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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.
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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

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