Angiotensin II protects fibroblast-like synoviocytes from apoptosis via the AT1-NF-κB pathway

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Objective. To evaluate the effects of angiotensin II (Ang II) treatment on apoptosis of fibroblast-like synoviocytes (FLS) from patients with osteoarthritis (OA) and rheumatoid arthritis (RA).

Methods. AT1 receptor expression was detected by western blotting and flow cytometry. Apoptosis induction was quantified by nucleosome ELISA and by TUNEL; cell proliferation was determined by a bromodeoxyuridine (BrdU) incorporation assay. Silencing of p65 NF-κB was obtained by using a specific siRNA. Caspase 3 activation was evaluated by a colorimetric assay and its cleavage by western blotting.

Results. AT1 expression resulted comparable in FLS from OA and RA patients. Ang II pre-treatment reduced FLS apoptotic response to serum starvation and nitric oxide (NO) exposure. This protective effect was reverted in the presence of the AT1 receptor antagonist losartan as well as after silencing the expression of NF-κB. Moreover, FLS treatment with the caspase inhibitor z-VAD-fmk cancelled this Ang II effect on apoptosis. Caspase 3 activation was reduced in presence of Ang II.

Conclusions. Ang II could represent an important mediator involved in FLS expansion, reducing their capacity to undergo apoptosis, through the activation of NF-κB and the blockage of caspase cascade.

Key words: Apoptosis, Angiotensin II, Fibroblast-like synoviocytes, NF-κB, Rheumatoid arthritis, Osteoarthritis.

Introduction

Rheumatoid arthritis (RA) is a systemic, autoimmune disorder that presents as a symmetric polyarthritis associated with swelling and pain in multiple joints, affecting 1% of the population worldwide [1]. It is characterized by infiltration of inflammatory cells and by hyperplasia of the synovial lining cellular components, in particular fibroblast-like synoviocytes (FLS) [2]. FLS play a pivotal role in the pathogenesis and progression of RA. In fact, due to their uncontrolled expansion, FLS actively contribute to the destruction of the articular tissue by aggressive invasion and erosion of cartilage and bone and by production of metalloproteases, which induce progressive joint damage [3]. Although the molecular bases of this expansion are not fully understood, lack of apoptosis induction in synovial tissue of patients with RA even in presence of apoptotic stimuli, such as DNA damage, has been observed [4]. A key role is played by tumour necrosis factor-α (TNF-α), which protects FLS from nitric oxide (NO)-induced apoptosis, as we have recently shown [5]. Moreover, it has been reported that FLS cells from patients with RA are more resistant to FasL-induced apoptosis, possibly due to an increased expression of two molecules, FLIP (FLICE inhibitory protein) and sentrin-1/SUMO-1, with respect to FLS from osteoarthritis (OA) patients [4]. The expression of Bcl-2, an anti-apoptotic factor, correlates with bone erosion and inflammation scores [6]. Among the different transcription factors involved in apoptosis regulation of RA FLS, a key role is played by NF-κB, which is over expressed and over activated in the course of RA. NF-κB acts as a homo- or hetero-dimer composed by different combinations of the rel/NF-κB family members. The most frequent combination is p50/p65, which in normal conditions is localized in the cytoplasm bound to the specific inhibitor I-κB [7]. The dissociation of this complex is mediated by an increased activity of the I-κB kinases, which recognize two serine residues of I-κB. Phosphorylation of these residues determines the ubiquitination and the proteasome-dependent degradation of I-κB and the release of NF-κB, which can in turn migrate to the nucleus and bind to specific recognition sequences on the DNA [8], thus modulating the expression of the genes involved in cell cycle control, inflammation and cell death [9]. In particular, NF-κB controls the transcription of pro- and anti-apoptotic factors [10], as well as of different caspase inhibitors [11].

Angiotensin II (Ang II), a highly active octapeptide produced by angiotensin converting enzyme (ACE) mediated cleavage of angiotensin I, regulates not only blood pressure and body fluid homeostasis, but actively contributes to different inflammatory reactions. By binding to two different receptors of angiotensin on the cell surface, AT1 and AT2 receptors, Ang II regulates cell proliferation and apoptosis, activates NF-κB, NADH oxidase, and induces the production of reactive oxygen species [12]. AT1 and AT2 receptors mediate, in some instances, opposite effects. In particular, AT1 receptor has been shown to play a proliferative and anti-apoptotic effect [13], whereas AT2 receptor seems to be involved in induction of apoptosis [14]. Thus, depending on the relative concentration of its receptors in various cell types and in the presence of different stimuli, Ang II can exert opposite effects. Since it has previously been reported that ACE activity is present in vascular endothelium and in synovia, and that locally produced angiotensinogen and renin activity are increased in synovial fluid of RA patients [15], we explored the role of Ang II in cultured FLS obtained from OA and RA patients.

In our study, we have evaluated the effect of Ang II on FLS protection from apoptosis.

Materials and methods

Reagents

NO, Ang II, collagenase I, staurosporine, FITC-conjugated anti-rabbit antibody and caspase 3 detection kit were purchased from Sigma (St Louis, MO, USA); anti-AT1 and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-p65 NF-κB siRNA, anti-p65 and anti-p42 antibodies from Cell Signaling Technology (Danver, MA, USA). The unpaired siRNA was from Ambion (Austin, TX, USA), anti-caspase 3 antibodies from AbCAM (Cambridge, UK), Anti-MAPK,
Nucleosome ELISA and bromodeoxyuridine (BrdU) cell proliferation ELISA kits were from Calbiochem (San Diego, CA, USA). In situ cell death detection kit was from Roche (Lewes, UK). HRP (horseradish peroxidase) conjugated antibodies and ECL detection system were from Amersham Biosciences (Amersham, UK). TransIT-TKO transfection reagent was purchased from Mirus (Madison, WI, USA). Intrastain kit was from DAKO (Glostrup, Denmark). Losartan was purchased from Merk & Dohme.

 Patients
FLS were obtained either from hip synovial tissue of OA patients or knee synovial fluid of RA patients as previously described [16, 17]. All patients fulfilled the American College of Rheumatology (formerly, American Rheumatism Association) 1987 revised criteria for hip OA [18] and RA [19] and gave their informed consent prior to study entry. This study was approved by the Local Ethical Committee. At the time of collection of synovial tissue, one of the OA patients was receiving nimesulide, codein and paracetamol, whereas another was being treated with etoricroxib and the third one with ketoprofen. One of the RA patients was receiving leflunomide and methylprednisolone, another methotrexate and methylprednisolone, and the third leflunomide alone.

 FLS culture and treatment
Synovial tissue specimens were cut in small pieces and exposed to type I collagenase (1 mg/ml) activity for 4 h at 37°C. Cells were then harvested and suspended in DMEM addition with 10% FBS and penicillin/streptomycin. Synovial fluid was spun for 5 min at 1300 r.p.m. The cell pellet was suspended in DMEM. The day after, culture medium of both OA and RA FLS was replaced with fresh medium. All the experiments were carried on using cells between passages 3 and 6.

To evaluate the effects of Ang II, FLS were pre-treated with 100 nM Ang II for 1 h, then exposed for 24 h to serum starvation (1% FBS) or NO exposure (0.75 mM). As a positive apoptosis control, cells were treated with 1 μM staurosporine.

AT1 detection by flow cytometry
FLS were fixed, then incubated for 15 min with the anti-AT1 antibody, washed and incubated with the FITC conjugated antibody. The samples were then analysed by a Coulter EPICS XL-MCL flow cytometer.

 Apoptosis detection
Quantitative determination of apoptosis induction was performed using the nucleosome ELISA kit. This assay allows the quantification of nucleosomes released from the nucleus to the cytoplasm and results directly correlated to apoptotic cell percentage [20]. TUNEL assay was performed on FLS cultured on glass slides, fixed in 4% paraformaldehyde for 30 min and washed with phosphate buffered saline. TUNEL assay was performed using the ‘Fluorescein in situ cell death detection’ kit, following the manufacturer instruction. Samples were then analysed under a fluorescence microscope.

 Proliferation assay
The determination of the proliferation rate was performed by using the BrdU cell proliferation assay.

FLS transfection and NF-κB silencing
To silence NF-κB expression in FLS, we plated 5 × 10⁴ cells in a 12-well plate. Twenty-four hours later, FLS were transfected by using the TransIT-TKO transfection reagent either with the anti-p65 NF-κB siRNA or an unpaired siRNA. Silencing was detected by semi-quantitative western blot.

 Cell lysis and western blotting
Western blots were performed as previously described [5]. The stripping procedure was performed by incubating the membrane in a 0.2 M glycine solution (pH = 2.5) for 30 min at 60°C and for 1 h at RT. All the western blot assays were performed twice.

 Caspase 3 activity assay
Caspase 3 activity was evaluated by caspase 3 assay kit following the manufacturer instruction.

 Semi-quantitative analysis
For semi-quantitative studies, the intensity of bands detected by western blotting was measured by densitometry using X-ray films exposed to ECL reagents and the Scion Images software.

 Statistical analysis
Results are presented as mean ± s.d. Each test was performed in duplicate and repeated twice. Wilcoxon test for unpaired data has been used for statistical analyses using SPSS 13.0 for windows (SPSS, Chicago).

Results
AT1 receptor is expressed on FLS surface
Since it has been reported that ACE is expressed in synovia obtained from OA and RA patients (15), we decided to explore the possibility that another component of the renin-angiotensin system, the Ang II receptor AT1, might be expressed on FLS. By means of western blotting, we evaluated the expression of AT1 on cultured FLS obtained from three patients with OA and RA and we found that FLS from both OA and RA patients expressed AT1 (Fig. 1A). The densitometric analysis of the obtained signals showed no difference between the expressions of AT1 on OA FLS compared with RA FLS. In fact, the mean ratio between the densitometric values obtained for the AT1 receptor band and that of β-actin bands was 1.17 ± 0.40 (mean ± s.d.) for OA and 1.38 ± 0.58 for RA. Likewise, flow cytometric evaluation showed no difference in the percentage of AT1 expressing FLS from OA and RA patients (96.07 ± 0.01 and 94.23 ± 0.02, respectively).

Ang II protects FLS from apoptosis
Through its binding to AT1 and AT2 receptors, Ang II can either induce or suppress apoptosis depending on the cell type studied [21, 22]. We evaluated the effects of Ang II on apoptosis of OA and RA FLS. As a positive control of apoptosis induction, we used FLS treated with 1 μM staurosporine for 8 h, whereas non-treated cells served as a negative control. Apoptosis induction was preceded by a 1 h-treatment with Ang II or water alone and was detected by two different methods, Nucleosome ELISA and TUNEL assay. Data obtained from this assay showed a significantly different induction of apoptosis in cells pre-treated with Ang II with respect to non-treated cells. In fact, the values (mean ± s.d.) obtained for serum-deprived OA samples were 0.48 ± 0.09 and 0.30 ± 0.11 in the absence or presence of Ang II pre-treatment, respectively, whereas for NO exposure the values were 0.80 ± 0.20 and 0.44 ± 0.18. In RA samples, the absorbance values were 0.37 ± 0.09 and 0.15 ± 0.02 for starvation and 0.32 ± 0.09 and 0.18 ± 0.04 for NO treatment, respectively (Fig. 2A). The value obtained for the positive control was 0.95 ± 0.05, whereas for non-treated cells the obtained value was 0.03 ± 0.01. TUNEL assay confirmed the results obtained by nucleosome ELISA (Fig. 2B).
We next investigated the possibility that this effect on apoptosis might be related to cell proliferation changes due to Ang II effects. To this purpose, we assayed BrdU incorporation rate by an ELISA. No statistically significant difference was observed between samples pre-treated with water and with Ang II (Fig. 2E). A lower BrdU incorporation has been detected in RA FLS with respect to OA FLS, in agreement with previous observations achieved during expansion passages.

**Ang II anti-apoptotic effect is reverted by AT1 inhibition**

Losartan is a specific AT1 receptor antagonist belonging to the Ang II receptor blockers family. To evaluate the role of this receptor on Ang II-mediated protection, OA and RA FLS were seeded and pre-treated with 3\( \mu \)M losartan for 1 h, then Ang II was added to the culture medium. One hour later, the apoptotic stimuli were added. Losartan pre-treatment reverted Ang II anti-apoptotic effects. In fact, the values obtained for OA FLS serum deprived and NO-treated, either in the absence or in the presence of Ang II, were 0.52 ± 0.08 and 0.63 ± 0.02, 0.36 ± 0.01 and 0.56 ± 0.01, respectively. For RA cells, the obtained values were 0.20 ± 0.01 and 0.23 ± 0.01; 0.17 ± 0.01 and 0.17 ± 0.01 (Fig. 3). Positive and negative controls were included in the experiment. No other toxicity was detected by morphological observation.

**Ang II protective effect is dependent on NF-κB**

We decided to explore the involvement of p65 NF-κB in Ang II mediated protection from apoptosis. NF-κB transient silencing was achieved by transfecting FLS with cationic liposome carrying a specific double strand siRNA molecule targeting the p65 subunit of NF-κB. This strategy allowed a reduction in p65 NF-κB expression >80% in both OA and RA FLS in 48 h, as observed by western blot (Fig. 4A). To determine the role of NF-κB in Ang II anti-apoptotic effect, cells were transfected with the siRNA and 48 h later, exposed first to Ang II for 1 h and subsequently to the apoptotic stimuli. NF-κB silencing cancelled the protective effect of Ang II on apoptosis, as observed by ELISA. In transfected cells, the values obtained for OA FLS were 0.21 ± 0.06 (in absence of Ang II) and 0.21 ± 0.05 (in presence of Ang II) in cells FBS deprived, and 0.19 ± 0.04 and 0.20 ± 0.04 for NO-treated FLS (in absence or presence of Ang II, respectively); for RA FLS the values were 0.11 ± 0.01 and 0.13 ± 0.01, and 0.09 ± 0.01 and 0.11 ± 0.01 (Fig. 4B). When cells were transfected with the
scrambled siRNA, no differences were observed with respect to non-transfected cells (Fig. 4C). Since NF-κB can affect cell proliferation, we tested the possibility that the effect of NF-κB silencing in FLS pre-treated or not with Ang II could be due to a change in cell proliferation rate by a BrdU incorporation assay. The differences were not statistically significant \((P > 0.05)\) for any treatment (data not shown). Staurosporine was used as a positive control and the negative control (no treatment) was included in the experiment.

**Ang II activated pathway in FLS is dependent on caspase activation**

To determine whether Ang II effect is mediated by caspase 3, the caspase inhibitor z-VAD-fmk (5 μM) was used. Ang II addition to z-VAD-fmk-treated OA FLS determined an increase in nucleosome release, thus in absorbance values from \(0.29 \pm 0.01\) to \(0.44 \pm 0.04\) and \(0.39 \pm 0.01\) to \(0.48 \pm 0.02\) (starved and NO-exposed cells), whereas for RA FLS the values were \(0.18 \pm 0.01\) and \(0.21 \pm 0.01\), and \(0.17 \pm 0.01\) and \(0.20 \pm 0.01\) (Fig. 5A). Positive and negative controls were included in the experiment. Caspase 3 activity was then measured by using a colorimetric assay. Ang II pre-treatment of FLS reduced the absorbance values (Fig. 5B). To confirm these data, we performed a semi-quantitative western blot assay, using an antibody raised against the pro-active form of caspase 3 and an anti-MAPK antibody as a loading control. Ang II treatment determined a decrease in caspase 3 activation in both OA and RA FLS cells serum deprived and NO-treated (Fig. 5C).

**Discussion**

In this study, we evaluated the effect of the octapeptide Ang II on FLS apoptosis. It has previously been reported [23] that ACE and renin concentrations are increased in synovial fluids obtained from RA patients with respect to OA patients. This difference has not been observed in the serum, supporting the hypothesis that the two enzymes are locally produced. Moreover, captopril, a thiolic ACE inhibitor sharing a structural similarity with d-penicillamine, has been shown to exert a beneficial effect on symptoms and signs of RA in a small open study [24]. This effect has been attributed to the thiolic function rather than its enzyme inhibiting activity [25]. In contrast with these findings, Dalbeth and colleagues reported [26] that the non-thiol ACE inhibitor quinapril as well as the AT1 receptor inhibitor candesartan suppress the severity of CIA (collagen induced arthritis). Interestingly, they observed that paw TNF-α concentration was reduced in mice receiving quinapril compared with those receiving water and that the decreased TNF-α levels were not a consequence of the suppressed disease activity. These results may suggest a direct influence on Ang II activated pathways in RA.

All these findings, together with our observation that FLS in culture express AT1 receptor, prompted us to explore the effects of Ang II exposure on the FLS capacity to undergo apoptosis. We chose two different apoptotic stimuli, serum starvation and NO treatment, mimicking the in vivo conditions of RA joints. In fact, although new blood vessels are produced, the neovascular
network is dysfunctional [27]; this leads to a limitation in available nourishment and oxygen. Moreover, NO concentration is increased in RA synovial fluid [28]. These two conditions represent apoptotic stimuli for FLS in vitro; nevertheless this cellular compartment undergoes an in vivo uncontrolled expansion.

To our knowledge, this is the first study in the literature demonstrating that Ang II protects FLS from apoptosis. We demonstrated that Ang II protects or at least delays FLS apoptosis by two different methods, one detecting nucleosome release from the nucleus to the cytoplasm, the other the presence of single strand-breaks of DNA, two characteristics of apoptosis. We showed that the two different assays lead to comparable results, thus we performed only the ELISA in the subsequent experiments.

We observed that Ang II effect is comparable for FLS from OA and RA patients; it thus seems to be fibroblast-specific rather than disease-specific. This finding is in agreement with the fact that the levels of AT1 are comparable in RA and OA FLS. However, we cannot exclude that the different environment in RA joints can determine a variation in Ang II effects between the two pathologies. In fact, it has been shown that ACE levels are higher in RA synovia with respect to OA. Thus it is conceivable that Ang II levels are more elevated in RA synovia.

OA FLS resulted more sensitive to NO than RA FLS both in the presence and in the absence of Ang II pre-treatment. The same effect, although less evident, has been observed for serum starvation. This difference could be due to a decreased rate of cell proliferation in RA FLS that may be attributable to a lack of proliferative signals in vivo, such as high concentrations of TNF-α, IL-1β and IL-6.

The BrdU assay showed that Ang II treatment does not affect cell proliferation. Thus, the decreased apoptosis induction after a pre-treatment with Ang II is not due to a modified rate of cell proliferation but to the activation of pro-survival pathways mediated by Ang II.

It has been reported that Ang II modulates NO-induced apoptosis in a cardiac fibroblast model by acting on AT1 receptor [29]. Based on this finding, we decided to evaluate the effect of the selective AT1 receptor antagonist losartan on FLS. We observed that losartan at the concentration reported in the paper published by Tian and coworkers [29] was able to revert the protective role of Ang II on FLS. This effect is more evident in OA FLS than in RA FLS. Ang II has been now recognized as a pro-inflammatory molecule, mainly for its receptor-dependant activation of NF-κB [30], a transcription factor involved in several inflammatory processes. NF-κB is overactivated in RA FLS where it plays a role in cell proliferation [31], apoptosis resistance [32] and production of inflammatory cytokines and metallo-proteases [33]. Moreover, NF-κB up-modulates inducible NO synthase and COX2, which have a pivotal role in inflammatory diseases. In our study, we silenced NF-κB expression with a siRNA strategy. Nowadays, siRNA technology is extensively used for functional studies, since it allows a specific inhibition of the mRNA transduction by inducing mRNA cleavage [34]. This method is highly specific and does not induce toxic effects, a feature particularly relevant to apoptosis studies. In particular, we targeted the p65 subunit of NF-κB, since this is part of the dimer p50/p65 most commonly involved in the regulation of transcription of inflammation-related molecules [35]. Using a specific RNA inhibitor, we obtained a high rate of silencing. Switching off this molecule reverted the anti-apoptotic effect of Ang II. Other transcription factors could be involved in Ang II mediated protection of FLS, but we chose to evaluate the effect of NF-κB since it has been extensively studied and its involvement has been demonstrated in RA development.

We then investigated the activation of caspase 3, one of the major executors of the apoptotic pathway, to which different apoptotic cascades converge. In the presence of the caspase inhibitor z-VAD-fmk, the protective effect of Ang II is abrogated. Since z-VAD-fmk can inhibit not only caspase 3, but also caspases 6 and 7, even if to a smaller extent, we verified that caspase 3 activation was indeed reduced by pre-treating cells with Ang II by using two different methods. The colorimetric assay allowed us to verify the state of activation of caspases; even if it is designed to specifically test caspase 3 activity, other caspases could contribute in part to the substrate cleavage. Thus, by means of western blotting, we observed that the levels of the inactive form of caspase 3 are higher in cells pre-treated with Ang II and that the active form is decreased in these cells. The results obtained using these three assays together suggest a strong implication of caspase 3 in this process, although we cannot exclude that Ang II may reduce the activation of other executioner caspases, nor can we exclude other mechanisms of cell death.

Based on our results, we propose that Ang II plays a role in FLS expansion in the course of RA, via its inhibitory effects on apoptosis. Moreover, we suggest a mechanism of action through which Ang II could protect FLS from apoptosis. The first step would be Ang II binding to its receptor AT1, followed by NF-κB activation and as a consequence inhibition of caspase 3 activation. However, other studies are required to better understand the molecular link between NF-κB and caspase 3. It has been observed that NF-κB regulates the transcription of the caspase inhibitors belonging to the IAP (inhibitors of apoptosis) family. IAPs represent thus a possible link between NF-κB and caspases.

Sagawa and colleagues [36] reported that the AT1 and AT2 receptor antagonist olmesartan ameliorates arthritis scores in a murine CIA model. This effect has been attributed solely to the effect of the drug on Th1 cells, particularly on their proliferation and cytokine production. Ikuni and coworkers [37] identified in MCP-1/CCL-2α a possible mediator, since its levels are induced by Ang II exposure; this effect is reverted by olmesartan. In contrast, we would like to suggest that the improvement obtained in this model of murine RA by inhibiting Ang II receptors might be due not uniquely to the effect on leukocytes, as proposed, but also on FLS.

Resistance to apoptosis induction by different stimuli is a characteristic feature of RA FLS, leading to their abnormal proliferation, cartilage and bone invasion and overproduction of metalloproteases. All these mechanisms contribute to the severity of RA synovitis. Nevertheless, the mediators involved in apoptosis resistance have not been yet fully determined. Since levels of ACE, the enzyme responsible for Ang II production, are more elevated in RA than in OA synovia, Ang II could be a candidate molecule taking part to this process, possibly by interacting with other molecular pathways. Ang II activated cascade could thus represent a potential target for RA therapy, particularly the AT1 receptor.

The traditional cardiovascular risk factors in general and hypertension in particular are more frequent in patients with RA [38]. Ang II can influence directly (hypertension) and indirectly (inflammation and insulin-resistance) the cardiovascular risk factors [39].

Our data, together with these previously reported studies, may provide support for considering the use of Ang II activated pathway as a possible target for RA therapy.

**Rheumatology key message**

- Ang II decreases FLS response to apoptotic stimuli. This effect requires AT1 and NF-κB activation, leading to caspase 3 inhibition. Ang II could be a mediator involved in FLS hyperplasia.
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