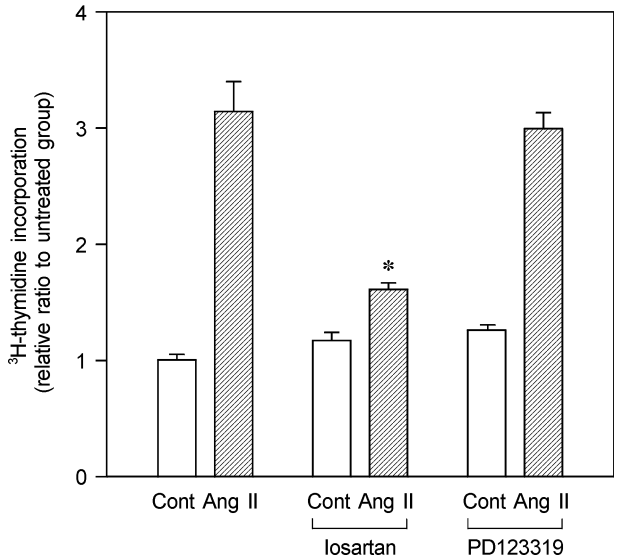

Fig. 5. Bar graphs show the effect of Ang II receptor antagonists on Ang II-induced ^{3}H-thymidine incorporation in AFs. Parallel control cultures received the antagonists with (hatched bar) or without Ang II (white bar). Losartan (10^{-6} M) significantly inhibited the response to Ang II (10^{-8} M), whereas PD123319 (10^{-6} M) had no effect. Data are expressed as mean±SEM obtained from three samples in each group. *$p<0.01$ vs Ang II without antagonists.

AT_1 receptor mediates Ang II-induced hyperplasia of AFs

We next examined which Ang II receptor subtype mediates the Ang II-induced mitogenic effect on AFs. As shown in Fig. 5, losartan, an AT_1 receptor blocker, prevented the Ang II-induced increase in ^{3}H-thymidine uptake, whereas PD123319, an AT_2 receptor blocker, did not. Thus, the Ang II-induced hyperplastic response of AFs was mediated primarily by the AT_1 receptor.

Ang II induces immediate early genes in AFs via the AT_1 receptor

It has been shown that Ang II causes the induction of immediate early genes in VSMCs and cardiac nonmyocytes (18, 21-25). Therefore, we examined the effect of Ang II on the immediate early gene expression in AFs. Ang II induced *c-fos*, *c-myc* and *jun B* (Fig. 6A). Induction of *c-fos* showed at approximately 30 min, whereas *c-myc* and *jun B* showed an earlier induction at approximately 15 min. The duration of the augmented expression of *c-fos* was shorter than that of *c-myc* and *jun B*, returning to the control level at 1 hr, whereas the elevated expression of *c-myc* and *jun B* was still present at 6 hr. Induction of *c-fos* expression by Ang II (10^{-7} M) was almost completely blocked by an AT_1 receptor antagonist losartan (10^{-5} M), but the same concentration (10^{-5} M) of PD123319 did not result in a significant inhibition (Fig. 6B). Thus, the induction of *c-fos* by Ang II was mediated primarily by the AT_1 receptor.

Effect on DNA synthesis of other growth factors alone or in combination with Ang II

Because it has been shown that EGF, PDGF-BB and bFGF are present at the site of vascular injury (26), the mitogenic actions of Ang II in the adventitia after vascular injury are likely to occur in the presence of other growth factors. Therefore, additional studies were conducted to evaluate whether other growth factors alone and in combination with Ang II increase DNA synthesis in AFs. We therefore tested mitogenic effects of these three growth factors alone and in combination with Ang II. AFs were exposed to increasing concentrations of EGF, PDGF-BB and bFGF for 12 hr, and the ^{3}H-thymidine incorporation was determined 4 hr later. EGF, PDGF-BB and bFGF increased ^{3}H-thymidine incorporation into DNA in a concentration dependent manner (Fig. 7). In order to examine whether Ang II potentiated EGF, PDGF-BB and bFGF-induced mitogenic responses, the effects of Ang II on the submaximal and maximal mitogenic growth-factor doses were compared. The results demonstrated that co-treatment with Ang II potentiates the proliferative response of AFs to both submaximal and maximal doses of EGF, PDGF-BB and bFGF (Fig. 8).

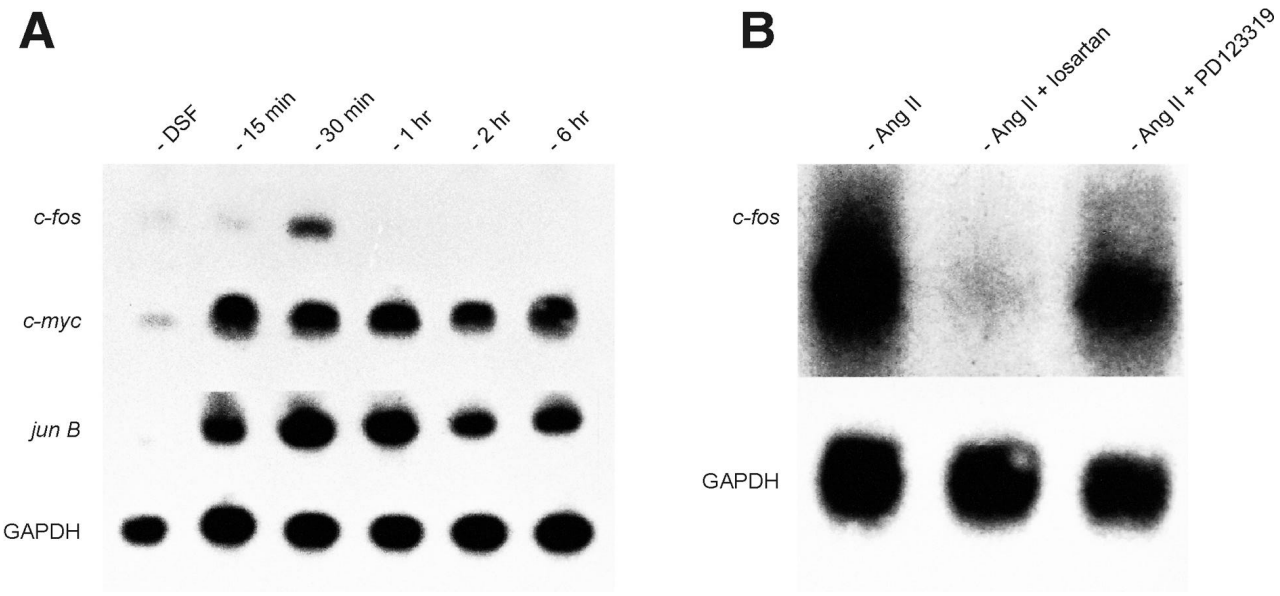

Fig. 6. Ang II-induced the expression of immediate early genes in AFs via AT_1 receptor. **A:** At confluence, the cells were grown in serum-free conditions for 48-72 hr by incubating them in a defined serum-free medium. They were then treated with Ang II (10^{-6} M) for the indicated time period. After Ang-II stimulation, *c-fos* showed a peak induction at approximately 30 min. The expression of *c-fos* was short, reverting to the control level after 1 hr. Immediate early genes *c-myc* and *jun B* were rapidly induced at 15 min after Ang-II stimulation and the duration of their expression was long. The increased expression persisted even at 6 hr. **B:** Cells were pretreated with antagonists (10^{-6} M) for 30 min, and then stimulated with Ang II (10^{-6} M) for 30 min in the presence of the antagonists (10^{-6} M). Ang-II-induced *c-fos* expression was fully inhibited by an AT_1 receptor antagonist (losartan), but not by an AT_2 receptor antagonist (PD123319).

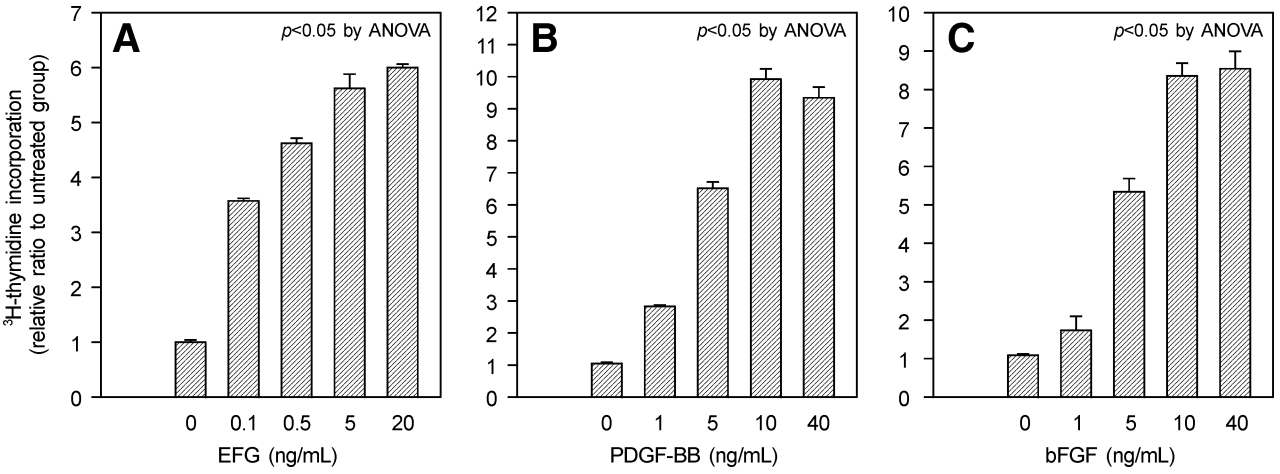

Fig. 7. Bar graphs show DNA synthesis in AFs treated with EGF, PDGF-BB or bFGF for 12 hr. The AFs were pulsed with ^{3}H-thymidine at 12 hr and assayed 4 hr later. Data are expressed as mean±SEM obtained from four samples in each group.

DISCUSSION

We analyzed the effects of Ang II on cultured AFs. The major findings are as follows. First, Ang II causes hyperplasia of AFs. Second, Ang II-induced hyperplasia of AFs is mediated by the AT_1 receptor. Third, Ang II induces the expression of a number of immediate early genes such as *c-fos*, *c-myc*, and *jun B*. Fourth, the induction of *c-fos* is primarily mediated by the AT_1 receptor. Fifth, other growth factors such as EGF, PDGF-BB, and bFGF induce DNA synthesis in AFs, and co-stimulation with Ang II and these growth factors enhances ^{3}H-thymidine incorporation into DNA.

Relatively little attention has been paid to the role of the adventitia in vascular remodeling following vascular injury, which is one of the primary pathological findings

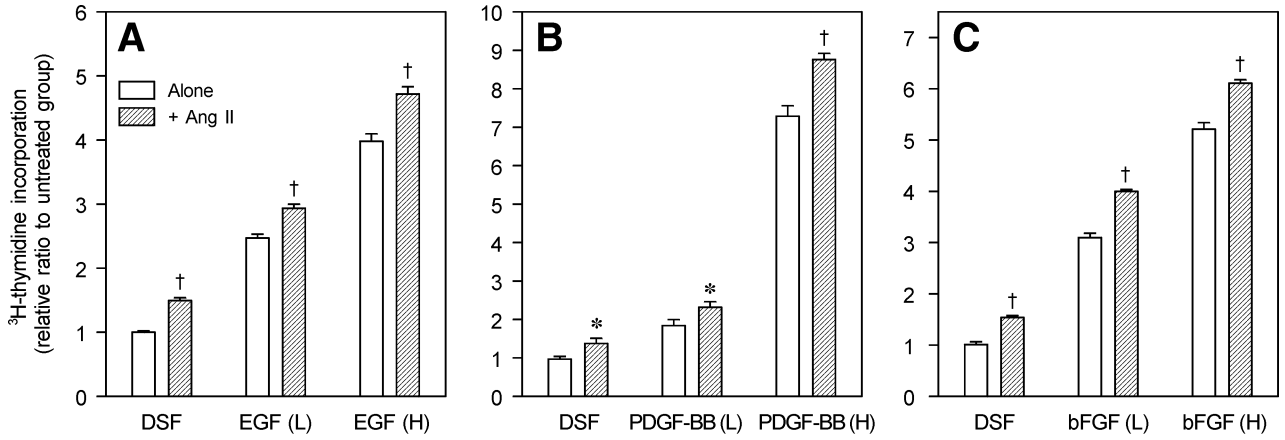

Fig. 8. Bar graphs show DNA synthesis in AFs treated with low (L) and high (H) concentrations of EGF, PDGF-BB or bFGF alone and in combination with Ang II for 12 hr. The AFs were pulsed with ^{3}H-thymidine at 12 hr and assayed 4 hr later. The concentrations of mitogens are as follows: Ang II, 10^{-6} M; EGF (L), 0.5 ng/mL; EGF (H), 20 ng/mL; PDGF-BB (L), 5 ng/mL; PDGF-BB (H), 40 ng/mL; bFGF (L), 5 ng/mL; bFGF (H), 40 ng/mL. Data are expressed as mean±SEM obtained from six samples in each group. *$p<0.05$, †$p<0.01$ vs DSF or growth factor alone.

in restenosis. More recently, evidence has accumulated that demonstrates the important role of the adventitia in the pathogenesis of restenosis after angioplasty. In porcine coronary vasculature, balloon-induced injury often results in medial dissection and exposure of the adventitia to the lumen (1, 3, 5). Under such pathological conditions, a chain of events reminiscent of the wound-healing process is initiated. Cellular proliferation is the early phenomenon. This involves the entire adventitia, with the majority of cells being activated fibroblasts. The adventitial damage leads to the formation of a neoadventitia and constrictive shrinkage. The important role of the adventitia is also emphasized in other vasculopathies (7, 27). The dramatic adventitial thickening of the pulmonary artery has been seen in vivo after hypoxic exposure in the neonatal calf (28). In a porcine model of a saphenous vein graft interposition in the carotid artery, the adventitia is transiently populated by myofibroblasts, which surround the vein (29, 30). The accumulation of mast cells and the inflammatory reaction in the adventitia are notable in patients with coronary vasospasm and fatal unstable coronary syndromes, respectively (31, 32). When all these are considered, the important role of AFs in the pathogenesis of vascular diseases should be evaluated. To study this issue at the cellular and molecular level, cell culture techniques for AFs are necessary and must be established. Reports of the successful culture of AFs are scarce. Raisanen et al. (33) and Bets et al. (34) reported fibroblast cultures from the adventitia of the rat aorta and the rabbit aorta, respectively. Using an explant technique, AFs could be reliably cultured using the techniques described in the present study. Although we cannot completely rule out contamination of the cultures of AFs with medial VSMCs, in view of the fact that the adventitia could be easily separated from the media and the morphology of the AFs is quite different from typical "hill and valley" pattern of the VSMCs, the majority, if not all, of cultured cells by our technique are AFs.

In our study, Ang II induced a hyperplastic response in AFs. This finding contrasts with the hypertrophic response of VSMCs in response to Ang II. However, a controversy exists as to the cellular response of VSMCs to Ang II administration (8, 26, 35, 36). It has been proposed that a balance of proliferative (such as PDGF and bFGF) and antiproliferative (namely TGF-β1) autocrine signals determines the growth response of VSMCs to Ang II (36). The temporal and spatial relationships between TGF-β1 and AMF formation in a porcine model of coronary artery injury by balloon overstretch suggest an important role of autocrine TGF-β1 in the phenotypic modulation of AFs (37). It is worthwhile to investigate the autocrine production of these growth factors in the Ang II-induced hyperplasia of AFs. Ang II has also been found to be mitogenic for neonatal rat cardiac fibroblasts and Swiss 3T3 cells (15, 38).

The Ang II-induced increase in DNA synthesis was greater than that induced by 10% FBS. This finding is quite striking, considering that in cardiac non-myocytes (mostly fibroblasts) Ang II is not as strong a mitogen as FBS, because the Ang II-induced increase in thymidine incorporation is only 8-10% of that seen with FBS (15). However, the prominent proliferative response of AFs to Ang II stimulation was also observed in other in vivo studies. Morphological analysis of the mesenteric resistance arterial vasculature of the rats, after continuous infusion of Ang II, showed massive fibrinoid necrosis and a marked fibroproliferative perivascular response in the adventitia (39). Local application of Ang II to the rat

carotid artery induces adventitial thickening (40). Perivascular fibrosis and fibroblast proliferation of intramyocardial coronary arterioles of the rat heart were noted after a continuous infusion of Ang II. This demonstrated that Ang II participated in the coronary vascular remodeling (41). These findings and the present observations suggest that fibroblasts in the adventitia may be a target of Ang II, and thus may contribute to the Ang II-induced vascular remodeling associated with a variety of physiological and pathological conditions.

The induction of immediate early genes by Ang II showed similar patterns compared with those in VSMCs and non-myocytes of the neonatal heart (15, 22-25). The induction of *c-fos*, *c-myc* and *jun B* occurred rapidly within 30 min after stimulation; the expression of *jun B* and *c-myc* was more prolonged than that of *c-fos*. The induction of immediate early genes and DNA synthesis were prevented by co-treatment with AT_1 receptor blocker and AT_2 receptor blocker did not show any effects, suggesting that these actions of Ang II are mediated through the AT_1 receptor in AFs.

The two major isoforms of the angiotensin receptor are the AT_1 and AT_2 receptors. It has been shown that both AT_1 and AT_2 receptors are expressed in fibroblasts such as rat neonatal skin fibroblasts and cardiac fibroblasts (42-44). Currently, the role of AT_2 receptor on the growth of fibroblasts is controversial. Our data are consistent with the report that the in vitro hyperplastic response of cardiac fibroblasts is mediated by the AT_1 receptor subtype and the AT_2 receptor does not play any role on the growth and protein synthesis of fibroblasts. However, Scheidegger and Wood (40) reported that the increased in vivo cellularity in the adventitia after local application of Ang II is stimulated via the AT_1 receptor and inhibited by the AT_2 receptor. In another report, chronic blockade of AT_2 receptor prevented the effects of Ang II on in vivo arterial hypertrophy and fibrosis (45). These experiments were performed under different conditions with different species and the physiological role of AT_2 receptor in AFs needs more investigation.

Our results demonstrated that Ang II increased the proliferative response of AFs to EGF, PDGF-BB and bFGF. Ang II has been reported to act synergistically with EGF and PDGF to induce proliferation in the VSMCs of spontaneously hypertensive rats (26). However, we could not find a synergistic action between Ang II and the other growth factors in terms of DNA synthesis. Ang II has been shown to induce PDGF, bFGF and TGF-β1 in smooth muscle cells (24, 37, 46). It is likely that the autocrine and paracrine production of growth factors by AFs, due to the combined effect of injury and Ang II, may induce further activation of AFs.

Balloon injury activates the vascular renin-angiotensin system, which may participate in the proliferation of AFs, leading to adventitial thickening and fibrosis with the resultant restenosis due to vascular remodeling. These findings may serve as a rationale for remedial therapy with ACE inhibitors or blockade of the renin-angiotensin system in patients with restenosis following angioplasty.

In summary, we have demonstrated that Ang II causes hyperplasia of AFs via AT_1 receptor stimulation and potentiates proliferative responses to other mitogens. These data suggest that Ang II might have important regulatory roles in the function of AFs under pathological conditions. Ang II-induced hyperplasia of AFs may represent an in vitro model that can be utilized to elucidate the cellular and molecular biology of a variety of vascular diseases including restenosis after angioplasty.

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