Increased Expression of Arginase II in Patients with Different Forms of Arthritis. Implications of the Regulation of Nitric Oxide

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ABSTRACT. ***Objective.*** To investigate the expression of arginase isoforms in patients with different forms of arthritis and the possible implications of the synthesis of nitric oxide (NO).

Methods. Arginase activity was measured in synovial fluid (SF) cells from patients with different forms of arthritis, either directly or after *in vitro* stimulation with cytokines. The identity of the isoform expressed was confirmed by reverse transcription polymerase chain reaction. We measured both arginase activity and NO production in SF macrophages and synovial membrane fibroblasts from patients with rheumatoid arthritis (RA).

Results. Arginase II was the isoform expressed in SF cells. In SF macrophages, dibutyryl-cAMP (dBt-cAMP), prostaglandin E_2 (PGE_2), and lipopolysaccharide (LPS) further increased the enzyme activity, while NO production was not detected even in the presence of Th1-like cytokines. In contrast, synovial membrane fibroblasts from patients with RA released NO into the culture media. Moreover, dBt-cAMP, PGE_2, and transforming growth factor-ß, which induced arginase II, reduced the levels of NO. Reciprocally, the induction of NO by Th1 cytokines inhibited arginase activity levels.

Conclusion. Arginase II expression is upregulated in RA and may increase cell proliferation by providing L-ornithine, which is the substrate of polyamine biosynthesis. In cells where both arginase II and inducible NO synthase activity occurs, there is a reciprocal regulation, suggesting that agents that induce arginase II in synovial cells could downregulate the levels of NO and divert L-arginine metabolism toward cell proliferation and/or tissue regeneration. (J Rheumatol 2002;29:2261–5)

Key Indexing Terms:
ARGINASE II
ARTHRITIS
INDUCIBLE NITRIC OXIDE SYNTHASE
HUMAN
CYTOKINES

Inflammatory arthritis is a disease of the joints characterized by chronic inflammation of the synovial tissue. The synovium is infiltrated by antibody-producing plasma cells, T cells, macrophages, and dendritic cells that together with resident fibroblasts produce cytokines such as interleukins (IL) 1, 4, 6, 8, 10, and 15, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-ß. This constitutes an invasive tissue capable of eroding cartilage and bone, leading to joint destruction[1].

One of the important proinflammatory mediators in the rheumatoid synovium is nitric oxide (NO)[2]. This radical, generated from arginine by the NO synthases (NOS), has been identified in different models of arthritis in animals, including adjuvant arthritis in the rat[3]. The first evidence of the involvement of NO in patients with arthritis came from Farrell, *et al*[4], who showed increased concentrations of this mediator in serum and synovial fluid (SF) from these patients. Further, the inducible form of NO synthase (iNOS) has been identified in human rheumatoid synovium by several groups, and it is now clear that NO is involved in the pathology of inflammatory arthritis[5].

The enzyme arginase catalyzes the hydrolysis of arginine to ornithine and urea and has been reported to modulate the levels of NO by substrate competition, because arginase and NOS share arginine as a substrate. At least 2 isoforms of mammalian arginase (EC 3.5.3.1) exist (types I and II); these differ in physiological function, tissue distribution, and subcellular localization[6]. Type I occurs in the cytosol and is found only in the liver, whereas type II is located in the mitochondria and is more widely distributed[7]. Both arginases can be induced by lipopolysaccharide (LPS) and by cAMP analogs, while only arginase I is known to be specifically induced by the Th2 cytokines IL-4 and IL-10 in mouse cells[8,9] and also possibly in human mononuclear cells after trauma[10]. In mouse macrophages, we have reported that arginase I and iNOS are differentially regulated by

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Th1/Th2 cytokines; the induction of arginase I by IL-4 and IL-10 could be inhibited by interferon (IFN)-γ and reciprocally, Th2 cytokines inhibited IFN-γ induced iNOS[11]. In human tissue, the regulation of arginase isoforms was until now poorly studied. In the past 2 years, however, an increased interest in the detection of human arginases has highlighted the relevance of L-arginine metabolic pathways in the regulation of several pathologies such as trauma[10], human colorectal cancer[12], diabetic cavernosal tissue[13], or glomerulonephritis[14].

With this premise, we investigated arginase isoforms in human inflammatory arthritis to study the regulation of arginase and iNOS by different mediators in the cells from these patients, and discuss the role of arginase II in the pathology of arthritis.

MATERIALS AND METHODS

Patients. SF cells were obtained from the knee joint of 25 patients with rheumatoid arthritis (RA) (14 women, 11 men; mean age 52.1 yrs, range 14–70) satisfying the American College of Rheumatology criteria for RA[15]. SF was also obtained from a small number of patients with other forms of arthritis, i.e., osteoarthritis (OA) (n = 3, age range 58–72), psoriasis (n = 2, age range 44–59), arthralgia (n = 1, age 39), and juvenile chronic arthritis (n = 1, age 35). Synovial tissue was obtained at knee or hip replacement from 10 patients (7 with RA, 3 OA; 7 women, 3 men; mean age 50.1 yrs, range 37–65).

Cell culture. Mononuclear cells from the SF were obtained by washing the SF twice in phosphate buffered saline (PBS; Gibco) at 1200 rpm for 10 min to remove debris. This procedure was followed by 2 density gradient centrifugation steps, the first using polymorphoprep (Nycomed, Pharma, Oslo, Norway) to collect neutrophils to be used for isolation of mRNA and the second using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) to isolate mononuclear cells. The adherent population of SF mononuclear cells was prepared by overnight incubation in RPMI 1640 (Gibco, UK) supplemented with 2% fetal calf serum (FCS) (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine following removal of nonadherent cells by washing twice with cold PBS. More than 70% of this adherent cell population were macrophages, as determined by anti-CD14 and anti-CD68 staining and flow cytometry.

Adherent cells were cultured at 10^6 cells/ml in 24 well plates and stimulated for 48 h with cytokines (1000 U/ml IFN-γ, 500 U/ml TNF-α, and 0.5 ng/ml IL-1ß, named cytokine mixture, CM) or 100 μM dibutyryl-cAMP (dBt-cAMP), or 1 μg/ml prostaglandin E_2 (PGE_2) or 1 μg/ml lipopolysaccharide (LPS) from *Salmonella minnesota* or TGF-ß (20 ng/ml), according to the experimental protocol.

Preparation of synovial tissue fibroblasts. Fibroblast-like synoviocytes were isolated from RA synovial tissues obtained at joint replacement surgery. The tissues were minced, incubated with 1 mg/ml collagenase and 0.15 mg/ml deoxyribonuclease I in serum-free RPMI 1640 for 2 h at 37°C, filtered through nylon mesh, washed extensively, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS in an atmosphere of humidified 5% CO_2. After overnight culture, nonadherent cells were removed and adherent cells were cultured in DMEM with 10% FCS. This population of fibroblast-like synoviocytes was used from passages 3 through 7, when they were a homogeneous population of cells (< 1% CD11b positive, < 10% CD14 positive).

Reverse transcriptase polymerase chain reaction (RT-PCR). Reverse transcription was carried out on poly A^+ mRNA extracted from SF neutrophils (purified by polymorphoprep and Ficoll gradients), SF mononuclear cells, and peripheral blood mononuclear cells and neutrophils from a healthy volunteer as a control. Fast Track reagents (Invitrogen, Leek, The Netherlands) were used. Poly A^+ mRNA was resuspended in 20 μl of diethyl pyrocarbonate treated water. Duplicate 2 μl aliquots of poly A^+ mRNA were reverse transcribed in a final volume of 20 μl and then pooled. Each RT reaction contained 100 ng random hexamers, 0.5 mM each of dATP, dTTP, dGTP and dCTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 200 U SuperScript II RNAse H^+ reverse transcriptase (Gibco BRL). PCR was conducted in a volume of 25 μl (containing 200 μM dNTPs, 10 pmol of specific forward and reverse primers, and 1 U of Taq polymerase).

The primers used for amplification of the GAPDH cDNA were 5′-AAGGTGAAGGTCGGAGTCAACG-3′ (sense) and 5′-GGCAGAGATGATGACCCTTTTGGC-3′ (antisense). The amplification reaction for GAPDH consisted of 35 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, followed by a final extension of 10 min.

The specific human arginase II primers were 5′-ATGTCCCTAAGGGGCAGCCTCTCGCGT-3′ (sense) and 5′-CACAGCTGTAGCCATCTGACACAGCTC-3′ (antisense). The specific human arginase I primers were 5′-CTCTAAGGGACAGCCTCGAGGA-3′ (sense) and 5′-TGGGTTCACTTCCATGATATCTA-3′ (antisense). The amplification reaction for arginase I and arginase II consisted of 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final extension of 5 min.

Each reaction contained 5 μl of cDNA and 1 μl of the specific primer.

The specificity of PCR primers was confirmed in reactions containing poly A^+ mRNA from human kidney (for arginase II) and human liver (for arginase I). Finally, 5 μl aliquots of the PCR reactions were separated on 1.5% agarose gels containing ethidium bromide.

Other measurements. Arginase activity was measured in cell lysates as described[8] and NO generation was measured in the supernatant of the same cells by the Griess reaction[16].

RESULTS

Arginase activity (30 to 72 mU/10^6 cells) was detected in SF cells from all RA patients tested (n = 13) (Table 1). Samples from 2 patients with psoriasis and from one with juvenile

Table 1. Arginase activity in total SF cells.

Patients	Duration, yrs	Drug Therapy	Arginase Activity, mU/10^6 Cells
RA1	19	AZA+S	33.77 ± 2.5
RA2	10	S+Indo	33.06 ± 3.3
RA3	6	MTX	52.08 ± 1.4
RA4	2	AZA	65.18 ± 2.4
RA5	3	None	38.7 ± 0.7
RA6	15	S	30.46 ± 1.0
RA7	16	S+D-Pen	37.44 ± 0.34
RA8	10	None	43.11 ± 3.8
RA9	2	S	32.17 ± 0.9
RA10	15	MTX	72.45 ± 0.4
RA11	20	AZA	55.88 ± 1.2
RA12	25	S	33.6 ± 0.7
RA13	16	Pred	65.3 ± 1.2
Psoriasis 1	7	None	91.3 ± 2.3
Psoriasis 2	5	MTX	70.1 ± 1.9
OA1	8	None	38.5 ± 1.6
OA2	10	None	41.6 ± 2.6
OA3	5	None	42.4 ± 1.1
Arthralgia	6	None	36.7 ± 1.6
JCA	2	Indo	83.3 ± 2.5

AZA: azathioprine; S: sulfasalazine; Indo: indomethacin; MTX: methotrexate; Pred: prednisolone; D-Pen: penicillamine; JCA: juvenile chronic arthritis.

chronic arthritis showed even higher values (70 to 90 mU/10^6 cells). Because the SF from healthy individuals contains very few cells, we used blood mononuclear cells from 3 healthy donors as controls. The arginase activity values from these cells were 5.72 ± 0.76 mU/10^6 cells, 10 times lower than those obtained from patients with RA. These results show that arginase was induced in SF cells from patients with all forms of arthritis tested. However, as the majority of the samples were from patients with RA, we were not able to determine if there was any statistically significant correlation between the levels of enzyme activity and the different forms of arthritis.

To identify which arginase isoform was upregulated in our patients, we separated the cells into neutrophil and mononuclear fractions and used RT-PCR with specific primers for arginase I and arginase II. The results revealed that arginase II was the specific isoform expressed in both the neutrophil and the mononuclear fractions of all samples tested (Figure 1). Arginase II was not expressed by blood neutrophils or mononuclear cells from a representative healthy donor (Figure 1, lines 1 and 2), suggesting that its expression must be disease related. The samples tested were negative for arginase I (data not shown), in agreement with previous measurements showing that arginase I expression was limited to the liver[7].

To investigate which mediators could be responsible for arginase II induction, we cultured the adherent population of SF cells (70% macrophages) in the presence of cytokines and agonists known to induce arginase in murine cells[8]. Figure 2 shows that arginase II could be induced *ex vivo* by dBt-cAMP, PGE_2, and LPS. All these agents are known to induce arginase in murine macrophages. However, the Th2 cytokines IL-4 and IL-10 (which are potent inducers of arginase I in murine macrophages) did not induce any significant elevation in enzyme activity, suggesting that human arginase II is not induced by Th2 cytokines, at least in macrophages from patients with arthritis.

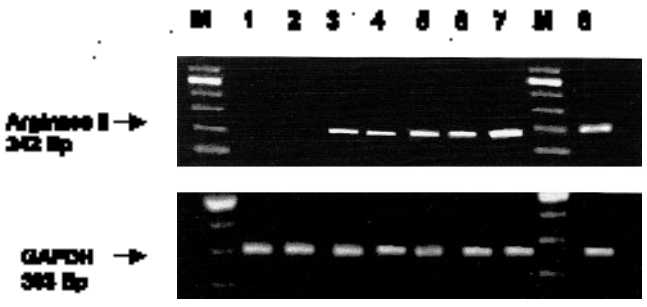

Figure 1. RT-PCR for human arginase II mRNA in purified neutrophils and mononuclear cells from patients with RA. Lane 1: blood neutrophils from a healthy donor; Lane 2: blood mononuclear cells from the same donor; Lanes 3 to 5: SF neutrophils from 3 patients with RA; Lanes 6, 7: SF mononuclear cells from 3 patients with RA; Lane 8: human kidney cDNA used as a positive control for arginase II. M: molecular weight markers.

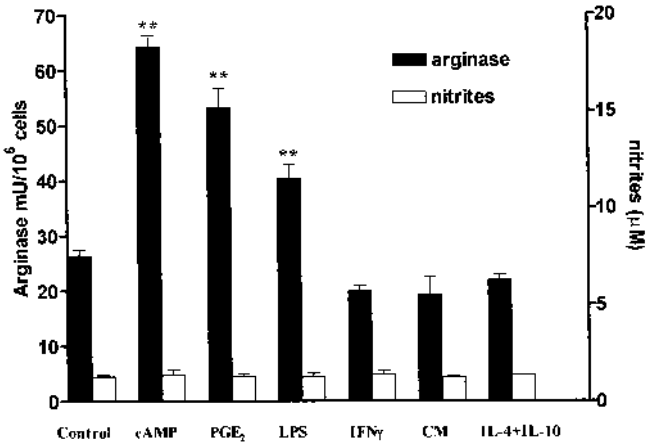

Figure 2. Induction of arginase and NO synthesis in synovial fluid macrophages from patients with arthritis. Cells (10^6/ml) were treated for 48 h with 100 μ dibutyryl-cAMP (cAMP); 1 μg/ml PGE_2; 1 μg/ml LPS; 1000 U/ml IFN-γ or CM (a mixture of 500 U/ml IFN-γ + 1 ng/ml IL-1ß + 500 U/ml TNF-α). Arginase activity was then measured in cell lysates and nitrite levels in culture supernatant as described. Results are the mean ± SE of triplicate cultures from 10 independent experiments. **$p < 0.01$ by Student paired t test, compared with the control.

In the same cultures, we determined nitrite levels in culture supernatant to determine whether there might be reciprocal regulation between the induction of arginase and iNOS in these cells. Control SF macrophages did not produce significant nitrite levels; similarly, they were negative by RT-PCR to iNOS mRNA (data not shown). Moreover, as can be seen in Figure 2, we found no significant increases in nitrite levels when the cells were activated with a mixture of Th1 cytokines (TNF-α + IFN-γ + IL-1ß; CM) known to induce iNOS in human cells[17].

Finally, we investigated the regulation of arginine metabolism towards arginase II or iNOS in cultures of fibroblasts obtained from the synovial tissue of patients with RA. We treated the cells with inducers of arginase II or iNOS and measured arginase activity into the cells and nitrite accumulation in culture supernatants of the same cells. The results are presented in Figure 3. In contrast with SF macrophages, cultured fibroblasts were positives for iNOS message (data not shown) and control cells already released nitrites into the culture media (Figure 3A). The activation with Th1 cytokines induced a significant increase in nitrite levels; interestingly, dBt-cAMP, PGE_2, and TGF-ß, which induced arginase, were at the same time inhibiting nitrite production. By contrast, arginase activity levels were significantly reduced by treatment with Th1 derived cytokines (Figure 3B). Thus, in synovial fibroblasts, arginase and iNOS appear to be reciprocally regulated.

DISCUSSION

We analyzed the expression of arginase isoforms in SF cells

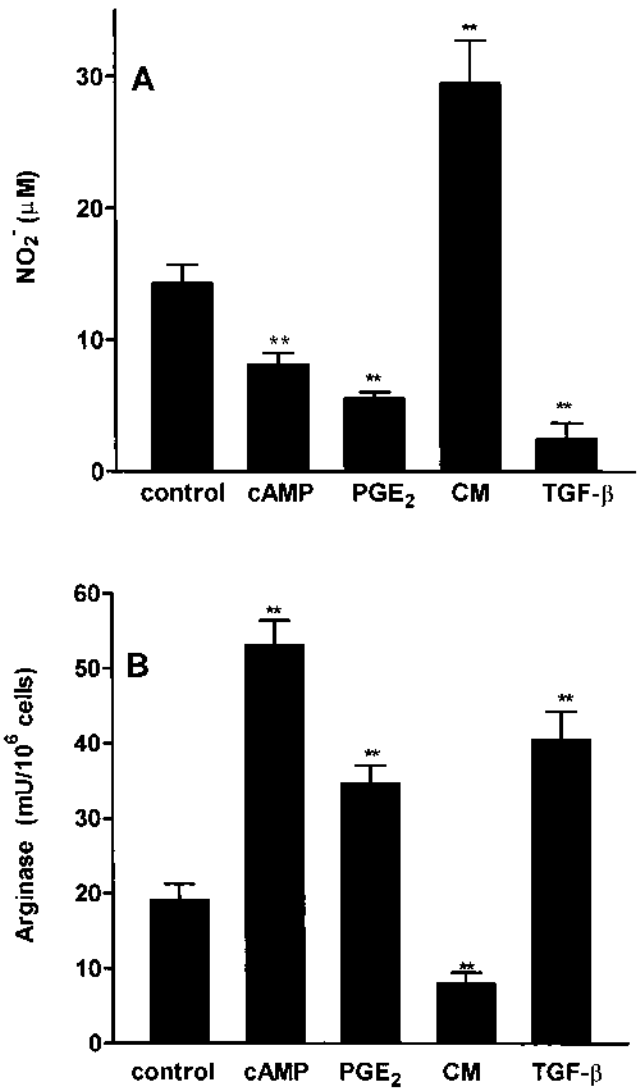

Figure 3. Nitrite production (A) and arginase activity (B) by fibroblasts from synovial membrane activated with 100 μM dibutyryl-cAMP (cAMP); 1 μg/ml PGE_2; CM (as in Figure 2); or 20 ng/ml TGF-ß. Cells were treated for 48 h with the agonists; supernatants were harvested for nitrite determination and cells were washed twice with PBS and lysed for arginase activity. Results are the mean ± SE of triplicate cultures from 4 independent donors. **$p < 0.01$ by Student paired t test.

and in fibroblasts from the synovial membrane of patients with different forms of arthritis.

The levels of enzyme activity in SF cells of the patients tested (Table 1) corresponded with the isoform arginase II as assessed by RT-PCR. We found induced arginase II in all forms of arthritis tested. However, as the majority of the samples were from patients with RA, it was not possible to make any correlation between the levels of enzyme activity and the different forms of arthritis.

Type II arginases are believed to function primarily in the net production of ornithine, which is used for polyamine biosynthesis or alternatively, for collagen biosynthesis via the production of proline[6]. Thus, the high expression of arginase activity could be involved in cell proliferation or tissue regeneration.

On the other hand, arginases can downregulate NO production by decreasing intracellular arginine concentrations. iNOS and arginase activities are regulated reciprocally in macrophages by cytokines, and this may guarantee the efficient production of NO[11]. Arginase isoforms have been found to be upregulated in other models of inflammation in humans. One of the best examples is the induction of arginase I in human mononuclear cells after trauma, in which the cytokine profile is balanced towards the induction of Th2-type cytokines together with a decrease in the production of NO[10]. In the same way, in glomerulonephritis, its expression has been associated with an increase in mesangial cell proliferation as well as a decrease in the levels of NO[14]. This radical, produced from L-arginine by NO synthases, is an important mediator in inflammation. When produced in high levels by the iNOS, it could lead to enhanced bone resorption, diminished bone proliferation and decreased proteoglycan synthesis, activate metalloproteases, and induce chondrocyte apoptosis. All of these effects contribute to joint damage[2].

Our results showed arginase II induction in SF macrophages by dBt-cAMP, PGE_2, and LPS, but no increase in nitrite levels in these cells. It is important to note that we did not use IFN-α, which has been identified as an inducer of iNOS in human mononuclear cells[18], and therefore it is possible that SF cells might be induced to produce nitrites *in vitro*. However, the levels of arginase activity were already high in all samples tested, suggesting that the metabolic fate of L-arginine in these cells could be preferentially diverted toward the production of polyamines. In this respect it has been shown that the levels of polyamines are highly increased in sera and SF from patients with RA[19]. Moreover, the cAMP responsive element binding protein (CREB), which responds to an increase in the intracellular cAMP concentrations, is expressed in synovial cells from patients with RA and is involved in cell proliferation in these patients[20], indicating that arginase II induction by cAMP analogs could provide the polyamines needed for cell proliferation.

In contrast with SF cells, fibroblasts from the synovial membrane were already positive for iNOS expression and released NO to the culture medium. This difference between cell types in the ability to generate NO could be due to a difference in cytokine profiles in the tissue versus SF, and also to better cell-to-cell contact inside the synovial membrane that might guarantee the induction of this enzyme.

In these cells, we found that all agents that induced arginase II activity were also able to reduce the levels of NO. This regulation appears to be particularly important at sites where there is an inflammatory process followed by a

phase of repair. An example of such is the regulation of arginine metabolism in wound healing[21], during which arginine is first metabolized through the iNOS pathway and then, as the healing process starts to occur, the inflammatory cells present at the site divert the arginine metabolism towards the arginase pathway, polyamine synthesis, and tissue repair.

This suggests that arginase upregulation may be involved in cell proliferation or recovery, since TGF-ß promotes fibroblast proliferation in RA[22] and also contributes to extracellular matrix remodeling, including stimulation of collagen synthesis by fibroblasts[23]. Ornithine, synthesized by arginase, is necessary for the production of collagen[6], which also occurs in RA, where collagen type II expression has been found[24].

In conclusion, we found arginase II induction in cells from patients with different forms of arthritis. In cells where both arginase II and iNOS are induced, there is a reciprocal regulation, which suggests that the relative activities of arginase and iNOS reflect different stages in the pathology of chronic inflammatory diseases. Regulation of arginase II induction in human arthritis could be very important in determining the progression of the disease; efforts are in progress in our laboratory to better assess the role of arginase in inflammatory joint diseases.

ADDED IN PROOF. Subsequent to acceptance of their revised manuscript, Drs. Corraliza and Moncada identified a new reference regarding increased levels of arginase activity in patients with RA. However, as only the abstract is in English, they have been unable to appropriately evaluate the work and cannot therefore include it in their discussion although they wish to acknowledge it among their references. Huang LW, Chang KL, Chen CJ, Liu HW. Arginase levels are increased in patients with rheumatoid arthritis. Gaoxioing Yi Xue Ke Xue Za Zhi 2001;17:358-63.

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