Effects of pro- and anti-inflammatory cytokines and nitric oxide donors on hyaluronic acid synthesis by synovial cells from patients with rheumatoid arthritis

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ABSTRACT

The aim of the present study was to investigate the effects of (i) the pro-inflammatory cytokines IL (interleukin)-1β, TNF-α (tumour necrosis factor-α), IFN-γ (interferon-γ) and anti-inflammatory cytokines IL-4 and IL-13, and (ii) NO (nitric oxide) donors on HA (hyaluronic acid) production by synovial cells from patients with rheumatoid arthritis. Synovial cells obtained from five patients with rheumatoid arthritis were incubated for 24 h without or with IL-1β, TNF-α, IFN-γ, or with this mixture for 24 h plus IL-4 or IL-13 for the last 6 h. The same cells were also incubated for 3–24 h without or with SNP (sodium nitroprusside) or SNAP (S-nitroso-N-acetyl-DL-penicillamine). HA secretion was determined by an immunoenzymic assay based on HA-specific binding by proteoglycan isolated from bovine cartilage. IL-1β, TNF-α and IFN-γ alone or in combination stimulated HA synthesis, whereas IL-4 and IL-13 dose-dependently inhibited HA production induced by Th1 cytokines. HA production was significantly increased by the presence of 1 mM SNP after 6 and 12 h (maximal effect). HA production was significantly increased by the presence of 0.01 and 0.1 mM SNAP after 12 h of incubation, and cells treated with 1 mM SNAP showed a maximal HA production after 24 h of incubation. In conclusion, the present study provides data concerning the regulatory role of pro- and anti-inflammatory cytokines and NO donors on HA metabolism in rheumatoid synovial cells and may help in understanding the pathophysiology of rheumatoid arthritis.

INTRODUCTION

RA (rheumatoid arthritis) is an autoimmune disease involving hyperproliferation of the synovial membrane and the accumulation of activated T-cells and macrophages that lead to progressive joint destruction. Synovial cells play an important role in the pathogenesis of this joint destruction [1]. IL (interleukin)-1β and TNF-α (tumour necrosis factor-α), pro-inflammatory cytokines present in large quantities in RA synovium, are potent stimulators of synovial tissue effector functions [2]; these inflammatory cytokines are more abundant than Th2 cytokines such as IL-4 and IL-13 in RA synovium. IL-4 can suppress the detrimental effects of IL-1 and

Key words: anti-inflammatory cytokine, hyaluronic acid, interleukin, nitric oxide donor, pro-inflammatory cytokine, rheumatoid arthritis.

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; HA, hyaluronic acid; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor antagonist; NO, nitric oxide; PG, proteoglycan; PNPP, p-nitrophenyl phosphate; RA, rheumatoid arthritis; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-DL-penicillamine; SNP, sodium nitroprusside; TNF-α, tumour necrosis factor-α.

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TNF-α by stimulating IL-1ra (IL-1 receptor antagonist) production [3], and down-regulating the TNF receptor, transcription of metalloproteinase genes and synovial cell proliferation [4–6]. Several studies also suggest that IFN-γ (interferon-γ), an essential mediator of Th1-type immune response, may be involved in the pathogenesis of RA [7,8]. In addition to this inflammatory process, RA synovium is exposed to oxidative [9] and nitrosative [10] stress. Indeed, ROS (reactive oxygen species) produced by leucocytes and synovial cells are responsible for collagen hydrolysis and increased expression of metalloproteinases, leading to the degradation of the extracellular matrix [11]. NO (nitric oxide) plays an important role in the catabolic cascade of biochemical events that lead to arthritis [12]: for example, it is synthesized by synovial cells and monocytes [13] and can also activate metalloproteinase [14]; NO has also been described as a mediator of apoptosis in the rheumatoid joint [15].

HA (hyaluronic acid) is a high-molecular-mass extracellular polysaccharide that contributes to synovial fluid viscosity, principally because of its lubricant properties. High quantities of HA are found in the synovial fluid (1400–3600 mg/l) [16]. Cultured synovial fibroblasts produce HA, and immunohistochemical studies of normal synovium show that HA surrounds the lining layer cells, but that it is much less abundant in deeper layers [17]. This suggests that one of the major functions of fibroblastic synovial lining cells is to produce, release and maintain constitutive concentrations of HA in the synovial fluid. HA contributes to the regulation of cell adhesion, migration and proliferation through its specific receptors, including CD44 [18], RHAMM (receptor for HA-mediated motility) [19] and LYVE-1 (lymphatic vessel endothelial HA receptor-1) [20].

The joint synovium of patients with RA contains considerable amounts of various CD44 isoforms as well as its specific ligand HA [21]. However, the regulation of HA production remains incompletely explained. Despite the overproduction of HA, RA joints show hypoviscosity [22]. Synovial cells are possibly subject to substantial inflammation and nitrosative stress that modifies HA metabolism.

The aim of the present study is to elucidate whether HA production by human synovial cells is regulated by IL-1β, TNF-α, IFN-γ, and subsequently IL-4 and IL-13, and how it is affected by NO donors.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco’s modified Eagle’s medium), FCS (fetal calf serum), trypsin and PBS were obtained from Eurobio (Les Ulis, France). Clostridium histolyticum type Ia collagenase, SNP (sodium nitroprusside), SNAP (5-nitroso-N-acetyl-DL-penicillamine), HA (potassium salt) from human umbilical cord, extravidin®-alkaline phosphatase conjugate, PNPP (p-nitrophenyl phosphate), Tris buffer and BSA were purchased from Sigma (St Louis, MO, U.S.A.). All recombinant human cytokines were obtained from R&D Systems (Abingdon, Oxon., U.K.). PG (proteoglycan) aggregate from bovine nasal cartilage and murine monoclonal anti-(keratan sulphate) antibody were purchased from ICN biomedicals (Aurora, OH, U.S.A.). Secondary biotinylated ovine polyclonal antibody anti-mouse IgG was obtained from Amersham Biosciences (Orsay, France).

Patients

Five patients who fulfilled the criteria of the American College of Rheumatology [23] (three women, two men; mean age, 63 years; range, 59–69 years; mean disease duration, 12 years; range, 11–15 years) were included in the study. At the time of the study, all patients were under treatment with non-steroidal anti-inflammatory drugs. Two of the RA patients were treated with azathioprine and two with methotrexate. A surgical synovectomy was performed for all patients. All patients gave written informed consent.

Synovial cell culture

Human synovial cells were isolated from surgical samples of synovectomies. The superficial layer of synovium was dissected and digested with collagenase and trypsin as described previously [24]. Cells were suspended in DMEM containing 10 % FCS, penicillin, streptomycin and amphotericin B, and were plated at a density of 5 × 10⁶ cells/75 cm² culture flask. Cells were cultured until confluence in DMEM supplemented with 10 % FCS at 37°C in an atmosphere containing 5 % CO₂. The medium was changed every 3 days. At confluence, the cells were passaged using trypsin. Cells were suspended in DMEM supplemented with 10 % FCS (7 × 10⁴ cells/ml) and placed in 12-well plates (1 ml/well) at 37°C (5 % CO₂). Experiments were performed with subconfluent cultures at first passage.

Synovial cell treatment

The growth medium of the synovial cell cultures was replaced with fresh medium without FCS. Cells were incubated for 24 h without or with IL-1β (0.25–1 ng/ml), TNF-α (0.125–0.5 ng/ml), or IFN-γ (2.5 × 10⁻¹–1 × 10⁶ units/ml) alone or in combination. In other experiments, cells were stimulated first with a combination of IL-1β (1 ng/ml), TNF-α (0.5 ng/ml) and IFN-γ (1 × 10⁶ units/ml) for 18 h, and then IL-4 (5 ng/ml) or IL-13 (5 ng/ml) were introduced into the medium for a further 6 h. In another experiment, cells were incubated for 6 and 12 h in the absence or presence of SNP (0.01–1 mM).
or SNAP (0.01–1 mM). Other cells were incubated with 1 mM SNP or 1 mM SNAP for 0, 3, 6 and 12 h.

**HA measurements**

HA concentrations in cultured cell supernatants were determined by an immunoenzymic assay based on the specific HA binding of PG isolated from bovine cartilage, according to the method of Kongtawelert and Ghosh [25]: the binding of PG to HA-coated microplates is inhibited by HA in proportion to the HA concentration in the solution assayed. Briefly, 100 µl of supernatant or HA solution (0–1000 µg/l in PBS) was incubated with 100 µl of a 10 mg/l PG solution (in PBS) for 90 min at 37°C in BSA-coated wells of a microplate [prepared previously by adding 200 µl of a 5% (w/v) BSA solution to each well and incubating for 1 h at room temperature]. The mixture was then transferred to the wells of a HA-coated microplate [prepared previously by adding 200 µl of a 100 mg/l HA solution to each well, incubating overnight, washing and then treating with 200 µl of 5% (w/v) BSA solution/well for 1 h at room temperature] and incubated for 90 min at 37°C. The monoclonal anti-keratan sulphate) antibody (1:2000 dilution in PBS) was added to the wells for 1 h at room temperature with gentle agitation. The wells were washed thoroughly and the secondary anti-mouse antibody (1:500 dilution in 1 M Tris/HCl buffer pH 10) was added to the wells for 1 h at room temperature. The mixture was then transferred to the wells of a microplate prepared previously by adding 200 µl of 1 M NaOH, and the absorbance was read at 405 nm on a Dynatech MR5000 spectrophotometer. Results are expressed in µg/l. Each measure was duplicated. Coefficients of variation were less than 10%.

**Statistical analysis**

Results are expressed as means ± S.E.M. The Mann–Whitney *U* test was used for comparisons. *P* values < 0.05 were considered statistically significant. The Statview statistical program (Abacus Concepts, Berkeley, CA, U.S.A.) was used.

**RESULTS**

**Effects of pro-inflammatory cytokines on HA synthesis**

Cells were treated for 24 h with various concentrations of IL-1β, TNF-α or IFN-γ. The highest concentration of each of these cytokines (1 ng/ml, 0.5 ng/ml and 1 × 10⁴ units/ml respectively) significantly increased the production of HA (Figure 1). The largest increase in HA production was with TNF-α. At lower concentrations, the cytokines used alone did not increase HA production significantly.

The combination of two cytokines at high concentrations (1 ng/ml of IL-1β, 0.5 ng/ml of TNF-α and 1 × 10⁴ units/ml of IFN-γ) did not have any greater effect than the cytokines alone, that is their effects were not additive. HA production after stimulation with IL-1β + TNF-α, IL-1β + IFN-γ or TNF-α + IFN-γ was 283±16, 496±98 and 605±283 µg/l respectively.

Cells were treated for 24 h with various concentrations of all three cytokines (IL-1β + TNF-α + IFN-γ). The increase in HA production was significant for the combination of 0.5 ng/ml IL-1β, 0.25 ng/ml TNF-α and 5 × 10³ units/ml IFN-γ (Figure 2). HA production was not substantially greater when cells were treated with higher concentrations.
Antagonist effects of Th2 cytokines on HA synthesis

As the Th2 cytokines IL-4 and IL-13 can have opposite effects to those of Th1 cytokines, we studied the action of these two cytokines on HA production. Cells were incubated with IL-1β (1 ng/ml), TNF-α (0.5 ng/ml) and IFN-γ (1 × 10^4 units/ml) for 18 h alone and then with this mixture and with IL-4 or IL-13 at the final concentrations indicated. Results are expressed as means ± S.E.M. * P < 0.01 and ** P < 0.001 compared with cells treated with the cytokine mixture only.

Dose-dependent effects of NO donors on HA synthesis

Cells were treated with various concentrations (0.01–1 mM) of SNP or SNAP for 6 or 12 h. After 6 h of incubation, HA production was significantly greater in the presence of 1 mM SNP than in untreated cells (Figure 4A); after 12 h of incubation, HA concentrations were significantly higher in the presence of 0.01, 0.1 and 1 mM SNP than in untreated cells (Figure 4A).

After incubation with SNAP for 6 h, HA production was higher at the lowest concentration of SNAP than in controls, but no dose-dependent effect was observed (Figure 4B). After 12 h of incubation, HA concentrations were significantly higher in the presence of 0.01, 0.1 and 1 mM SNAP than in untreated cells (Figure 4B).

Time-dependent effects of NO donors on HA synthesis

Cells were treated with 1 mM SNP or 1 mM SNAP for 0, 3, 6 and 12 h (Figure 5). Treated cells showed the greatest HA production after 12 h of incubation for both SNP and SNAP.

DISCUSSION

The present study demonstrates that (i) human RA synovial cells produce increased amounts of HA after stimulation with the pro-inflammatory cytokines IL-1β (1 ng/ml), TNF-α (0.5 ng/ml) and IFN-γ (1 × 10^4 units/ml) and this production is inhibited by IL-4 and IL-13, and (ii) NO donors increase HA production by synovial cells. There has been little work addressing
HA synthesis by synovial cells. Haubeck et al. [26] and Kawakami et al. [27] investigated lining cells and found that HA production was higher following IL-1β than TNF-α treatment. In the present study, we used human synovial cells and also studied the effects of IFN-γ and NO donors in an attempt to reproduce the conditions in vivo: the effect of TNF-α was larger than that of IL-1β. This may be because TNF-α and IL-1β do not act through the same pathway [28]. There was no evident additive effect of the combination of two cytokines (IL-1β + TNF-α, IL-1β + IFN-γ or TNF-α + IFN-γ), but the combination of IL-1β, TNF-α and IFN-γ, corresponding to the conditions of stimulation in vivo, stimulated HA production substantially. A similar effect has been described in other metabolic pathways such as the regulation of NO production reported by Grabowski et al. [13]. The maximal effect was with the following cytokine concentrations: 0.5 ng/ml IL-1β, 0.25 ng/ml TNF-α and 5 × 105 units/ml IFN-γ, i.e. at concentrations similar to those observed in RA [29]. Our present study thus demonstrates that HA production is up-regulated by the major pro-inflammatory cytokines present in RA.

Despite the excessive production of IL-1β and TNF-α in RA synovium, Th2 cytokines, in particular IL-4 [30] and IL-13 [31], have also been described in RA synovium and articular fluids. However, these Th2 cytokines are produced in very low quantities, leading to an imbalance between Th1 and Th2 cytokines [32]. In the present study, we have shown that IL-4 and IL-13 at pharmacological concentrations inhibit HA production by RA synovial cells. IL-4 and IL-13 have the same anti-inflammatory properties [33], although they do not act through the same receptor on synovial cells [34]. IL-4 or IL-13 may possibly increase expression of IL-1ra, the competitive IL-1 inhibitor, by synovial cells [35]. This would be consistent with our present results showing that the combination of these two cytokines had no additive effect on HA production in that, when IL-1ra inhibits the effects of IL-1β, cells are only stimulated by TNF-α and IFN-γ.

RA synovium is highly exposed to nitrosative stress, and NO can activate metalloproteinases [10,14]. However, although HA is a major constituent of the extracellular matrix, the effects of NO on HA metabolism have not been studied previously, and we considered it would be valuable to assess the effects of NO on HA metabolism. We studied NO production by two different donors to assess the effects of this radical in our in vitro cellular model. We used SNP, an NO donor, after exposure to light or thiols, and SNAP, a spontaneous NO donor, at concentrations that reflect the high production of NO observed in pannus [36,37]. Both SNP (1 mM) and SNAP (1 mM) increased HA production by RA synovial cells after 6–12 h of incubation. SNP may generate nitrosoy ions that are more stable over time [38]. ROS can generate HA fragmentation [39,40] and NO products can degrade chondroitin sulphate in vitro [41]. Our findings thus provide new information about the effects of nitrosative stress in RA.

In conclusion, we assessed the regulatory role of pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ) and anti-inflammatory cytokines (IL-4 and IL-13), independently and in combination, and NO donors (SNP and SNAP) on HA production by RA synovial cells. We report data that will help our understanding of HA metabolism in these cells. IL-1 potentiates the synthesis of low-molecular-mass HA [42], and thus it is possible that IL-1-β and TNF-α induce NO production by synovial cells, leading to the post-synthesis degradation of high-molecular-mass HA into smaller fragments.

REFERENCES


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