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Coexpression of AT1 and AT2 receptors by human fibroblasts is associated with resistance to angiotensin II

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Abstract

Angiotensin II (AngII) is considered as a cytokine-like factor displaying a variety of proinflammatory and profibrotic cellular effects. Most of these effects seem mediated by AT1 signaling, whereas AT2 expression and function in adult human cells remain unclear. We have studied AT1 and AT2 expression in different human adult fibroblasts types and analyze their response to AngII. AngII did not induce thymidine incorporation, apoptosis nor collagen gene or protein expression in human fibroblasts. Specific AT1 or AT2 inhibitors did not modify this apparent resistance to AngII. We found abundant expression of both AT1 and AT2 receptors in all human fibroblasts studied, whereas vascular smooth muscle cells (VSMC) which only expressed AT1 receptor, displayed a clear AT1-dependent proliferative response to AngII. These data demonstrate that cultured human adult fibroblasts express both AT1 and AT2 receptor types and this phenomenon is associated with a lack of growth or collagen synthesis responses to AngII.

Keywords: Angiotensin II; Apoptosis; Collagen; Inflammation; Fibroblast

1. Introduction

Angiotensin II (AngII) is the major effector peptide of the rennin–angiotensin system, which plays a prominent role in vascular and renal homeostatic balance. Nowadays, AngII is also considered as a true cytokine displaying a large variety of cellular effects involved in cell growth, inflammation and extra cellular matrix (ECM) synthesis [27]. The effects of AngII are mediated by specific receptors, AT1 and AT2 being the main subtypes. Both AT1 and AT2 receptors are seven transmembrane-spanning G protein-coupled receptors [5]. Most of the physiological effects of AngII are mediated by AT1 receptor, which is widely expressed by most cell types, whereas AT2 expression is more restricted and it is predominant in fetal tissues where it has been suggested to play a role during development [7].

Under physiological conditions, AT1 receptor regulates blood pressure and water and electrolyte homeostasis, whereas in pathology, AT1 function has been linked to vascular, renal and cardiac fibroproliferative diseases [4,24,29]. In contrast, the physiopathological roles of AT2 receptor are less clear. Based on its pattern of expression, studies in transgenic and knockout animal models, and cellular in vitro studies, AT2 receptor appears to modulate tissues development and repair by counterbalancing AT1 mediated effects [3,12,35–37]. The therapeutic impact of AT1 specific inhibitors has confirmed its participation in vascular and renal fibroproliferative diseases [14,18]. However, the multiple functions of this receptor in vascular and renal homeostasis, inflammatory cells behavior and resident fibroblasts or smooth muscle cells, make the specific contribution AT1 to these interdependent processes difficult to dissect in humans or animal models of disease.

The potential contribution of the AngII receptor subtypes to the different AngII-induced cellular effects has been analyzed in different cells either transfected or naturally express-

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ing AngII receptors using specific AT1 or AT2 inhibitors [13,16,26]. In spite of the proposed role for AT1 in fibroproliferative lesions, there is scarce information regarding the cellular effects of AT2 signaling in human adult fibroblasts. Most studies in adult fibroblasts are consistent with exclusive expression of AT1 receptor, which can mediate cell proliferation and ECM synthesis [2,9–11,15,22,29]. However, some data suggest that human adult renal, cardiac and dermal fibroblasts can also express AT2 receptor, although its function has not been well characterized [16,29,32]. Different studies suggest that coexpression of AT1 and AT2 receptor antagonizes the cellular effects of AT1 receptor activation by two mechanisms, first by specific AT2 intracellular signaling that results in a final antagonistic effect (i.e. growth inhibition) [6,33], and second, by abrogating AT1 signaling through the formation of AT1/AT2 heterodimeric receptors [1]. The later mechanism has been characterized in fetal fibroblasts and human myometrial biopsies where coexpression of both receptor types has been demonstrated. In these models, AT1 is insensitive to AngII stimulation and the effect is not dependent on AT2 signaling by AngII. AT2 receptor expression can also participate in apoptosis independently of its ligand AngII [17]. In spite of the described counterbalancing effects of AT2 receptor on AT1 responses, there is little information regarding their role in human cells. In cardiac fibroblasts from failing hearts, an increase in AT2 expression has been shown to inhibit AngII-induced mitogenic signaling [32].

The aim of this study was to analyze AT1 and AT2 receptors expression in different types of human adult fibroblasts and the biological effects of specific AT1 and AT2 inhibition. We have specifically analyzed the potential role of AngII in cellular proliferation, collagen synthesis and apoptosis, which seem the most relevant cellular effects described so far regarding fibroproliferative diseases.

2. Material and methods

2.1. Cell cultures

Dermal fibroblasts (DF) were obtained from skin biopsies from fibrotic skin of three patients with diffuse systemic sclerosis (SSc) and three sex- and age-matched healthy controls. Normal skin biopsies were obtained from the normal margins of benign cutaneous lesions during minor cosmetic surgery. Synovial fibroblasts (SF) were isolated from synovial tissue obtained from patients with osteoarthritis at the time of knee prosthetic replacement surgery. Adult primary lung fibroblasts (LF) were isolated from lung tissue obtained from the normal margins of a nodular lesion at the time of diagnostic biopsy by thoracotomy. Vascular smooth muscle cells (VSMC) obtained from rat thoracic aorta were a gift from Dr. Sánchez-Pernaute (Renal and Vascular Research Laboratory, Fundación Jiménez-Díaz, Madrid, Spain). VSMC were grown in DMEM with 10% non-heat inactivated FCS and penicillin/streptomycin and used between passages seven and nine. All fibroblasts types were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and penicillin/streptomycin and used between passages 3 and 10.

2.2. Cell proliferation and apoptosis

Cell proliferation was evaluated by determining DNA synthesis rates by [^{3}H] thymidine incorporation. The effect of different AngII (Sigma–Aldrich, Madrid, Spain) concentrations under different serum conditions was analyzed. To study the potential role of AT1 and AT2 receptors, losartan (losartan potassium, Merck & Co. Inc., Rahway, NJ) or PD123319 (PD123319 ditrifluoroacetate, Sigma–Aldrich) were added to the cultures at different concentrations 30 min before AngII stimulation. Subconfluent fibroblasts or VSMC cultured in 24-well plates were synchronized in DMEM supplemented with 0.1% FCS for 48 h and then treated for 24 h with 0.1 μM AngII, 10 μM losartan, 10 μM PD123319, or 10% FCS and pulsed with 1 μCi/ml [^{3}H] thymidine during the last 4 h. Cell extracts were precipitated with 10% trichloroacetic acid for 45 min at 4 °C. After centrifugation, pellets were washed with 10% trichloroacetic acid and solubilized in 0.3N NaOH/1% sodium dodecyl sulphate for liquid scintillation counting.

The potential effect of AngII to modulate DF apoptosis induced by serum deprivation was analyzed by direct counting of TUNEL-labeled nuclei in fibroblasts grown on coverslips. After 48 h of FCS starvation, cultures were exposed during 24 h to 0.1 μM AngII with or without 10 μM losartan or 10 μM PD123319. Coverslips were fixed with 4% formaldehyde and labeled by TUNEL with fluorescein-dUTP as previously described [28]. Nuclei were simultaneously labeled with 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were observed under a fluorescence microscope and the proportion of apoptotic cells was calculated by dividing the number of positive-TUNEL cells by the total number of DAPI-labeled nuclei.

2.3. Collagen expression by fibroblast cultures

Procollagen α1(I) mRNA expression was analyzed by Northern blot as previously described [34]. Three normal and three SSc DF lines were analyzed under basal conditions (DMEM with 10% FCS) and after exposure for 48 h to 0.1 μM AngII. The autoradiograms were quantified by scanning laser densitometry. The level of procollagen α1(I) mRNA in each sample was normalized to the level of GAPDH.

Quantitative determination of collagen synthesis was performed by procollagen type I C-peptide (PIP) specific enzyme immunoassay (EIA), according to manufacturer procedure (Takara Biomedicals, Takara Shuzo Co., Japan). Briefly, PIP present in the media derived from DF cultures was bound to peroxidase-labeled anti-PIP antibody, with

color development proportional to the amount of PIP. The amount of PIP was determined by measuring the absorbance in an EIA plate reader. Duplicate cultures from three normal and three SSc DF grown in 0.5% FCS DMEM, after exposure to 0.1 μM AngII with or without pretreatment with 10 μM losartan or 10 μM PD123319 were analyzed.

2.4. AngII receptor subtypes expression

The AT1 and AT2 protein expression was analyzed by Western blot. Protein from 10^6 fibroblasts or VSMC was extracted in ice-cold lysis buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 2 μg/ml of pepstatin A and 0.5 mM phenylmethylsulfonyl fluoride). The supernatant was used for electrophoresis on SDS-10% polyacrylamide gels and transferred electrophoretically to nitrocellulose filters. After blocking 2 h with 5% non-fat dried milk in Tris buffered saline containing 0.05% Tween 20 (TBST), the membranes were incubated overnight at 4 °C with 3 μg of anti-AT1 (polyclonal N-10), 3.34 μg of anti-AT2 (polyclonal C-18) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actin antibodies (clone AC-15; Sigma–Aldrich) in 5% non-fat dried milk in TBST. The filters were washed and incubated for 1 h with goat anti-rabbit IgG (for anti-AT1) or donkey anti-goat IgG (for anti-AT2) horseradish peroxidase conjugated antibodies at 1:2500 dilution (Santa Cruz Biotechnology). Bands were visualized by enhanced luminol chemiluminescence in the presence of hydrogen peroxide (SuperSignal system; Pierce, Rockford, IL).

3. Results

3.1. Proliferation of human adult fibroblasts treated with AngII

Exposure to 0.1 μM AngII for 24 h did not induce thymidine incorporation in any of the studied human fibroblast lines (Fig. 1A). As positive control, cells were exposed to 10% FCS and a significant proliferative effect was obtained in all fibroblasts lines. In contrast, a significant increase in thymidine incorporation was detected in rat VSMC stimulated with 0.1 μM AngII (Fig. 1A). Different AngII concentrations (0.1, 1 and 10 μM) under different serum conditions (0.1, 5 and 10% FCS) were analyzed and no AngII-dependent thymidine incorporation was detected under any condition (data not shown).

To analyze AngII differential effects after specific blocking of either AT1 or AT2 receptor, similar experiments were performed in DF by adding 0.1 μM AngII after pretreatment with 10 μM losartan or 10 μM PD123319. Similarly to untreated cells, no proliferative responses were detected in the presence of either inhibitor (Fig. 1B). In rat VSMC, losartan but no PD123319 inhibited thymidine incorporation induced by AngII stimulation.

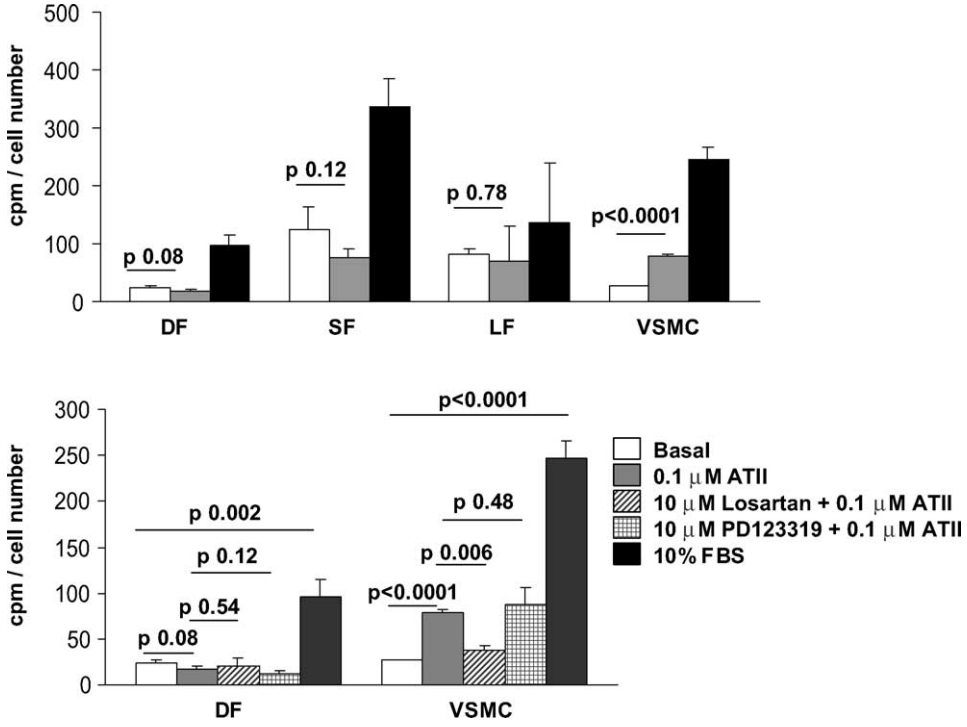

Fig. 1. Proliferation of human adult fibroblasts as determined by [^{3}H] thymidine incorporation. Mean (cpm/cell number) and standard deviation from triplicate cultures are reflected. (A) Different human fibroblasts and rat VSMC maintained under basal conditions (0.1% FCS) and exposed to 0.1 μM AngII or 10% FCS for 24 h. (B) Mean (cpm/cell number) and standard deviation from triplicate cultures of a DF and VSMC lines exposed to 0.1 μM AngII in the presence or absence of 10 μM losartan and 10 μM PD123319.

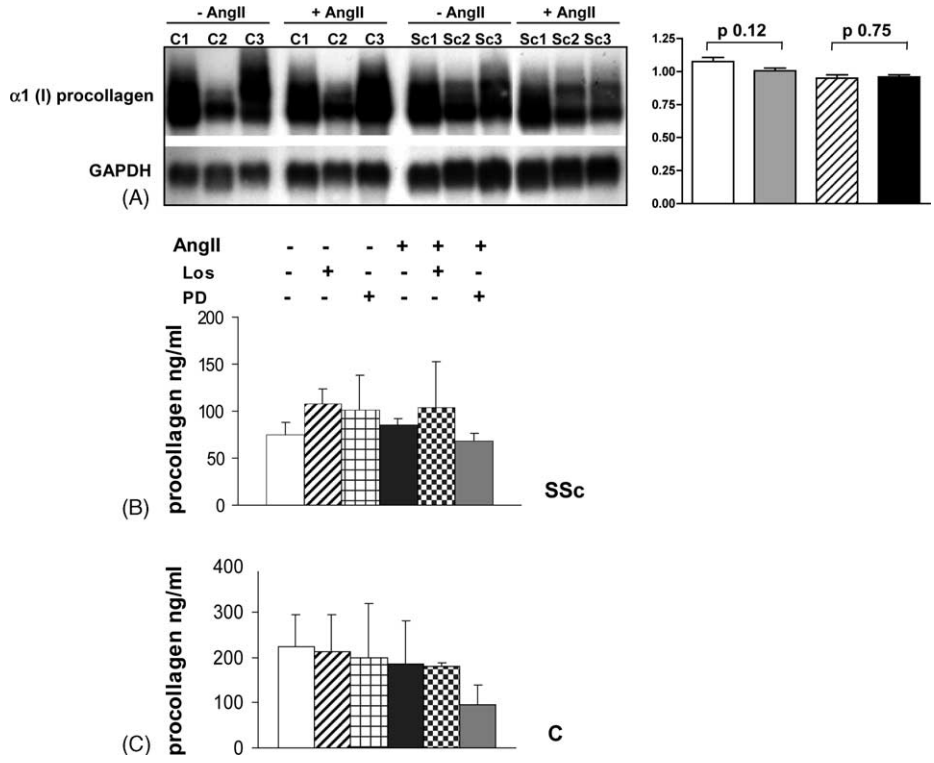

Fig. 2. Collagen expression in fibroblast cultures. (A) Procollagen α1(I) and GAPDH mRNA from three normal (N) and three SSc DF lines grown under basal conditions (10% FCS) and exposed to 0.1 μM AngII for 48 h. On the right, mean densitometric quantification in triplicate cultures corresponding to procollagen α1(I) mRNA normalized to the level of GAPDH is shown. (B) Quantitative determination of α1(I) collagen protein synthesis (ng/ml) in duplicate cultures of three normal (C) and three SSc DF.

3.2. Collagen expression by human DF treated with AngII

The expression of α1(I) procollagen mRNA in DF from three healthy controls and from three patients with skin fibrosis due to SSc was analyzed by Northern blot. Treatment with 0.1 μM AngII for 48 h did not increase α1(I) procollagen mRNA levels normalized to GAPDH neither in normal nor in SSc fibroblasts (Fig. 2A).

The α1(I) collagen protein synthesis was evaluated by PIP specific EIA in normal or SSc DF cultures. Duplicate cultures from three normal and three SSc DF were analyzed. We analyzed DF cultures under basal or 0.1 μM AngII stimulated conditions, in the presence or absence of 10 μM losartan or 10 μM PD123319. Stimulation with AngII did not induce a significant increase in collagen synthesis, and AT1 and AT2 specific inhibitors did not modify collagen synthesis under basal or AngII stimulated conditions ($p > 0.05$ for all comparisons) (Fig. 2B).

3.3. Apoptosis in DF treated with AngII

DF from healthy controls and SSc patients were grown on coverslips and, after 48 h of FCS starvation, they were exposed for 24 h to 0.1 μM AngII with or without 10 μM losartan or 10 μM PD123319. The proportion of apoptotic fibroblasts was analyzed by direct TUNEL analysis of fibroblasts fixed on coverslips. Serum starvation induced a significant increase in the proportion of apoptotic fibroblasts after 72 h ($p < 0.0001$). Treatment with 0.1 μM AngII either in the presence or absence of 10 μM losartan or 10 μM PD123319 did not modify the apoptotic response to serum deprivation nor induced apoptosis in 10% FCS cultures (Fig. 3).

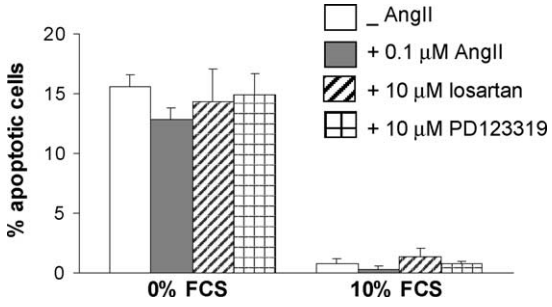

Fig. 3. Apoptotic response to serum deprivation of fibroblasts treated with AngII. The proportion of apoptotic fibroblasts was analyzed by direct TUNEL analysis of fibroblasts cultured on coverslips. Mean percent of apoptotic cells and standard deviation from triplicate cultures of DF grown in DMEM with 0% FCS or 10% FCS in the presence or absence of 0.1 μM AngII, 10 μM losartan and 10 μM PD123319 for 24 h are represented.

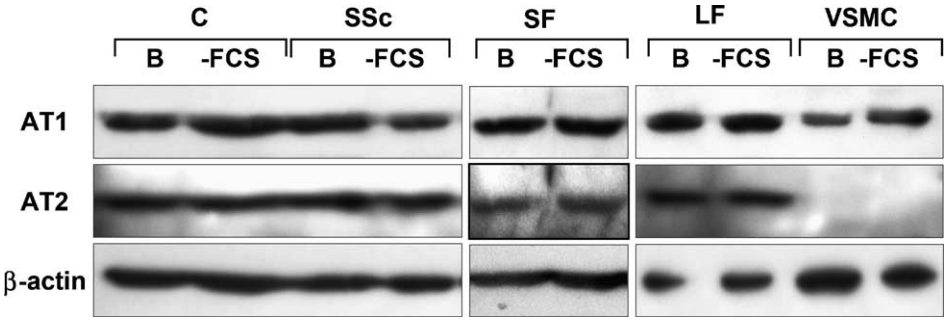

Fig. 4. AT1 and AT2 receptors protein expression in human fibroblasts by Western blot. Different human adult fibroblasts types and rat VSMC grown under 10% FCS (B) and after 48 h serum starvation (−FCS) are represented. Normal (C) and systemic sclerosis (SSc) dermal fibroblasts, synovial fibroblasts (SF), lung fibroblasts (LF) and vascular smooth muscle cells (VSMC).

3.4. AngII receptor subtypes expression

The expression of AT1 and AT2 protein was analyzed by Western blot. Anti-AT1 antibodies specifically detected a 45 kDa protein, and anti-AT2, a 40 kDa protein, consistently with previous studies [25]. All studied fibroblast types and rat VSMC expressed AT1 receptor. All fibroblast types did also express AT2, whereas it was not detectable in VSMC. No differences in AT1 or AT2 expression were detected after serum starvation after adjusting to β-actin expression (Fig. 4).

4. Discussion

Recent studies have highlighted the potential role of AngII as a cytokine-like mediator besides a vasoactive hormone. Accordingly, AngII is considered as a growth factor that mediates a variety of cellular effects involved in the pathogenesis of inflammatory and fibroproliferative cardiovascular and renal diseases. AngII has been reported to mediate proinflammatory effects and ECM synthesis in several cellular and animal models [21,23,31].

However, in vitro data regarding AngII effects on fibroblasts are controversial. Although, most reports suggest that AngII increases cell proliferation and synthesis of ECM proteins in cultured fibroblasts via AT1 receptors, different studies in human fibroblasts do not consistently demonstrate proliferation, collagen synthesis or a specific role for AT1 receptors [2,8,9,20,32]. AT2 expression has been described in renal and dermal fibroblasts and in cardiac fibroblasts obtained from failing hearts [16,29,32], but its function has not been systematically addressed. Cellular effects induced by AngII via AT2 are not well defined and, although antiproliferative and proapoptotic effects have been suggested, the effects of AT2 signaling in human fibroblasts are unclear. Expression of AT2 has been found to induce apoptosis in R3T3 fibroblasts or in different transfected cells independently from AngII stimulation [17].

Our data show that human adult fibroblasts from different lineages do not proliferate nor increase collagen synthesis in response to AngII stimulation, whereas in parallel studies in VSMC AngII induced strong proliferative responses. Endogenous AngII synthesis has been demonstrated in human fibroblasts [10], but our results using AT1 or AT2 inhibitors in non-stimulated cells also rule out a role for autocrine AngII in cell proliferation or collagen synthesis.

Our data demonstrate that all studied human fibroblast lineages coexpress AT1 and AT2 receptors at the protein level. Interestingly, rat VSMC, which display AngII mediated proliferation did only express AT1 receptor and consistently, cell proliferation was specifically blocked by losartan. This suggests that similarly to other cellular models of AT1 and AT2 receptors coexpression, AngII does not induce functional effects in human fibroblasts. In AT2 transfected cells, overexpression of AT2 reduces proliferation by counteracting AT1 receptor signaling [19,30]. In pathological conditions, such as heart failure, up-regulation of AT2 inhibits AngII-induced mitogen signals [32]. Finally, heterodimerization of the AT1 with the AT2 receptor has been described in human myometrial biopsies, where AT2 antagonizes the activation of the AT1 receptor by direct binding therefore stabilizing or inducing conformational changes in AT1 hindering its activation [1]. However, since we have only addressed the most relevant cellular effects regarding fibroproliferative diseases we cannot rule out that AngII may signal through AT1 or AT2 receptors leading to alternative cellular effects in human fibroblasts.

Cultured fibroblasts may not represent quiescent tissue fibroblasts and may be similar to activated fibroblasts during reparative or fibrotic processes. In this regard, AT2 expression has been demonstrated in mesenchymal cells, including fibroblasts, in human tissues, particularly in reparative or fibrotic processes [12,32,37]. Under these circumstances, AT2 expression may provide a mechanism of restraining their response to AngII [32], but we do not exclude that AngII may initiate fibroproliferative responses either by indirect mechanisms or by promoting cell proliferation or ECM matrix in resting tissue fibroblasts which seem to predominantly express AT1 receptor [10].

In conclusion, human fibroblasts can express both receptor types under culture conditions and this phenomenon is associated with a lack of response to AngII that may be rele-

vant to understand their behavior during reparative or fibrotic processes.

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