

RESEARCH ARTICLE

Contact with stimulated T cells up-regulates expression of peptidylarginine deiminase 2 and 4 by human monocytes

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ABSTRACT. Objective: The antigenic targets of the rheumatoid arthritis (RA)-associated autoantibodies to “citrullinated” proteins are generated following citrullination by a peptidylarginine deiminase (PAD). Of the five PAD isotypes, two – PAD2 and PAD4 – are expressed in RA synovial tissue. Within this tissue, the activation of macrophages and fibroblasts mediated by T-cell contact or driven by cytokines plays a prominent part in the pathogenesis. We wanted to determine whether cytokine stimulation and contact with T cells could play a role in PAD expression by peripheral blood monocytes and fibroblastic synoviocytes. **Methods:** Human monocytes and T lymphocytes were derived from the blood of healthy donors. HUT-78 cells and T lymphocytes were stimulated with PHA and PMA. Human synovial fibroblasts were isolated after surgical synovectomy. The expression of PAD was determined by real-time PCR and immunoblot. **Results:** In monocytes, the *PADI2* and *PADI4* mRNAs were transiently up-regulated by contact with stimulated HUT-78 and/or T lymphocytes. Positive modulation of the PAD2 and PAD4 proteins were also observed upon contact with stimulated HUT-78 T cells. Stimulation by IL-1 β or IFN- β did not modify the *PADI2* and *PADI4* mRNAs, but enhanced PAD4 protein expression. No isotype of PAD was detected at the mRNA or protein level in resting or stimulated synovial fibroblasts. **Conclusion:** Contact between stimulated T cells and monocyte-macrophages or cytokine-activated monocyte-macrophages constitutes a highly likely source of PAD2 and PAD4, which are observed in inflamed synovial tissues. In contrast, it is most unlikely that fibroblastic synoviocytes contribute to PAD expression in rheumatoid synovial membranes.

Keywords: peptidylarginine deiminase (PAD), rheumatoid arthritis, T cell contact, monocytes, synovial cells

Rheumatoid arthritis (RA) is characterized by hyperplasia of the synovial tissue and destruction of the joint structure. The synovium thickens due to the infiltration of inflammatory cells – mainly monocyte-macrophages and lymphocytes [1] – and to the local proliferation of resident

synovial and endothelial cells. Cytokines, produced in particular by fibroblast-like and macrophage-like synoviocytes, mediate the interaction between immune and inflammatory systems. In particular, IL-1 β and TNF- α up-regulate to a marked extent the production of PGE₂ and metalloproteinase-1 (MMP-1) by fibroblasts [2, 3], and contribute to destruction and remodeling of the extracellular matrix.

T lymphocytes and macrophages also infiltrate the target tissue at an early stage of the disease, frequently to be followed by joint damage. It is therefore likely that these cells interact, particularly in perivascular and sublining regions [4]. Like others, we previously reported that upon contact with stimulated T cells, monocytes produce high levels of IL-1 β and TNF- α as well as IL-6, IL-8, IL-10 and MMP [5-9]. Moreover, high levels of PGE₂ and MMP-1 are produced by fibroblasts and synoviocytes upon their contact with stimulated T cells [3, 10, 11].

Abbreviations

FCS	fetal calf serum
HUT-78 cells	a T cell line
IL-1Ra	IL-1 receptor antagonist
MMP-1	metalloproteinase-1
PAD	peptidylarginine deiminase
PBS	phosphate-buffered saline
PHA	phytohemagglutinin-L
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
TNFsR	TNF soluble receptor
7-AAD	7-amino-actinomycin D

RA is also characterized by the presence of autoantibodies to "citrullinated" (deiminated) proteins (anti-citrullinated protein autoantibodies/ACPA). These autoantibodies are highly specific for RA, detectable in the serum years before the onset of symptoms, and predictive of a more aggressive disease course (reviewed in [12]). In addition, ACPA are produced and concentrated in the rheumatoid synovium [13], where citrullinated fibrin is a major autoantigen target [14]. Citrullination, i.e. post-translational deimination of arginyl residues mediated by peptidylarginine deiminase (PAD), is essential to the formation of the epitopes recognized by ACPA as these consist of citrullinyl residues in the context of a specific amino-acid environment [15-17]. Five human PAD isotypes (PAD1, 2, 3, 4, and 6) have been described, each of which exhibits a particular pattern of expression (for a review see [18]). We recently analyzed the synovium of patients with RA and other arthritides for the presence of the five PAD isotypes. Transcripts of *PADI* 2, 4 and 6 genes were detected, but, at the protein level, only the presence of PAD2 and PAD4 enzymes was evidenced, both being probably involved in the citrullination of fibrin deposits [19]. Moreover, a close relationship between the levels of expression of PAD2 and PAD4 and the degree of tissue infiltration by inflammatory cells was observed, pointing to these cells as a source of the enzymes. In addition, PAD4 was frequently observed in the synovial lining layer, suggesting that cells in this hyperplastic tissue area also provide a supply of this PAD isotype [19]. However, to date, neither the nature of the cells nor the factors involved in the synovial expression of PAD2 or PAD4 have been precisely identified.

The crosstalk between monocyte-macrophages, fibroblasts and T cells is largely responsible for the pathogenesis and maintenance of chronic, destructive, inflammatory reactions in RA. In order to determine if these inflammatory processes also play a role in PAD expression by fibroblasts and macrophages in the rheumatoid synovium, we analyzed the expression of PADs in adherent synoviocytes and peripheral blood monocytes at the basal level, after stimulation with cytokines or contact with T cells.

DONORS AND METHODS

Reagents

RPMI-1640 medium, DMEM, fetal calf serum (FCS), polymyxin B sulfate, PMSF, pepstatin, leupeptin, and phorbol myristate acetate (PMA) were purchased from Sigma Chemicals (St. Louis, MO, USA). Phosphate-buffered saline (PBS), penicillin/streptomycin, L-glutamine, non-essential amino acids and sodium pyruvate were obtained from Gibco-Invitrogen (San Diego, CA, USA). Phytohemagglutinin-L (PHA) was purchased from EY Laboratories (San Mateo, CA, USA). Ionomycin was obtained from Calbiochem (Darmstadt, Germany). EDTA was purchased from Roche Diagnostics (Basel, Switzerland). Human AB serum was provided by the Blood Bank of the University Hospital (Geneva, Switzerland). Ficoll-PaqueTM Plus was from Amersham Biosciences (Uppsala, Sweden). Recombinant human IL-1 β and recombinant human TNF- α were obtained from Biogen (Geneva, Switzerland) and recombinant human IFN- β -1a (abbreviated IFN- β) a gift from ARES-Serono [20].

T cells and T-cell membranes

HUT-78, a human T-cell line [21], was obtained from the American Type Culture Collection. T lymphocytes from the peripheral blood of healthy donors were isolated by nylon column as described previously [5]. HUT-78 cells and peripheral blood T lymphocytes were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 50 IU/mL penicillin, 50 μ g/mL streptomycin and 2 mM L-glutamine (referred to as complete medium) in a 5% CO₂-air, humidified atmosphere at 37°C. For stimulation, HUT-78 cells (1×10^6 cells/mL) and peripheral blood T lymphocytes (4×10^6 cells/mL) were cultured for 6 h and 48 h, respectively in the presence of 1 μ g/mL PHA and 5 ng/mL PMA (stimulated), or in their absence (unstimulated) [9]. Cells were then harvested and plasma membranes were prepared as previously described [9, 22].

Isolation and stimulation of human monocytes and fibroblastic synoviocytes

Monocytes from blood buffy coats from healthy donors were isolated by density-gradient centrifugation as previously described [23], adding polymyxin B sulfate (2 μ g/mL) to all solutions during the isolation procedure. The purity of the isolated monocytes, assessed by FACS analysis, was >92%. Monocytes were cultured in 24-well plates at 10×10^6 cell/mL RPMI complete medium containing 5 μ g/mL polymyxin B sulfate (medium with polymyxin, referred to as control). Synoviocytes were isolated by protease treatment of surgical synoviectomy specimens obtained from patients with mechanical, articular lesions. Different lines of synoviocytes that had not been subjected to more than six passages were used in this study. Synoviocytes were cultured in Petri dishes at 1×10^6 cells/dish in DMEM complete medium. After 48 h, monocytes or synoviocytes were stimulated by different cytokines, by plasma membranes from unstimulated or stimulated HUT-78 T cells or peripheral blood T lymphocytes (5×10^6 cell equivalents/well), as previously described [3]. For cytokine stimulation of synoviocytes, IFN- β was added at 10^4 U/mL, IL-1 β at 250 pg/mL and TNF- α at 10 ng/mL. For monocytes, IFN- β was used at 10^4 U/mL and IL-1 β at 500 pg/mL. At different time points, cells and supernatants were harvested for mRNA and protein analyses as described below. As PAD expression may be linked to cell death, we assessed the mortality of monocytes in our culture conditions by means of 7-amino-actinomycin D (7-AAD). FACS analysis revealed that after 24 h, mortality was similar in all conditions and was consistently lower than 10% (data not shown).

Determination of cytokines and metalloproteinases

Synoviocyte and monocyte culture supernatants were subjected to enzyme-linked immunoassay (ELISA) to determine production of MMP-1, IL-1Ra (Quantikine; R&D Systems, Minneapolis, MN, USA) and IL-1 β (Immunotech, Marseille, France) as described previously [24, 25]. The sensitivity of protein assays ranged between 10 to 30 pg/mL.

Western blot analysis

Monocytes were transferred from plates to polypropylene tubes, washed with 1 mL of ice-cold PBS and centrifuged. Synoviocytes were harvested by 10-min incubation on ice with 1.5 mL of ice-cold PBS containing 1 mM EDTA and detached with a scraper, moved to polypropylene tubes and centrifuged. Monocytes and synoviocytes were stored at -80°C until proteins were extracted by addition of 100 μL of 40 mM Tris-HCl pH 7.4 containing 150 mM NaCl, 40 mM EDTA, 10 mM N-ethylmaleimide, 0.5% NP-40, 1 mM PMSF and 1/100 of a tablet of protease inhibitor cocktail (#11836170, Roche Applied Science) for $1.6-5 \times 10^6$ cells, followed by sonication on ice at 8-10 W (three times for 15 seconds) and centrifugation. Supernatants were stored at -80°C until western blot detection of PADs, essentially as described previously [19]. Briefly, validated isotype-specific rabbit antibodies to human PAD2, PAD4 or PAD6 were used at concentrations allowing the detection of at least 1 fmol of the matching PAD target [19, 26]. As controls, PAD2 purified from rabbit skeletal muscle (Sigma-Aldrich), a protein extract from HL-60 cells containing PAD4 [27], and recombinant human PAD6 [19] were immunodetected simultaneously by the relevant anti-PAD antibodies. Peroxidase-conjugated goat antibody to rabbit IgG (H+L) (SouthernBiotech, Inc., Clinisciences, Montrouge, France) was used as secondary antibody.

Real-time PCR analysis

Total RNA was isolated by lysing cells with Trizol reagent (Life Technologies) according to the supplier's instructions, and the concentration of RNA samples determined on a Beckman Coulter Spectrophotometer DU640. One microgram of RNA was reverse-transcribed and its quality controlled for the expression of the housekeeping gene GAPDH [28]. Subsequently, the relative amounts of mRNAs encoding PAD2, PAD4, PAD6, IL-1 β , IL-1Ra and MMP-1 were determined by TaqMan real-time PCR analysis on an ABI prism 7900 sequence detection instrument and normalized to the housekeeping gene 18S. The expression of each condition was related to the control, to which an arbitrary value of 1 was assigned [29]. Primers and probes were purchased from Applied Biosystems (Foster City, CA, USA).

Statistical analysis

Data were analyzed using the two-factor ANOVA test (Statview 5.1, SAS Institute, Inc., Cary, NC, USA, and GraphPad Prism 3.02); $p < 0.05$ being considered significant, * denotes $p < 0.05$ and ** denotes $p < 0.0001$.

RESULTS

Transcription of PADI genes in human monocytes upon stimulation by T-cell contact

To determine whether the expression of PADI genes in human monocytes would be affected by contact with activated T cells, monocytes purified from the peripheral blood of healthy donors were incubated with plasma membranes of cells from the HUT-78 T-cell line prepared after stimulation with PMA and PHA (mbHUTs), and the levels of transcripts for PAD2, PAD4 and PAD6 (PADI2, PADI4

and PADI6 mRNAs) assessed by real-time PCR (figure 1). Stimulation was also performed with optimal concentrations of IFN- β or of IL-1 β . After 4 h of contact with mbHUTs, the levels of PADI2, PADI4 and PADI6 mRNAs had increased as compared to unstimulated monocytes (figure 1A). On the other hand, contact with membranes prepared from resting HUT-78 cells (mbHUTns) or stimulation with IL-1 β had no significant effect on the mRNA expression of the 3 PADI genes. When monocytes were stimulated with IFN- β , only PADI6 mRNA was induced. As a control, we analyzed the modulation of IL-1 β mRNA after stimulation under the same conditions (figure 1B). As expected, mbHUTs and IL-1 β induced a massive production of IL-1 β mRNA, while IFN- β had no effect [25].

The kinetics of PADI mRNA expression induced by contact with stimulated HUT cells was explored by analyzing monocytes at different time points after stimulation. After exposure to mbHUTs for 4 h, all three PADI mRNAs were significantly increased (figure 1C), but declined rapidly, and the levels of PADI2, PADI4 and PADI6 mRNA were below those of resting monocytes after 12 h and 24 h of contact. Another time-course experiment revealed that a return to levels found in resting monocytes had already occurred after 6 h (data not shown). By contrast, following stimulation by mbHUTs, the kinetics of IL-1 β mRNA expression were different since the levels of the cytokine messenger kept increasing to a marked extent, at least up to 24 h of contact (figure 1D).

To determine whether membranes prepared from human peripheral blood T lymphocytes would also affect the production of PADI mRNAs, monocytes were cultured in the presence of membranes from unstimulated (mbTns) or PHA- and PMA-stimulated (mbTs) T lymphocytes purified from the peripheral blood of healthy donors. Contact with mbTs resulted in a very transient increase in levels of PADI2 mRNA as after 1 h it was significantly higher than in unstimulated monocytes, but had returned to baseline after 4 h. However, neither the level of PADI4 mRNA nor that of PADI6 mRNA was affected (figure 2A). Once again, IL-1 β mRNA served as control and, as expected, was found to increase over the 4 h time-course upon stimulation with mbTs (figure 2B).

Expression of PAD proteins in human monocytes upon stimulation by contact with T-cells

The expression of PAD in monocytes stimulated by T-cell membranes was also analyzed at the protein level by immunoblotting analysis of low-salt protein extracts of the cells using antibodies specific for PAD2, for PAD4 and for PAD6. Figure 3 shows the results obtained after detection of these PAD isotypes in extracts of the monocytes from three different blood donors following different incubation periods with mbHUTs.

In monocytes from two blood donors (figures 3A-B) incubated with mbHUTs, the PAD2 enzyme was up-regulated compared to levels in resting monocytes and monocytes stimulated with mbHUTns. In monocytes from the first donor, this up-regulation was observed after incubation for 18 h (figure 3A). Note that the vertical band in the PAD4 control lane is an artifact. In monocytes from the second donor, it was visible after incubation for 12 h (figure 3B). However, the basal levels of PAD2 in unstimulated monocytes diverged in the two donors, PAD2 being

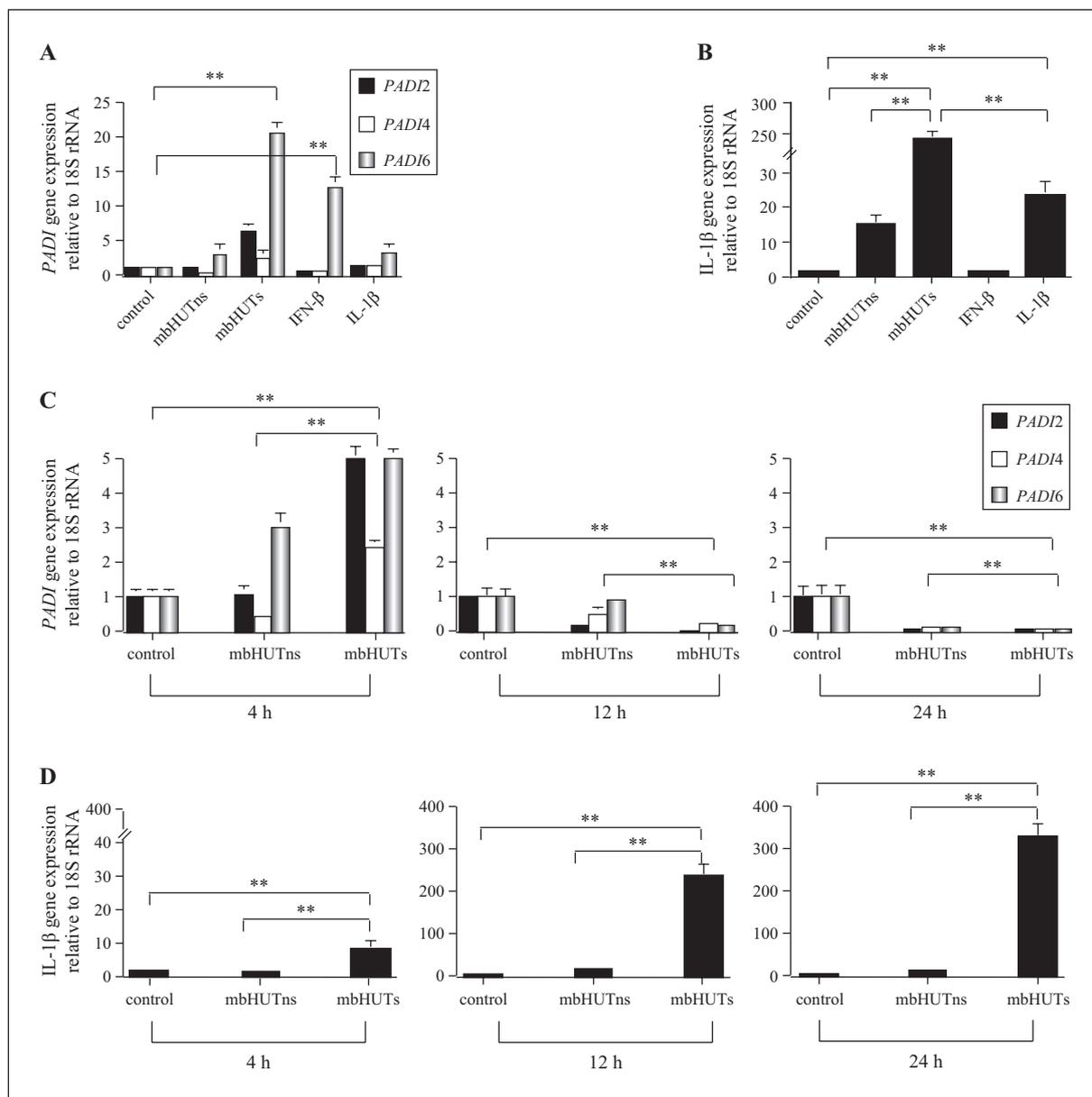


Figure 1

mRNA levels of PAD and of IL-1 β in monocytes stimulated with plasma membranes of resting or PMA- and PHA-stimulated HUT-78 cells, or in the presence of IFN- β or IL-1 β .

Relative mRNA levels (A) of *PADI2*, *PADI4* and *PADI6* and (B) of IL-1 β were assessed after 4 h of stimulation. Each gene under the various conditions was related to the control, to which an arbitrary value of 1 was assigned. Bars represent the median \pm SEM of relative gene expressions of three independent experiments with monocytes from different blood donors. Comparisons of the expression of *PADI2*, of *PADI4* and of *PADI6* between controls and mbHUTs consistently yielded p values of <0.0001 ; for *PADI6* the difference between control and IFN- β was also significant ($p < 0.0001$), for IL-1 β , comparison of control with mbHUTs, of mbHUTns with mbHUTs, of control with IL-1 β and of mbHUTs with IL-1 β consistently yielded p values of <0.0001 . The relative mRNA levels (C) of *PADI2*, *PADI4* and *PADI6* and (D) of IL-1 β were analyzed over a 24-h period. Bars represent the median \pm SEM of relative gene expressions in four separate experiments. For *PADI2*, *PADI4*, *PADI6* and IL-1 β , comparison of control with mbHUTs and of mbHUTns with mbHUTs yielded exclusively p values of <0.0001 at all time points.

almost undetectable in the first donor while in the second donor it was easily detected after 4 h of culture and persisted over time. Stimulation with mbTns was also tested in the first donor and, in comparison with levels in control monocytes and those stimulated with mbTns, was found to induce an up-regulation of the PAD2 enzyme after 18 h (data not shown). In monocytes from the third donor, PAD2 remained undetectable, regardless of culture conditions, including stimulation with mbHUTs (figure 3C). Of note, the vertical band in lane 4 h for PAD2 is an artifact. Finally, incubation of monocytes from donor 1 with IFN- β or IL-1 β did not affect the levels of PAD2 after 18 h (figure 3A). Culture supernatants from that donor, tested by

ELISA for IL-1 β and IL-1Ra production, served as control. The test showed, as expected, that the production of IL-1Ra was stimulated by mbHUTns, mbHUTs and IFN- β , and that mbHUTs induced a massive production of IL-1 β (figure 3D).

Although variable in intensity and duration, expression of PAD4 in unstimulated monocytes was shown, and declined over time in all donors. Stimulation with mbHUTs did induce a slight enhancement of PAD4 levels as compared to controls (unstimulated monocytes or monocytes stimulated with mbHUTns) in the monocytes at 18 h in the first donor and at 12 h in donors 2 and 3. In monocytes from the first donor, treatment with IFN- β or IL-1 β prevented

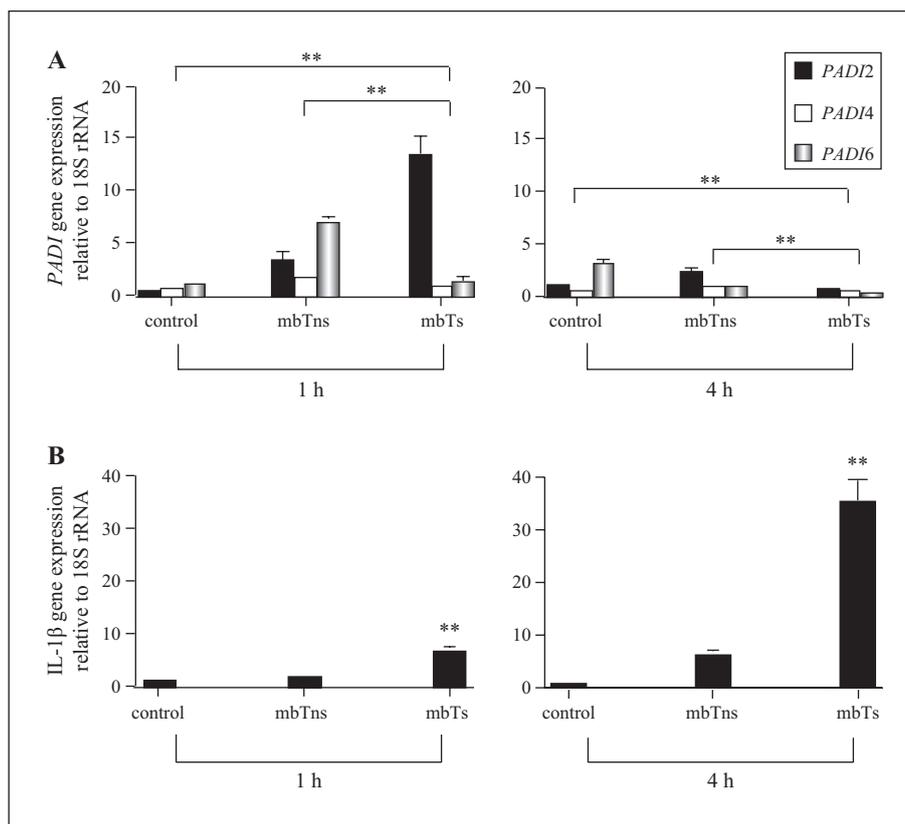


Figure 2

mRNA levels of PAD and of IL-1 β in monocytes stimulated with plasma membranes of resting or PMA- and PHA-stimulated peripheral blood T lymphocytes.

Relative mRNA levels (A) of *PADI2*, *PADI4* and *PADI6* and (B) of IL-1 β were assessed after 1 h and 4 h of stimulation. For (A), gene expression was expressed relative to the value of the control. Bars represent the median \pm SEM of relative gene expressions in three separate experiments performed on monocytes from different blood donors. For *PADI2* and IL-1 β , comparison of control with mbTs and of mbTns with mbTs yielded $p < 0.0001$ at all time points.

any decline in the PAD4 enzyme (figure 3A). As shown above, mRNA results obtained on monocytes from different donors were similar, but the production levels of PAD2 or PAD4 protein differed from mRNA data (figures 1 and 2). These differences may be due to the time point chosen or to a dissociation between mRNA expression of PAD2 or PAD4 and their protein production.

PAD proteins seem to be absent in CD3+ peripheral blood T lymphocytes [30]. However, to rule out that the detection of PAD2 and PAD4 enzymes in monocyte extracts stimulated with mHUTs was due to the presence of PAD in the plasma membranes of T cells, two different mHUT preparations were analyzed by western blot using anti-PAD antibodies. These tests confirmed that neither PAD2 nor PAD4 was present (data not shown).

Finally, on testing monocytes from two donors for the presence of PAD6, the latter was also found to be absent (figures 3A,C).

Transcription of *PADI* genes and expression of PAD proteins in resting human fibroblastic synoviocytes and after T-cell contact or cytokine stimulation

To determine whether the expression of *PADI* genes in human fibroblastic synoviocytes would be affected by contact with activated T cells, synovial fibroblasts from enzyme-dissociated synovial tissue from patients with mechanical joint lesions were cultured in the presence of mbHUTs. Levels of *PADI2*, *PADI4* and *PADI6* mRNAs

were assessed by real-time PCR (figure 4A), and expression of the corresponding proteins was analyzed by immunoblot of low-salt protein extracts of the cells (figure 4C). In basal conditions, none of these mRNAs or PAD proteins were detected. Stimulation with mbHUTs, as well as incubation with IFN- β or with IL-1 β and TNF- α – both cytokines known to stimulate synovial fibroblasts – did not change the mRNA levels of *PADI2*, *PADI4* or *PADI6* (figure 4A), or of *PADI1* and *PADI3* (data not shown), neither did it change the corresponding protein levels (figure 4C and not shown). In contrast, and as expected, MMP-1 mRNA levels were significantly enhanced upon stimulation with mbHUTs or IL-1 β and TNF- α (figure 4B) and, under these conditions, the concentration of MMP-1 protein increased in culture supernatants (figure 4D).

DISCUSSION

Since immune reactions to citrullinated antigens in the synovial tissue are likely to play a significant part in the persistence of RA synovitis, it seems appropriate to understand the mechanisms underlying the expression of PAD enzymes in this tissue. An exhaustive analysis of the five PAD isotypes expressed in the synovial tissue of patients with established disease led to the detection of *PADI2*, *PADI4* and *PADI6* transcripts, without any evidence of *PADI1* and *PADI3* transcripts [19]. At the protein level, PAD2 and PAD4 were the only PAD isotypes

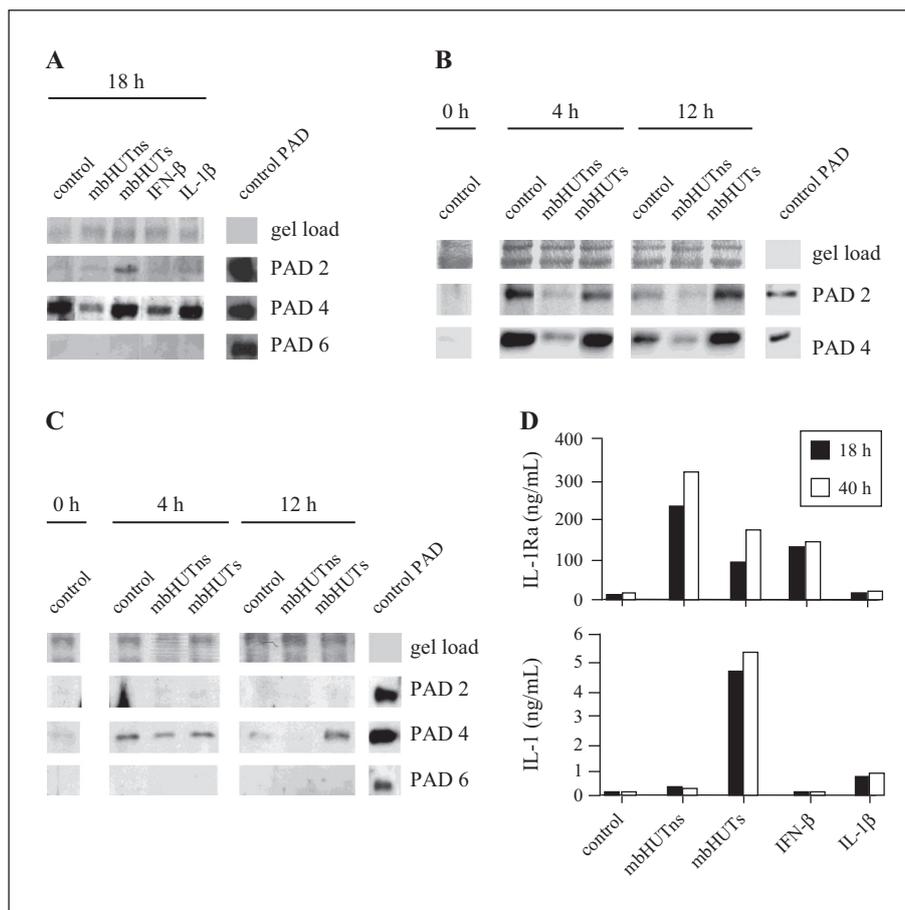


Figure 3

Production of PAD, IL-1Ra and IL-1 β by monocytes stimulated with plasma membranes of resting or PMA- and PHA-stimulated T cells, in the presence of IFN- β or of IL-1 β .

Expression of PAD2, PAD4 and PAD6 enzymes was analyzed by immunoblot in extracts of the monocytes from three different blood donors (A, B, C, respectively) following different periods of incubation with mbHUTs. "Control PAD" refers to the lane where the relevant control PAD was immunodetected simultaneously. (D) As to the donor analyzed in (A), culture supernatants were tested by ELISA for the production of IL-1Ra and IL-1 β after 18 h and 40 h of stimulation. Bars represent the median cytokine concentration measured in three separate experiments performed after ELISA determinations.

detected. Both enzymes were observed directly or in the close vicinity of citrullinated fibrin deposits, a major target of ACPA in the RA synovium. In addition, PAD2 was detected in mononuclear cells scattered in the sub-lining or deep synovium, and PAD4 was detected in generally grouped mononuclear cells located in all tissue areas including the synovial lining. Since the morphology of both PAD2- and PAD4-positive cells tends to be similar to that of macrophages, and PAD-expressing cells were frequently CD68-positive, it is probable that monocyte-macrophages constitute an important cellular source of PAD [19]. Therefore, to better understand the mechanisms underlying PAD expression in RA synovium, we analyzed monocyte expression of PAD mRNA and proteins under various conditions likely to arise in the synovium of RA patients, focusing on the three isotypes for which transcripts had been detected in the RA synovium, *i.e.* PAD2, PAD4 and PAD6 [19].

PAD6 corresponds to the most divergent isotype of the family of PAD enzymes. Its mRNA has been detected in oocytes and early cleavage-stage embryos, and its murine ortholog has been shown to play a role in early embryonic development. Detection of the mRNA in a human fetal brain library, and in peripheral blood leucocytes nonetheless suggests that it could also fulfill other functions in other tissues. However, although we found evidence that

stimulation of monocytes with mbHUTs or IFN- β could up-regulate the *PADI6* transcript, the corresponding protein was never detected, suggesting a tight regulation of the protein concentration at a post-transcriptional level.

Cytokines are implicated in the pathogenesis of RA (reviewed by Brennan *et al.* [31]). An imbalance between IL-1 β and IL-1Ra levels may, depending on which way the balance tips, lead to either perpetuation or prevention of articular inflammation, [32]. IFN- β inhibits the production of the pro-inflammatory cytokines IL-1 β and TNF- α , while inducing the release of the anti-inflammatory cytokines IL-1Ra and IL-10 [33, 34]. It was therefore expedient to determine whether PAD expression by monocytes would be modulated by IL-1 β or IFN- β . Although we did not observe any modulation of either *PADI2* or *PADI4* transcripts after 4 h of stimulation, analysis of PAD expression at the protein level revealed that both cytokines prevented the decline in PAD4 that occurred over time in resting monocytes. Although the latter observation needs confirmation, since it results from analysis of monocytes from a single blood donor, it suggests that the two cytokines contribute to the expression of this enzyme at the protein level.

Direct cellular contact with stimulated T cells is considered an important pathway triggering the activation of monocyte-macrophages in the absence of infectious agents

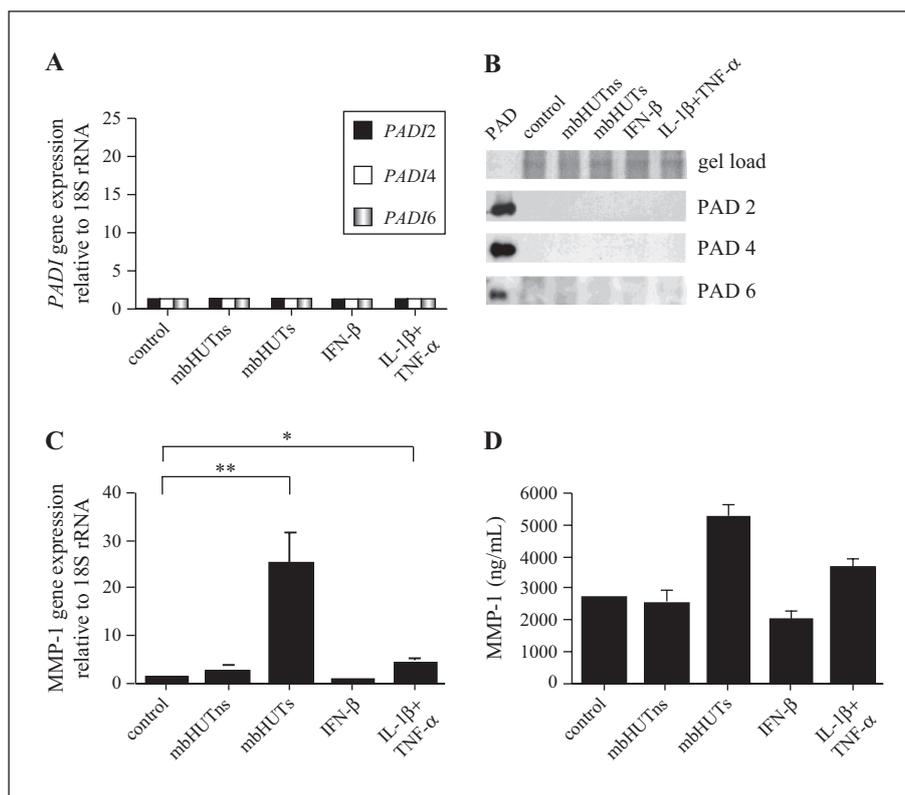


Figure 4

Expression of PADs and MMP-1 at mRNA and protein levels in fibroblastic synoviocytes stimulated with plasma membranes of resting or PMA- and PHA-stimulated HUT-78 cells, with IFN- β or with IL-1 β and TNF- α .

(A) Relative mRNA levels of *PADI2*, *PADI4* and *PADI6* and (B) of MMP-1 were assessed after 4 h of stimulation. Each gene in the various conditions was related to the control. Bars represent the median \pm SEM of relative gene expressions in two separate experiments performed with different extractions of fibroblastic synoviocytes. For MMP-1, comparison of control with mbHUTs yielded $p < 0.0001$ and of control with IL-1 β + TNF- α $p < 0.01$. (C) Synoviocytes were stimulated for 40 h before immunoblot was carried out on low-salt extracts of the cells to determine the expression of PAD2, PAD4 and PAD6 enzymes, and (D) culture supernatants were tested by ELISA for the production of MMP-1.

(reviewed by Dayer and Burger [35]). Does this cell-cell interaction also play a role in PAD production? The present data show that PAD2 was up-regulated upon contact with activated T cells. Indeed, *PADI2* transcripts accumulated rapidly for a short while after stimulation with membranes of either stimulated HUT-78 or stimulated peripheral blood T lymphocytes, and with either type of membrane the PAD2 enzyme was up-regulated. Results were less clear-cut for PAD4, but they also suggest that its expression is affected by contact with activated T cells. Indeed, elevated levels of mRNA transcripts were detected when using membranes of stimulated HUT-78 cells, with similar results on the enzyme but at lower levels.

The production levels of PAD protein may be slightly different in freshly isolated human cells (*figure 3*), highlighting the variability that was found in healthy donors, but in all cases PAD proteins were induced when stimulated – as opposed to unstimulated – T cells were in contact with monocytes.

Recently, Makrygiannakis *et al.* reported the beneficial effect of glucocorticoids on the decrease of T-cell infiltration into the synovium [36], and on its specific inhibition of protein citrullination [37]. The use of glucocorticoids may produce beneficial effects in RA through independent yet interactive processes, by inhibiting PAD enzyme expression and citrullination during increased T-cell/monocyte interaction in the inflamed synovium. Therefore, the inhibition of the PAD enzyme or T-cell/monocyte interaction

may be a key approach to treatment, or the revival of an existing treatment of RA [38]. This seminal, *in vitro* study could represent a sound basis for clinical studies.

Several surface molecules including CD69 and receptor/ligand interactions including LFA-1/ICAM-1, CD2/LFA-3 and CD40/CD40L, have been reported to play a part in the T-cell activation of monocytes/macrophages [5, 35, 39]. They could be involved in the contact-mediated modulation of PAD expression, although the implication of other, as yet unidentified, surface-expressed molecules cannot be ruled out [24].

Upon cell-cell contact with stimulated T cells, fibroblastic synoviocytes produce substantial amounts of PGE₂ and MMP-1 in the inflamed joint [3, 10, 11]. We and colleagues have also reported that this process was partly due to T cell-membrane-associated IL-1 β and TNF- α [3]. Having observed PAD4-expressing cells in the lining of inflamed synovial tissues, we tested synovial cells from patients with mechanical articular lesions for the expression of any PAD isotypes during interaction with stimulated T cells or cytokines. No transcripts of *PADI2*, *PADI4* or *PADI6* genes, or the corresponding proteins were detected after contact with stimulated T cells, following stimulation by IFN- β , IL-1 β or TNF- α . Moreover, regardless of the culture conditions, neither PAD1 nor PAD3 was expressed at the mRNA or protein level (data not shown).

In conclusion, our study demonstrates that fibroblastic synoviocytes are very unlikely to participate in PAD

expression in the synovial membrane in either physiological or inflammatory situations. On the other hand, monocyte-macrophages definitely constitute a source of PAD. We confirm a marked and very stable down-regulation of PAD6 expression at the post-transcriptional level. Basal expression of PAD2 and PAD4 was variable, and this may be related to a varying degree of differentiation into possibly phenotypically heterogeneous macrophages during the isolation procedure, as the differentiation of monocytes into macrophages has been claimed to influence the expression of both enzymes [19, 40]. Nevertheless, contact with activated T cells constituted an up-regulating factor for both isotypes, and stimulation by IL-1 β or IFN- β enhanced PAD4 expression. The present work describes, for the first time, the modulation of PAD by monocytes and immune cells. Although further studies are needed to understand the precise molecular pathway(s) leading to variations in the basal levels of the enzymes and to their stimulus-induced up-regulation, our results provide some explanation as to how monocyte-macrophages activated by contact with T cells could constitute a source of PAD in inflamed synovial tissues.

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