Angiotensin II AT₂ receptor inhibits smooth muscle cell migration via fibronectin cell production and binding

Catherine Chassagne, Christophe Adamy, Philippe Ratajczak, Bruno Gingras, Emmanuel Teiger, Emmanuelle Planus, Patricia Oliviero, Lydie Rappaport, Jane-Lise Samuel, and Sylvain Meloche. Angiotensin II AT₂ receptor inhibits smooth muscle cell migration via fibronectin cell production and binding. Am J Physiol Cell Physiol 282: C654–C664, 2002. First published November 27, 2001; 10.1152/ajpcell.00318.2001.—To explore the vascular function of the angiotensin II (ANG II) AT₂ receptor subtype (AT₂R), we generated a vascular smooth muscle cell (SMC) line expressing the AT₂R (SMC-vAT₂). The involvement of AT₂R in the motility response of SMCs was examined in SMC-vAT₂ cells and their controls (SMC-v) cultured on substrates; cellular fibronectin

Angiotensin II AT₂ receptor inhibits smooth muscle cell migration via fibronectin cell production and binding.

vvascular remodeling is generally described as a consequence of growth and/or phenotype alterations of the vascular smooth muscle cells (SMCs) (20). Migration of medial SMCs within the vessel wall, however, is another essential aspect of the process of vascular remodeling (4, 55, 59). Identification of the environmental signals susceptible to control SMC migration has been derived largely from studies in cultured SMCs. These include growth factors, such as platelet-derived growth factor or fibroblast growth factor, cytokines, components of the extracellular matrix (ECM) (1), and nitric oxide (8) and endothelial nitric oxide synthase (25). It also is well documented that matrix metalloproteinases (MMPs), by cleaving ECM components, participate in promoting SMC migration (36, 61, 73).

Normal or pathological vessel remodeling is generally described as a consequence of growth and/or phenotype alterations of the vascular smooth muscle cells (SMCs) (20). Migration of medial SMCs within the vessel wall, however, is another essential aspect of the process of vascular remodeling (4, 55, 59). Identification of the environmental signals susceptible to control SMC migration has been derived largely from studies in cultured SMCs. These include growth factors, such as platelet-derived growth factor or fibroblast growth factor, cytokines, components of the extracellular matrix (ECM) (1), and nitric oxide (8) and endothelial nitric oxide synthase (25). It also is well documented that matrix metalloproteinases (MMPs), by cleaving ECM components, participate in promoting SMC migration (36, 61, 73).

Angiotensin II (ANG II) is an important mediator of systemic vascular remodeling. ANG II has been shown to promote hyperplasia and/or hypertrophy of vascular SMC in vitro as well as in vivo in the normal arterial wall (31) and to participate in the myointimal proliferation response to vascular injury (13, 47, 50). ANG II also stimulates migration of SMCs both in vitro (6) and during restenosis formation after vascular injury (46, 51). Two major subtypes of ANG II receptors, designated AT₁ and AT₂, have been identified on the basis of their affinity for selective receptor antagonists (reviewed in Ref. 15). It is currently admitted that the stimulatory effects of ANG II on either growth or migration of SMCs are attributable to the AT₁ subtype (18, 19, 29, 31, 34, 46, 51, 71). Much less is known about the physiological roles of the AT₂ receptor, which is abundantly expressed in the developing vascular system (45, 60, 66) and is reexpressed in adult SMCs in response to vascular injury (45), arteriogenesis after myocardial infarction (70), or hypoxia-induced lung vessel remodeling (11). In vitro investigations of the function of AT₂ receptor have proven difficult, mainly because of low or lack of expression of this receptor in cultured SMCs (3, 31, 39, 46), which contrasts with the abundant expression of the AT₁ subtype. Ectopically expressed AT₂ receptor in cultured SMCs has been

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shown to exert either an antiproliferative (45) or apoptotic (72) effect, thereby counteracting the growth-promoting action of the AT1 receptor. Whether such an effect is accompanied by a modulation in the migratory properties of SMCs has not been explored in cultured SMCs.

In the present study, we have generated a vascular SMC line expressing the human AT2 receptor by retrovirus-mediated gene transfer to compare the effect of the AT2 receptor on SMC migration on either laminin or fibronectin substrate. To further define the molecular basis of the action of the AT2 receptor on SMC migration, we also have investigated the effect of AT2 receptor activation on SMC growth, cytoskeletal stiffness, and expression of several ECM and cytoskeleton components.

MATERIALS AND METHODS

Reagents. Human ANG II was purchased from American Peptide Company. The radioligand 3-[125I]iodotyrosyl[Sar1, Ile8]-ANG II was obtained from Amersham (specific activity 2,000 Ci/mmol) or prepared by radiiodination of [Sar1, Ile8]ANG II (sarile) using a solid-phase method. The AT1 and AT2 receptor-selective antagonists used were losartan (Merck Sharp & Dohme Research Laboratories), PD-123319 (kindly provided by Prof. B. Levy, Univ. Diderot, Paris, France), and CGP-42112A (Neosystem Laboratory, Strasbourg, France). The GRGDTP and GRGETP peptides were obtained from Sigma. Dulbecco’s minimum essential medium (DMEM), fetal calf serum (FCS), I-glutamine, antibiotics, geneticin, trypsin-EDTA, phosphate-buffered saline (PBS), bovine serum albumin (BSA), and mouse laminin (Lam) were from Life Technologies, and human plasma fibronectin (FN) was from Sigma.

Infection of vascular SMC with the human AT2 receptor gene. A 1.3-kb genomic fragment containing the entire coding sequence of the human AT2 receptor gene (10) was subcloned into the retrovirus expression vector pLNCX (40) to generate pLNCX-AT2. Empty pLNCX vector and pLNCX-AT2 were transfected with Lipofectamine (Life Technologies) into the amphotropic retrovirus packaging cell line CxCRIP (14), and stably transfected cells were selected in complete medium containing 0.4 mg/ml G418 (Life Technologies). For infection of vascular SMCs, virus supernatants were harvested from helper-free retrovirus producer cell lines and applied to 30% confluent cultures of rat aortic SMCs in the presence of 8 M; unlabeled sarile, 10^{-5} M losartan, in the absence or presence of the nonselective ANG II receptor antagonist sarile (10^{-4} M) or the AT2-selective antagonist PD-123319 (3 × 10^{-6} M). Bound 125I-sarile was determined by rapid filtration on GF/B filters. Binding data are expressed as fmol of 125I-sarile bound per mg of membrane protein. Each value represents the mean ± SE of triplicate determinations.

Receptor binding assays. For whole cell binding assays, infected cells were grown to confluence in six-well culture plates washed twice with DMEM and incubated with ~2 × 10^{-10} M 125I-labeled sarile and 10^{-5} M losartan, in the absence or presence of competing agents (PD-123319, 3 × 10^{-6} M; unlabeled sarile, 10^{-6} M), for 90 min at 25°C in a total volume of 1 ml of DMEM, 25 mM HEPES (pH 7.4), and 0.1% heat-inactivated BSA. After incubation, the cells were washed rapidly three times in ice-cold PBS. Bound 125I-sarile was determined by counting the cell-associated radioactivity in an Auto-Gamma counter after solubilization in 0.1 M NaOH.

Preparation of membranes from SMC-v and SMC-vAT2 cells and competitive binding studies were performed as previously described (38). Competition-binding curves were analyzed by nonlinear least-square curve fitting using the SCAFIT computer program (16). Equilibrium binding constants are reported as dissociation least-square curve fitting using the SCAFIT computer program (16). Equilibrium binding constants are reported as dissociation least-square curve fitting using the SCAFIT computer program (16). Equilibrium binding constants are reported as dissociation least-square curve fitting using the SCAFIT computer program (16). Equilibrium binding constants are reported as dissociation least-square curve fitting using the SCAFIT computer program (16).
receptor antagonists PD-123319 (3 × 10^{-6} M) or CGP-42112A (10^{-6} M). For migration assays, the GRGDTP peptide that competes with FN for binding to cells (23) was used at a final concentration of 10^{-4} M.

**Cell migration assay.** The cell migration assay was similar to those previously described by Varani and Ward (65) and Kiernan and ffrench-Constant (30), with a few modifications (49). Cells were resuspended at 40 × 10^6 cells/ml in a 0.3% agarose-DMEM solution containing 0.2% FCS and 10^{-5} M losartan. Drops (2.5 μl) of the cellular agarose suspension were plated on 24-well plates precoated with either Lam/H2O or FN/PBS and kept at 4°C for 20 min to allow gelation of the agarose. The drops were then incubated at 37°C for 24 or 48 h in a 0.5-ml volume of medium as described in **Cell culture and treatments.** Afterward, the medium was removed and the samples were fixed and stained with the Diff Quick kit (Dade). To assess the degree of radial migration of cells from drop side to external environment, we recorded light microscopy images of drops onto a computer after digitization using image analysis software (Perfect Image, Paris, France) that enables measurement of surface areas by enclosing them in a cell-by-cell freehand curve. For each sample, both the area enclosed by the drop side (i.e., the drop surface) and the area enclosed by the cell front line (i.e., the drop surface plus the area between the drop side and the cell front line) were measured. The subtraction of the former from the latter gave a relative value representing cell population migration. Because of the possibility of slight variations in the surface area of the original drops, values were normalized to the surface area of each corresponding original drop to obtain cell migration indexes that were comparable among drops according to the following formula: cell migration index = [(drop area +

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**Fig. 2.** Mobility of SMC-v and SMC-vAT_{2} cells on fibronectin (FN; A) or laminin (Lam; B) substrate. Cells were resuspended at 40 × 10^6 cells/ml in culture medium containing agarose and 10^{-5} M losartan (Los). Drops from each cell-type suspension were plated in the center of a 24-well tissue culture plate coated with either FN or Lam and then incubated at 37°C for 24 h in medium containing 10^{-5} M losartan and supplemented with either 10% fetal calf serum (FCS), 0.2% FCS (control), or 10^{-7} M ANG II (All). After incubation, cells were fixed, stained, and examined by light microscopy. Representative microphotographs are shown.
Staining of cellular fibronectin on migrating cells by immunocytochemistry. Cellular drops were plated in the center of eight-well cover slides precoated with Lam/H2O and incubated as described above. At the end of incubation, cells were fixed with 4% paraformaldehyde and rinsed twice with ice-cold PBS. Immunocytochemistry was performed by using the biotin-avidin-peroxidase technique with 3-amin-9-ethylcarbazol (AEC) chromogen as a substrate (Sigma). Briefly, samples were preincubated for 30 min at 25°C in PBS supplemented with 5% BSA and then incubated at 4°C overnight with anti-cellular fibronectin (c-Fn) monoclonal antibody (MAb) (clone FN-3E2, Sigma) diluted 1:100 in PBS-2% BSA. After being washed in PBS, they were incubated for 30 min at 25°C with mouse biotinylated Ig (Dako) diluted 1:100 in 5% serum-PBS, washed with PBS, and treated with 3% H2O2 for 10 min. After being washed in PBS, the samples were incubated for 30 min at 25°C with avidin-peroxidase and then for 10 min with AEC substrate to allow adequate chromogen development. The cover slides were mounted and observed by microscopy.

Immunoblot analysis. The cellular contents in desmin, tenascin, smooth muscle actin, and fibronectin (c-Fn) were determined by Western blot analysis. Lysate proteins (4–10 μg) were resolved by electrophoresis on 7.5% SDS-acrylamide gel and electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham). The membranes were incubated for 1 h 45 min at 25°C with either anti-smooth muscle actin MAb (1:7,000; clone 1A4, Dako), anti-c-Fn MAB (1:7,000; clone FN-3E2), anti-tenascin C MAB (1:3,000; clone BC-24, Sigma), or anti-desmin polyclonal antibody (1:4,000) (Sigma) in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20. After being washed, the membranes were incubated with anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) (Amersham). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry using a computer-based imaging system (Gel Doc 1000; Bio-Rad).

Analysis of c-Fn secretion activity. Cells were stimulated with ANG II as described. After 12 h, [35S]methionine was added at a final concentration of 100 μCi/ml, and the cells were incubated for another 12 h. The medium was diluted with 3 vols of immunoprecipitation (IP) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM sodium orthovanadate, 5 mM EGTA, and 0.1% SDS], and c-Fn was immunoprecipitated at 4°C overnight with 5 μl of rabbit anti-plasmatic Fn polyclonal antibody (Chemicon). Immune complexes were collected by incubation with protein A/G-Sepharose beads for 2 h at 4°C. The beads were washed four times with IP buffer, and immunoprecipitates were analyzed by SDS-gel polyacrylamide on 7.5% acrylamide gels and visualized with a PhosphorImager.

Measurements of DNA and protein contents. Cells were plated (40 × 10^4 cells/cm²) on six-well plates and allowed to attach in DMEM-0.2% FCS for 24 h at 37°C. They were preincubated for 15 min with losartan in the absence or presence of PD-123319 and then treated for 24 or 48 h as described in Cell culture and treatments. The DNA total content was measured by fluorometry using the bisbenzimide dye Hoechst 33342 as described previously (53). After measurement, the medium containing the Hoechst 33342 dye was removed, and the plates were rinsed with PBS, frozen with liquid nitrogen, and stored at −80°C. Protein measurement was performed as already described.

Statistical analysis. Results are expressed as means ± SE. The statistical significance of differences between the various cell treatments was determined by one-way analysis of variance, and group-to-group comparison was made by Scheffé’s F test. The accepted level of significance was P < 0.05.

![Figure 3](http://ajpcell.physiology.org/)

**Fig. 3.** Mobility of SMC-vAT2 cells on Lam substrate in the absence or presence of CGP-42112A or PD-123319. Agarose drops were incubated for 24 (A) or 48 h (B) in 0.2% FCS-DMEM containing 10−6 M Los in absence (C, control) or presence of 10−7 M ANG II, added alone or with 3 × 10−8 M PD-123319 (ANG II + Los) or 10−10 M CGP-42112A (ANG II + CGP). Bar graphs show the migratory indexes of SMC-vAT2 cells after 24 (C) and 48 h (D) of incubation in the media described above. Controls with PD-123319 and CGP-42112A also are shown. For each time, data were obtained from 2 independent experiments (N = 2; 6–9 drops/condition in each experiment) and are expressed as means ± SE in arbitrary units (AU). **P < 0.01 and ***P < 0.001 vs. control condition.
RESULTS

Expression of AT₂ receptor in rat vascular SMCs. Vascular SMCs infected with the human AT₂ receptor gene display a high increase in binding activity in contrast to cells infected with empty vector (Fig. 1A). The AT₂ receptor binds the radioligand in a saturable manner with a binding capacity of 770 fmol/mg protein (data not shown). Binding assays were conducted in the presence of a saturating concentration of losartan to prevent binding of the radioligand to the AT₁ receptor subtype (Fig. 1B). The potency order of the ligands in competing for ¹²⁵I-sarile binding was sarile \( (K_d = 0.7 \text{ nM}) \) > CGP-42112A \( (K_d = 1.2 \text{ nM}) \) > ANG II \( (K_d = 4.6 \text{ nM}) \) > PD-123319 \( (K_d = 10.0 \text{ nM}) \), similar to that reported previously for the endogenous or cloned AT₂ receptor (44). These results indicate that the stably expressed recombinant receptor exhibits the typical pharmacological properties of the AT₂ receptor.

AT₂ receptor activation inhibits SMC migration. The migratory properties of SMC-v and SMC-vAT₂ cells on FN and Lam substrates were analyzed in the presence of a saturating concentration of losartan by using the agarose drop technique. As shown in Fig. 1, incubation with \( 10^{-5} \text{ M losartan} \) completely blocked the binding of ANG II to the AT₁ receptor subtype, thereby allowing study of the action of the AT₂ receptor. Figure 2 shows representative pictures of cell drops incubated for 24 h under diverse conditions on either FN (A) or Lam (B) substrate. In the presence of serum, SMC-v and SMC-vAT₂ cells migrated out from their drops on either substrate, whereas under control conditions both cell lines displayed migration on Lam only, indicating that under our conditions Lam, but not FN, substrate is able per se to promote cell migration.

Importantly, on Lam substrate, the migration of SMC-vAT₂ but not SMC-v cells was clearly diminished upon treatment with ANG II for 24 h compared with control conditions (Figs. 2B, 3A, and 4). The basal migration of SMC-vAT₂ cells on Lam substrate was further increased by 48 h but was still reduced when ANG II was added to the media (Fig. 3B). Quantitative analysis of the drops indicated that the migration of

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Fig. 4. Mobility of control SMC-v cells on Lam substrate in the absence or presence of CGP-42112A or PD-123319. A: agarose drops were incubated for 24 h in the same media as described in Fig. 3. Controls with PD-123319 and CGP-42112A also are shown. Bar graphs show the migratory indexes of SMC-v cells after 24 (B) and 48 h (C) of incubation in the media described above. For each time, data were obtained from 2 independent experiments \( (N = 2; 4–6 \text{ drops/condition in each experiment}) \) and are expressed as means ± SE in AU. *** \( P < 0.001 \) vs. control condition.
SMC-vAT<sub>2</sub> cells was significantly inhibited in the presence of ANG II by 23.1% (P < 0.001) after 24 h (Fig. 3C) and by 18.2% (P < 0.01) after 48 h (Fig. 3D). High concentrations of CGP-42112A, which per se has no effect on SMC-vAT<sub>2</sub> migration (see Fig. 3, C and D), completely prevented the inhibitory effect of ANG II on cell migration (Fig. 3, A–D), confirming that the action of ANG II is specifically mediated through the AT<sub>2</sub> receptor subtype. On the other hand, PD-123319 not only failed to prevent the inhibitory effect of ANG II but also reduced SMC-vAT<sub>2</sub> cell migration by 25% (P < 0.001) after 24 h and by 39% after 48 h (P < 0.001) (Fig. 3, C and D) in the absence of ANG II stimulation. Surprisingly, PD-123319 similarly inhibited the migration of control SMC-v cells after 24 and 48 h (Fig. 4). We concluded from these results that PD-123319 exerts an AT<sub>2</sub>-independent inhibitory action on SMC migration that masks its antagonistic effect on the migration inhibition caused by AT<sub>2</sub> receptor activation.

Table 1. Effects of ANG II on DNA and total protein contents in SMC-vAT<sub>2</sub> cells plated on laminin

<table>
<thead>
<tr>
<th></th>
<th>24 h Control</th>
<th>ANG II</th>
<th>ANG II + PD</th>
<th>48 h Control</th>
<th>ANG II</th>
<th>ANG II + PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA content, µg</td>
<td>25.6 ± 1.0</td>
<td>23.1 ± 1.2</td>
<td>22.5 ± 2.0</td>
<td>38.7 ± 0.5</td>
<td>39.1 ± 2.2</td>
<td>43.3 ± 1.3</td>
</tr>
<tr>
<td>Total protein content, µg/µg DNA</td>
<td>7.6 ± 1.6</td>
<td>6.8 ± 2.2</td>
<td>9.4 ± 2.6</td>
<td>9.5 ± 0.8</td>
<td>8.4 ± 0.8</td>
<td>8.5 ± 1.3</td>
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Smooth muscle cells expressing the angiotensin II (ANG II) type 2 receptor (SMC-vAT<sub>2</sub>; 40 × 10<sup>3</sup> cells/cm<sup>2</sup>) plated on laminin were incubated for 24 or 48 h with 10<sup>−5</sup> M losartan in absence (control) or presence of 10<sup>−7</sup> M ANG II added alone or with 3 × 10<sup>−6</sup> M PD-123319 (ANG II + PD). The total DNA and protein contents were measured by fluorometry and colorimetry, respectively, as described in MATERIALS and METHODS. Data are expressed as means ± SE of 2 independent experiments with triplicate determinations in each experiment.
**Inhibition of cell migration by the AT$_2$ receptor is concomitant with increased c-Fn secretion and fiber formation.** To define the molecular basis of the inhibitory action of the AT$_2$ receptor on SMC migration, we examined the impact of AT$_2$ receptor activation on both SMCv-AT$_2$ cell growth and expression of cytoskeletal (actin, desmin) and ECM (tenascin, c-Fn) proteins. Total DNA and protein contents of SMCv-AT$_2$ cells plated on Lam substrate were unchanged after either 24 or 48 h of ANG II treatment compared with controls (Table 1), indicating that inhibition of SMC migration in response to AT$_2$ activation was not associated with changes in cell growth properties. Thus the lower number of cells observed around ANG II-treated drops results from a decrease in the motility, not the proliferation, of SMCv-AT$_2$ cells.

Western blot analysis revealed that expression of smooth muscle actin, desmin, and tenascin by SMCv-AT$_2$ cells did not vary upon AT$_2$ receptor activation (not shown). By contrast, ANG II increased both the cell expression and secretion of c-Fn (Fig. 5, A and B).

The c-Fn content in ANG II-treated cells was increased 1.7-fold at 24 h $[0.69 \pm 0.05 \text{ AU} (N = 2 \text{ independent experiments}, n = 3 \text{ determinations})$ vs. $0.41 \pm 0.11 \text{ AU} (N = 2, n = 3), P < 0.05]$ and $2$-fold at $48 \text{ h} [0.63 \pm 0.12 \text{ AU} (N = 2, n = 3) \text{ vs. } 0.31 \pm 0.05 \text{ AU} (N = 2, n = 3), P < 0.01]$ (Fig. 5A). The amount of newly synthesized c-Fn secreted into the medium at $24 \text{ h}$ was increased $2.4$-fold over control level $[0.59 \pm 0.12 \text{ AU} (n = 3) \text{ vs. } 0.25 \pm 0.01 \text{ AU} (n = 3), P < 0.05]$ (Fig. 5B). These changes in c-Fn levels were prevented by the addition of CGP-42112A or PD-123319 to the medium. Figure 5C shows typical pictures of c-Fn staining within the border zone of drops after $48 \text{ h}$ in migrating conditions; in the presence of ANG II, c-Fn staining was increased, forming numerous fibers at the surface of cells compared with untreated cells. This process was inhibited by the addition of CGP-42112A or PD-123319 to the incubation medium. Together, these data indicate that AT$_2$ receptor activation by ANG II contributes to the synthesis and secretion of c-Fn that accounts for the increase in c-Fn binding to cell surface; this effect is
concomitant with inhibition of the migrating capacity of SMCs.

AT<sub>2</sub>-mediated inhibition of SMC migration is due to the attachment of newly synthesized c-Fn. To determine whether the increased binding of c-Fn is responsible for the inhibition of cell migration, we examined the effect of the GRGDTP peptide on the expression of c-Fn and the migration properties of SMC-vAT<sub>2</sub> cells. GRGDTP, as well as GRGETP control peptide, had no effect on either basal or ANG II-stimulated c-Fn synthesis and secretion in SMC-vAT<sub>2</sub> cells (Fig. 6, A and B). However, GRGDTP markedly reduced the ANG II-dependent increase in c-Fn staining at the cell surface (Fig. 6C vs. 5C), indicating that this peptide specifically prevents cell attachment of c-Fn.

Importantly, the GRGDTP peptide, which per se had no effect on SMC-vAT<sub>2</sub> cell basal migration, completely prevented the inhibitory effect of ANG II on cell migration (Fig. 7). These results demonstrate that the AT<sub>2</sub>-dependent inhibition of SMC migration is directly due to c-Fn attachment to the cells.

**DISCUSSION**

In this study, we have demonstrated that AT<sub>2</sub> receptor activation inhibits vascular SMC migration through the secretion and subsequent cell attachment of c-Fn. These results provide the first direct evidence for a negative influence of the AT<sub>2</sub> receptor on SMC migrating capacity through a process involving modifications of proximal matrix environment.

Previous studies have documented the promoting effect of ANG II on migration of vascular SMCs in culture (6, 18, 19, 29, 34, 46, 71) or in vivo during neointimal thickening after balloon injury (46, 51). The blockade of ANG II-stimulated SMC migration by various AT<sub>1</sub> but not AT<sub>2</sub> receptor-selective antagonists (18, 19, 29, 34, 46, 71) has led to the conclusion that migration-promoting effects of ANG II are mediated through AT<sub>1</sub> receptors. The role of the AT<sub>2</sub> receptor had remained elusive in this setting.

Although AT<sub>2</sub> receptors have been found to be expressed in the vasculature of adult rats (37), their expression is barely detectable in cultured SMCs (3, 39, 46, 64), presumably because AT<sub>2</sub> receptors are easily lost after subculturing as postulated by Moriguchi et al. (42). In agreement with these observations, we noted in this study the scarcity of endogenous AT<sub>2</sub> receptors in both the empty virus-infected SMC-v (Fig. 1A) or the untransfected parental cells (not shown). To circumvent this problem, we ectopically expressed the AT<sub>2</sub> receptor in aortic SMCs by retrovirus-mediated gene transfer and conducted all experiments in the presence of a saturating concentration of losartan to inactivate AT<sub>1</sub> receptors. This “gain-of-function” strategy enabled us to demonstrate that activation of AT<sub>2</sub> receptor by ANG II increases the synthesis, secretion, and cell binding of c-Fn. To our knowledge, this is the first direct evidence for a stimulatory effect of AT<sub>2</sub> on c-Fn synthesis, which thereby extends previous results that blockade of AT<sub>1</sub> receptors in adult rat increases aortic levels of c-Fn, presumably through the increased expression of the “accessible” AT<sub>2</sub> vascular receptors (58). Further support for our finding is provided by reports that 1) in SMCs, AT<sub>2</sub> activates the nuclear transcription factor NF-κB (56), which enhances c-Fn expression in gliostama multiform-derived cell lines (54) and 2) after arterial injury, both AT<sub>2</sub> receptors and c-Fn increase at the site of injury (5, 45). It is noteworthy that AT<sub>2</sub> receptor also stimulates collagen synthesis in cultured SMCs (39) and in vasculature in vivo (9, 33). It is noteworthy that CGP-42112A, which per se had no effect on c-Fn synthesis (data not shown), completely inhibited the stimulatory effect of ANG II on c-Fn production (Fig. 5), indicating that this compound acts as a full antagonist in our cell system. There have been conflicting reports on the pharmacological properties of CGP-42112A, with some studies describing it as an antagonist (7, 35, 41, 56, 62) and others as a partial or full agonist (28, 63).

Because MMPs regulate the cell matrix environment by cleaving ECM components (36, 61, 73), we also examined the effect of AT<sub>2</sub> receptor activation on MMP cell activity. We found that SMCV-AT<sub>2</sub> cells produce mainly MMP-2, in agreement with previous reports that MMP-2 is the prevalent isozyme expressed in cultured (21) as well as in situ SMCs (67). Activation of
the AT2 receptor did not change the levels of activated MMP-2 (data not shown). Thus the AT2-dependent increase in c-Fn deposition is not related to a decrease in cell MMP activity.

Newly synthesized c-Fn is secreted as a soluble monomer and polymerizes in a insoluble, multimeric complex that modifies cell properties (57). Changes in the strength of linkages between cell and ECM components can be inferred from the measurement of cytoskeletal stiffness by either magnetocytometry (32, 68, 69) or by optical trapping (12). To determine whether the increased c-Fn binding modifies cell-substrate interactions, we measured the cytoskeletal stiffness of SMC-vAT2 cells by a magnetocytometry approach, which is based on the application of controlled mechanical stresses directly to cell surface integrins using RGD-coated microbeads. Activation of AT2 receptor did not change cytoskeletal stiffness of SMC-vAT2 cells (data not shown), suggesting that cellular binding of newly polymerized c-Fn is not enough to alter the strength of linkages between integrins and the cytoskeletal apparatus (68). However, the observation that GRGDTP peptide prevented both AT2-dependent cell binding of c-Fn (Fig. 6) and inhibition of cell migration (Fig. 7) indicates that the inhibitory action of AT2 is due to cell attachment of c-Fn. This is in agreement with the lack of migration of SMCs on FN substrate (Fig. 2A). These results also are consistent with the demonstration that “superfibronectin,” which resembles matrix fibers, greatly enhances cell adhesive properties and suppresses cell migration (43) and that accumulation of c-Fn within ECM suppresses the motility and growth potential of fibrosarcoma tumor cells, most likely through α5β1-integrins (2). Thus we propose that the twofold increase in c-Fn synthesis in response to AT2 activation results in an increase in the adhesive properties of SMC-vAT2 cells, with this effect leading to inhibition of SMC-vAT2 cell migration. The observation that PD-123319 is able to inhibit SMC migration in an AT2-independent manner (Fig. 4) provides a unifying explanation for the paradoxical results that PD-123319 fails to antagonize the inhibitory action of AT2 receptor on cell migration despite its inhibitory effect on c-Fn binding.

The amplitudes of the inhibitory effects of AT2 receptor on cell migration are comparable to the range of stimulated migration responses by AT1 receptor activation reported in the literature (6, 18, 29, 34), indicating that our results with this “artificial” expression system may be physiologically relevant. However, the observations that engagement of both AT1 and AT2 receptor stimulates c-Fn production and yet that the two receptors have apparently opposite effects on SMC migration, raise questions. Interestingly, DiMilla et al. (17) reported that the migration speed of human vascular SMCs on FN depends in a biphasic manner on both ECM surface density and attachment strength. It is maximal at an intermediate level of cell-substratum adhesiveness and minimal when the attachment strength is either weak or high. Thus it is conceivable that the increase in c-Fn synthesis that occurs in response to AT1 receptor activation results in an intermediate level of cell adhesiveness that allows cell migration, whereas that observed upon AT2 receptor activation is incompatible with migration stimulation.

In conclusion, we have shown that AT2 receptor activation enhances both the synthesis and secretion of Fn and its binding to SMCs, resulting in the inhibition of SMC migration. This novel insight into AT2 receptor function may provide a new basis for understanding the exact role of AT2 in the structural changes occurring during normal and pathological growth of blood vessels, especially during postangioplasty restenosis. Indeed, AT2 receptors have been implicated in the reduction of neointimal formation after vascular injury (28, 45). Interestingly, Pickering et al. (48) reported that a subpopulation of α5β1-integrin-bearing SMCs orchestrates integrin-mediated Fn assembly in the repairing artery wall. It is therefore conceivable that AT2 receptors might, through the mechanism described in this study, participate in inhibiting vascular SMC migration from media to neointima, thus resulting in the reduction of arterial thickening.

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