Abstract
Objective—To determine the contribution made by synovial fluid (SF) neutrophils to the augmented expression of macrophage inflammatory protein 1 α (MIP-1α) in rheumatoid arthritis (RA).

Methods—Neutrophils were isolated from samples of SF from RA patients and peripheral blood (PB) samples from RA patients and healthy controls. Cell associated MIP-1α was visualised immunohistochemically, and cell associated MIP-1α as well as MIP-1α secreted into the SF was assayed by ELISA. Steady state expression of MIP-1α mRNA was assessed by reverse transcription polymerase chain reaction (RT-PCR).

Results—Freshly isolated SF neutrophils contained significantly higher concentrations of both MIP-1α protein and its transcript than PB neutrophils from either RA patients or healthy controls; incubation in the absence or presence of tumour necrosis factor α for 24 hours resulted in a significant increase in MIP-1α secretion by RA SF neutrophils compared with neutrophils obtained from either normal PB or RA PB; and expression of MIP-1α by SF neutrophils was well correlated with both RA disease activity and SF mononuclear cell (MNC) counts.

Conclusion—Expression and secretion of MIP-1α by SF neutrophils may be indicative of local and systemic inflammation in RA. Moreover, this C-C chemokine may contribute to the recruitment of MNCs from the bloodstream into synovial joints and tissues.

The progression of rheumatoid arthritis (RA) is characterised by the appearance of inflammatory cells in both the pannus and the joint fluid and by eventual tissue destruction. Various mediators, including inflammatory cytokines and adhesion molecules, have been implicated in the pathogenesis of RA, and specific cell-cell interactions, especially between synovial cells and invading cells, seem to play pivotal parts during the progression of the disease.

Recently identified is a group of factors belonging to a gene superfamily of chemotactic cytokines that share substantial homology, in particular four conserved cysteine amino acid residues. The C-X-C family of chemokines (for example, IL8, GRO, and ENA-78), in which the first two cysteines are separated by another amino acid residue, is chemotactic for neutrophils and T cells; whereas the C-C chemokine family (for example, macrophage inflammatory protein (MIP)-1, macrophage chemoattractant protein (MCP)-1, and RANTES), in which the first two cysteine residues are in juxtaposition, is chemotactic for monocytes and subpopulations of T cells. These chemokines seem to play key parts in inflammatory and immune responses mediated by their respective affected cell populations; for instance, release of chemokines from inflammatory cells is a crucial step in the recruitment of the cells required to establish local inflammatory responses (for example, rheumatoid synovitis). Thus, the coordinated production of chemokines and proinflammatory cytokines is likely to be important in the orchestration of the inflammatory responses observed in patients with RA.

Activated neutrophils produce several polypeptide inflammatory mediators: the cytokines, IL1α–10 IL1 receptor antagonist, IL6α–11 and tumour necrosis factor (TNF), the chemokines, IL8 and MIP-1α, and the chemokines, IL8 and MIP-1α. Because most infiltrating leucocytes present in the synovial fluid (SF) are neutrophils, and because rheumatoid SF contains a variety of humoral factors produced by monocytes and synoviocytes, neutrophils must be considered to be an important source of the factors mediating RA disease activity. In that context, the aim of this study was to examine the expression of MIP-1α by SF neutrophils in patients with RA, and to characterise the relation between the concentration of neutrophil derived MIP-1α and RA disease activity.

Methods

Reagent preparation

Recombinant human TNFα with a specific activity of 1.6 × 10⁶ U/mg was purchased from Pepro Tech EC (London, England). Complete medium consisted of RPMI 1640 (Dai-nippon Pharmaceutical Co Ltd, Japan) supplemented with 2 mM L-glutamine, 25 mM Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO Laboratories, Grand Island, NY), and 5% heat inactivated fetal calf serum (GIBCO Laboratories). Monoclonal antibody (MA-291–5) and polyclonal antibody for MIP-1α were purchased from Austral Biologicals (San Ramon, CA), and R and D Systems Inc, (Minneapolis, MN), respectively. The
antibodies to human MIP-1α did not cross react with each other, or with other known members of the chemokine family, including RANTES, MCP-1, platelet factor 4, connective tissue activating peptide III, beta-thromboglobulin, NAP-2, ENA-78, or IP-10. All reagents were tested for endotoxin contamination using a limulus amebocyte lysate test kit (Limulus HS-F test; Wako Chemicals, Tokyo, Japan). The concentration of endotoxin was consistently below 0.03 EU/ml in all reagents.

ISOLATION AND CULTURE CONDITIONS OF PERIPHERAL BLOOD AND SYNOVIAL FLUID NEUTROPHILS

RA SF neutrophils and MNC, RA peripheral blood (PB) and normal PB neutrophils were obtained from knee puncture of 25 patients (mean age: 56 years, range 36–72, 16 women and nine men) with RA who fulfilled the 1987 ACR criteria, and from 20 of 25 patients with RA and age and sex matched 15 healthy controls (mean age: 50 years, range 29–69, nine women and six men), respectively, by Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc, Piscataway, NJ), centrifugation, subsequently separated from erythrocytes by lysis of erythrocytes in a solution of 0.15 M NH₄Cl, 0.01 M NaHCO₃, and 0.01 M tetra EDTA. Most RA patients were receiving a non-steroidal anti-inflammatory drug (NSAID) and a slow acting anti-rheumatic drug. Of those receiving a slow acting anti-rheumatic drug, three were taking methotrexate, six were taking D-penicillamine, seven were taking sulphasalazine, and four were taking gold. No patient was receiving either >5 mg oral prednisolone/day or intra-articular injection of glucocorticoids within one month from SF sample aspiration. The recovered neutrophils were washed three times and resuspended at a density of 5 × 10⁶ cells/ml in complete medium. The final cell preparation contained more than 98% neutrophils by morphology and viability was more than 98% by trypan blue dye exclusion, and monocyte contamination was below 2% by non-specific esterase staining. Neutrophils were incubated in the presence or absence of TNF-α (10 ng/ml) and then cell free supernatants of neutrophil cultures were collected and immediately frozen. To assess cell associated chemokine, neutrophil lysates were obtained by addition of same volume of complete medium to the cells, followed by three freeze/thaw cycles. Furthermore, SF samples were aspirated from patients with RA or osteoarthritis (OA) under aseptic condition,
and then centrifuged (2500 rpm for 10 minutes) to remove cell debris. The supernatants were immediately frozen. C reactive protein (CRP) was measured by the nephelometric standard method.

All human experiments were performed in accordance with protocols approved by the Human Subjects Research Committee at our institution, and informed consent was obtained from all patients and volunteers.

ASSESSMENT OF MIP-1α CONCENTRATIONS BY SPECIFIC ELISA

Specific neutrophil derived MIP-1α was quantified using a modification of a double ligand ELISA method, as previously described. Monoclonal murine antihuman MIP-1α antibody (0.5 μg/ml) as first antibody and polyclonal rabbit anti-MIP-1α antibody (1 μg/ml) as second antibody were used. This ELISA method consistently detected MIP-1α in a linear fashion between 20 pg/ml and 5 ng/ml.

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Neutrophil derived MIP-1α was visualised by immunohistochemistry as previously described. Briefly, neutrophils deposited on a glass slide by using a Cytospin II (Shandon Southern Instruments, Inc, Sewickley, PA). The slides were incubated with polyclonal rabbit anti-MIP-1α antibody (1:1000 dilution) or pre-immune mouse IgG. Biotinylated rabbit antimouse IgG (1:200; Vector Laboratories Inc, Burlingame, CA) and peroxidase conjugated streptavidin were used as second and third reagents, respectively. The substrate for red colour reaction was 3-amino-9-ethylcarbazole in N,N-dimethylformamide (Sigma Chemical Co, St Louis, MO). After rinsing with distilled water, the slides were stained with Mayer’s haematoxylin.
extracted with the use of chloroform-phenol and alcohol precipitated.

Semiquantitative RT-PCR was performed as previously described. Briefly, 5 µg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (GIBCO BRL). The PCR reaction was performed with the primers 5’-ACT-GCT-GCC-CTT-GCT-GTC-CTC (sense) and 5’-ACT-CAG-CTC-CAG-GTC-GCT-GAC (anti-sense) for MIP-1α,23 and 5’-GGC-ATC-CGG-ACG-TTC-TAC-GG (sense) and 5’-ATG-GTG-AAG-GTC-GGT-GTG-AAC (anti-sense) for GAPDH,24 as an internal control. The PCR reactions were separated on 2% agarose gel containing 0.3 µg/ml of ethidium bromide, and were visualised and photographed using ultraviolet transillumination.

STATISTICAL ANALYSIS
Data were analysed by Power Macintosh computer using a statistical software package (Statview 4.5; Abacus Concept, Inc, Berkeley, CA) and expressed as the mean (SEM). Data were evaluated by Mann-Whitney test and the Spearman rank correlation coefficient where appropriate. A p value of less than 0.05 was considered significant.

Results

EXPRESSION OF CELL ASSOCIATED MIP-1α BY ISOLATED NEUTROPHILS AND CONCENTRATIONS OF MIP-1α IN SYNOVIAL FLUID
We hypothesised that the neutrophils present in the SF of RA patients (RA SF neutrophils) are an important cellular source of the C-C chemokine, MIP-1α. To test this hypothesis, we first determined whether freshly isolated neutrophils contained MIP-1α protein. Neutrophils were isolated from the PB of patients with RA, from the PB of healthy control subjects, and from the SF of patients with RA. Cell associated MIP-1α was then assayed by ELISA. As shown in figure 1A, freshly isolated RA SF neutrophils contained substantial quantities of MIP-1α (58.6 pg/million cells), whereas MIP-1α was undetectable in neutrophils isolated from PB. Moreover, in agreement with previous reports (fig 1B),25 concentrations of MIP-1α in the SF of patients with RA (729 pg/ml) were significantly greater than those in the SF of patients with OA (63 pg/ml).

The specific cell type expressing MIP-1α protein was verified immunohistochemically: freshly isolated RA SF neutrophils were positively labelled by anti-MIP-1α antibodies (fig 2B). RA SF neutrophils exposed to preimmune control IgG (fig 2A) exhibited no MIP-1α labelling, nor did PB neutrophils obtained from healthy controls and RA patients (data not shown).

Consistent with the expression of MIP-1α protein, PT-PCR revealed substantial steady state expression of MIP-1α mRNA in freshly isolated RA SF neutrophils; indeed, there was a significant correlation between the levels of MIP-1α transcript and the levels of cell associated MIP-1α protein (fig 3A and B). MIP-1α mRNA was not detected in PB neutrophils.

The acute phase response, as measured by CRP concentration, is commonly used in clinical practice to monitor RA disease activity. Increased CRP concentrations are indicative of disease progression and the absence of a positive response to treatment. We, therefore, examined the extent to which disease activity correlated with MIP-1α concentrations in RA SF neutrophils and in the SF of RA patients.

Figure 5 Basal and TNF-α induced MIP-1α secretion by neutrophils obtained from RA SF, RA PB, or normal PB. Neutrophils were incubated for 24 hours in the absence (medium) or presence of TNF-α (10 ng/ml), after which MIP-1α in the supernatants was assessed by ELISA. Data are expressed as the mean (SEM) of triplicate cultures of three independent experiments. * p values < 0.05 v medium alone.

Figure 6 Correlation between SF neutrophil associated (A) or SF MIP-1α (B) and SF MNC counts. Each point represents an individual RA patient.
Synovial fluid neutrophil expression of MIP-1α

Figure 4 shows that there was a significant correlation between serum CRP and both cell associated MIP-1α levels ($r_s=0.503$, $p=0.022$) and MIP-1α concentrations in SF ($r_s=0.509$, $p=0.021$). In contrast, there was no correlation between MIP-1α and CRP concentrations in either SF or serum from OA patients ($r_s=0.003$, $p=0.903$; data not shown); nor was there a correlation between MIP-1α levels and the use of NSAIDs, oral or intra-articular glucocorticoids, or slow acting antirheumatic drugs (data not shown).

**EFFECTS OF TNF-α ON THE SECRETION OF NEUTROPHIL DERIVED MIP-1α**

We next examined the effects of TNF-α on MIP-1α secretion. In the absence of TNF-α, RA SF neutrophils secreted 809 pg/ml of MIP-1α (fig 5) over the course of a 24 hour incubation period. Neutrophils obtained from normal and rheumatoid PB, by contrast, secreted only 22 pg/ml and 29 pg/ml, respectively, over the same period. Twenty four hours in the presence of TNF-α increased MIP-1α secretion from all three neutrophil populations; none the less, secretion from RA SF neutrophils remained significantly higher (928 pg/ml) than from normal (174 pg/ml) or rheumatoid PB neutrophils (131 pg/ml).

**CORRELATION BETWEEN MONONUCLEAR CELL INFILTRATION OF THE SF AND THE LEVELS OF NEUTROPHIL ASSOCIATED AND SECRETED MIP-1α**

MIP-1α, which is a member of the C-C chemokine family, is a lipopolysaccharide (LPS) inducible, heparin binding polypeptide first isolated from LPS treated murine RAW 264.7 cells.21 22 In a recent study, we showed that MIP-1α was present in arthritic joints during the evolution of murine collagen induced arthritis (CIA), an experimental model of RA; moreover, exacerbation of CIA could be prevented by administration of neutralising anti MIP-1α antibodies.22 This strongly suggests that MIP-1α plays a pivotal part in the development of RA.

Chemokines (for example, IL8, MCP-1, and MIP-1α) are readily detectable in rheumatoid SF, and their values correlate with RA disease activity.25 29–32 These findings led us to speculate that the presence of chemokines in rheumatoid SF might, in part, depend on SF neutrophils, which are the principal cell type accumulated in the SF of RA patients. The main finding of this study was that freshly isolated RA SF neutrophils express significant quantities of MIP-1α and its transcript, and that this expression was well correlated with RA disease activity. In addition, secretion of MIP-1α was significantly augmented by TNF-α, suggesting that in vivo, RA SF neutrophils are primed by exposure to mediators (for example, TNF-α) within rheumatoid joints. Clinically, synovial inflammation in RA is characterised by marked infiltration by MNCs and neutrophils into the synovial space and into synovial tissue. Although MNCs isolated from the SF of patients with RA contained higher levels of MIP-1α (325.7 (111.9) pg/million cells; data not shown) than did RA SF neutrophils (58.6 (20.2) pg/million cells), the latter probably contribute more significantly to the increased MIP-1α levels in RA, because neutrophils are generally present in much larger numbers. In addition, we observed that both SF neutrophil associated MIP-1α and secreted MIP-1α were well correlated with the numbers of MNCs present in the rheumatoid SF. Thus, it seems probable that increased concentrations of neutrophil associated and secreted MIP-1α may be indicative of both local and systemic inflammation in RA, and that MIP-1α may stimulate recruitment of MNCs from the bloodstream into synovial joints.

In conclusion, the data presented here shed new light on the function of SF neutrophils in perpetuating inflammation in RA. Increased understanding of the role of SF neutrophils in RA may provide the basis for a new approach to therapeutic immunointervention in the disease process, whereby activated neutrophils and neutrophil derived MIP-1α in the synovial space are targeted.

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Macrophage inflammatory protein 1 alpha expression by synovial fluid neutrophils in rheumatoid arthritis

Yoshihito Hatanaka, Tsuyoshi Kasama, Hideaki Iwabuchi, Ryosuke Hanaoka, Hiroko T Takeuchi, Lu Jing, Yoshiaki Mori, Kazuo Kobayashi, Masao Negishi, Hirotsugu Ide and Mitsuru Adachi

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