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The Participation of IL-8 in the Synovial Lesions at an Early Stage of Rheumatoid Arthritis

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TAKAHASHI, Y., KASAHARA, T., SAWAI, T., RIKIMARU, A., MUKAIDA, N., MATSUSHIMA, K. and SASAKI, T. *The Participation of IL-8 in the Synovial Lesions at an Early Stage of Rheumatoid Arthritis.* Tohoku J. Exp. Med., 1999, **188** (1), 75-87 —— Synovial tissues from Rheumatoid Arthritis (RA) were divided into three groups based on their histopathological findings and compared for their expression of IL-8 and monocyte chemotactic and activating factor (MCAF) by using immunohistochemistry and in situ hybridization. The levels of IL-8 as well as those of MCAF were markedly higher in the synovial fluid from RA joints. Synovial lining cells (SLC) and macrophages had an ability to produce IL-8 at an early phase of the disease. The presence of MCAF was restricted in macrophages at this stage. On the other hand, the production of IL-8 as well as MCAF were prominent in most components of the joints such as SLC, migrated monocytes, sublining fibroblastoid cells, endothelial cells or migrated neutrophils at an active phase. The expression of IL-8 or MCAF was low in fibrotic synovitis of RA. These data indicate that IL-8 generated from SLC and macrophages may participate to the inflammatory process in the early synovitis of RA. ——
rheumatoid arthritis; interleukin-8; monocyte chemotactic and activating factor; in situ hybridization; immunohistochemistry

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Rheumatoid arthritis (RA) is a chronic inflammatory synovitis characterized by marked hyperplasia of the synovial lining cells and by extensive infiltration of macrophages and leukocytes (Krane and Simon 1986). It has been well documented that various types of cytokines (Feldmann et al. 1990) or growth factors (Kumkumian et al. 1989), including interleukin-1 β(IL-1β) (Fontana et al. 1989), IL-6 (Waage et al. 1988), tumor necrosis factor-α (TNF-α) (Digiovini et al. 1988), or others, are responsible for the process of inflammation in RA (Xu et al. 1989). A large number of mononuclear cells and neutrophils may also participate in the inflammatory and destructive phase (Jones et al. 1991; Chathan et al. 1993). It has also been demonstrated that the destruction of joints is attributed to the prolonged migration of chemotactic factor-secreting cells into the inflamed joints.

Neutrophils show chemotaxis which is caused by stimulants such as small peptides (Schiffmann et al. 1975) and complements (Fernandes et al. 1978). IL-8, one of recently discovered chemokines (Matsushima et al. 1988) also has the ability to cause chemotaxis and activate neutrophils (Mukaida et al. 1989). This cytokine is known to cause the emigration of neutrophils into synovial fluid (Brennan et al. 1990), resulting in the promotion of joint inflammation and neutrophil-mediated cartilage degradation (Seitz et al. 1991). In this process, recruitment of macrophage into the synovial tissues (Walz et al. 1987) is necessary for the continuous cytokine release which induces persistent synovitis. The secreted chemokine, monocyte chemotactic and the activating factor (MCAF), induce the production of superoxide anion, and the release of a lysosomal enzyme from monocytes though the autocrine and/or paracrine mechanisms. It has been also demonstrated that various types of cells from RA joints can produce IL-8 and MCAF in vitro (Cushing et al. 1990; Yoshimura and Leonard 1990), and IL-8 or MCAF exist in a high concentration in the synovial fluid of the RA joint (Koch et al. 1991, 1992). Furthermore, in the cases studied, IL-8 is present in the synovial lining cells in 87% of RA, and in 62% of osteoarthritis (OA) when determined by immunohistochemical techniques (Deleuran et al. 1994). However, it is not yet elucidated whether these cytokines are produced in synovium in vivo.

Materials and Methods

Subjects

Synovial tissues were obtained from 24 RA and 6 OA patients who underwent arthroscopic biopsy or total knee joint replacement. Patients with RA met the American College of Rheumatology's criteria for the diagnosis of RA (Arnet et al. 1988), and all including OA gave an informed consent for the study. Normal synovial tissues were obtained from patients undergoing knee arthroscopy for traumatic ligament lesion.

IL-8 and MCAF assay

The levels of IL-8 and MCAF in serum and synovial fluid from RA or OA cases were determined by a sensitive EILSA described previously (Ko et al. 1992) or by a competitive inhibition radioimmunoassay (Kasahara et al. 1991).

Antibodies

Monoclonal anti-IL-8 antibody, WS-4 or anti-MCAF antibody, 26j was obtained according to the method described in an earlier paper (Kasahara et al. 1991; Ko et al. 1992). KP-1 (DAKO Japan Co., Ltd., Kyoto), monoclonal anti-macrophage antibody (Pulford et al. 1989) was used for the identification of macrophages in the synovium. UCHL-1 (DAKO Japan Co., Ltd.) (Norton et al. 1986) or L-26 (DAKO Japan Co., Ltd.) (Norton and Isaacson 1987) was also used for the identification of T or B lymphocytes in the synovium. Cell types of IL-8 and MCAF expression cells are determined on serial sections by using monoclonal antibodies (KP-1, L-26 and UCHL-1).

Tissue preparation

The synovial tissues obtained were fixed in 4% paraformaldehide (PFA) for 2 hours at room temperature (RT). They were dehydrated in ethanol and embedded in paraffin.

Immunohistochemistry

Immunohistochemistry was performed by a biotin-streptavidine method. Briefly, after deparaffinization, sections were treated with 0.3% H_2O_2 in methanol for 30 minutes to minimize any endogeneous peroxidase activity. Then nonspecific protein binding was blocked with 10% normal rabbit serum for 30 minutes at RT. Consequently, the sections were treat with 0.1% trypsin (Sigma Chemical Co., St. Luis, MO, USA) 0.05 M Tris-HCl, pH 7.6 for 5 minutes at RT. After rinsing in 0.01 M phosphate buffer saline (PBS), the primary antibody (anti-IL-8 antibody, anti-MCAF antibody) was applied on each specimen, and slides were incubated overnight at 4°C. Subsequently, the slides were added on biotinized anti-mouse IgG+IgM (DAKO Japan Co., Ltd.), incubated for 60 minutes at RT, and rinsed in 0.01 M PBS. The slides reacted with streptavidine (Histfin, Nichirei Co.) for 30 minutes at RT, and then developed in a solution of 0.05% 3,3' diaminobenzidine detrahydrochloride (Grade III, Sigma Chemical Co.) in 0.05 M Tris-HCl buffer, pH 7.6 and 0.01% H_2O_2.

Histological classification of RA synovial tissues

RA synovial tissues were classified in three groups, histologically (Yates and Scott 1975). (A) early synovitis (exudative phase): Slight synovial lining cell proliferation and mononuclear cell infiltration within one year from onset the RA.

(B) active synovitis: Marked synovial lining cell proliferation and mononuclear cell infiltration. (C) fibrotic synovitis: Predominant fibrosis without mononuclear cell infiltration.

In situ hybridization

In situ hybridization (ISH) for IL-8 or MCAF mRNA was performed as described in our previous paper (Miyamasu et al. 1995). Namely, the section was treated in 0.2 N HCl for 10 minutes. After rinsing in 0.01 M PBS, the slides were dehydrated and dried. They were then treated with predigested pronase (250 ng/ml) (Calbiochem Co., San Diego, CA, USA) for 15 minutes at RT. After post fixation was performed in 4% PFA for 20 minutes at RT, the sections were treated with 0.25% (w/w) acetic anhydride/0.1M triethanolamine for 10 minutes. After rinsing in 2×SSC (standard saline citrate buffer) for 4 minutes twice, the sections were dehydrated and dried. The hybridization was carried out in a mixture containing 50% formamide, 0.3 M NaCl, 1 mM EDTA, 10 mM Tris HCl, 1xDenhardt's solution, 80 μg/ml salmon sperm DNA, 550 μg/ml yeast tRNA, and 50 pmol/ml degoxigenin-labeled IL-8 oligonucleotide probe (Oncogen Science, Union dale, NY, USA) or 30 μg/ml degoxigenin-labeled MCAF cDNA probe for 15 hours at 45°C. Sections were rinsed with 2×SSC twice, each time for 10 minutes, and with 2×SSC for 1 hour at RT, and with 0.1×SSC twice, each time for 30 minutes at 45°C.

Immunological detection

Immunological detection was performed according to the recommendations made by Boehringer (Mannheim, Germany). Briefly, the slides were incubated with the alkaline phosphatase conjugated anti-digoxigenin antibody for 2 hours and developed by nifroblue tetrazolium salt and 5-bromo 4-chloro-3-indolylphosphate containg levamisole (Sigma Chmical Co.) using a DIG Nucleic Acid Detection Kit (Boehringer).

We estimated the expression of protein or mRNA on the cell types of synovium by grading as 0 (positive cells <0), $1+(0<$positive cells$<25\%)$, $2+$ $(25\%<$positive cells$<50\%)$, $3+(50\%<$positive cells). In all cases, five to ten high-power fields ($\times 400$) were examined and a minimum of five handred cells were counted independently by two observers.

Statistical analysis

Values are presented as means$\pm$S.E. Statistical evaluation of difference was performed by Student's t-test. The criterion for statistical significance was $p<0.05$.

Probe presentation

The IL-8 oligonucleotide probe is 40 bases in length (Mukaida et al. 1989) and

was labeled by Oligonucleotide Labeling Kit (Boehringer).

The MCAF cDNA probe employed was a PST-1-digested fragment, with 400 base-pair from pUC9 (Kasahara et al. 1991), and labeled with a DNA Labeling Kit (Boehringer).

Result

The levels of IL-8 and MCAF in serum and synovial fluid

IL-8 was undetectable (<20 pg/ml) in RA sera as well as in OA sera. On the other hand, the levels of IL-8 were markedly higher in RA synovial fluid than those in OA (RA: 4493.5 ± 8387.7 pg/ml; OA: 34.23 ± 7.33 pg/ml), as shown in Fig. 1.

The levels of MCAF were higher in sera as well as in synovial fluid from RA compared to those from OA (RA: sera 2.06 ± 1.38 ng/ml, synovial fluid 6.60 ± 5.11 ng/ml; OA: sera 0.61 ± 0.48 ng/ml, synovial fluid 1.75 ± 1.34 ng/ml (Fig. 1).

Immunohistochemical localization of IL-8 and MCAF

According to the described method, we tried to stain IL-8 in synovial cells of RA or OA. The results showed positive for various types of cells except lymphocytes in RA (Figs. 2A and B) and (Table 1). In contrast with RA, we failed to detect antigenic IL-8 in OA in synovial tissues.

Anti-MCAF-reactive cells were also demonstrated on synovial lining cells, migrated monocytes, sublining fibroblastoid cells, and entothelial cells in RA synovial tissues (Figs. 2C and D) and (Table 2). The distribution of MCAF-positive cells was compatible with that of IL-8-positive cells at an active phase of RA.

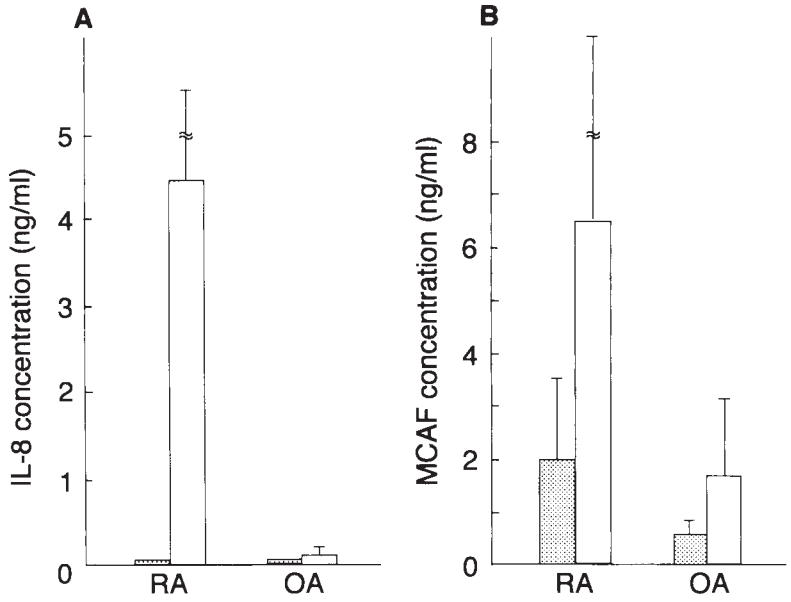

Fig. 1. Antigenic IL-8 and MCAF levels in sera ( ) or synovial fluid ( ) from rheumatoid arthritis (RA) and osteoarthritis. Results represent the mean $\pm$ S.E. Six replicate determinations/patients were performed.

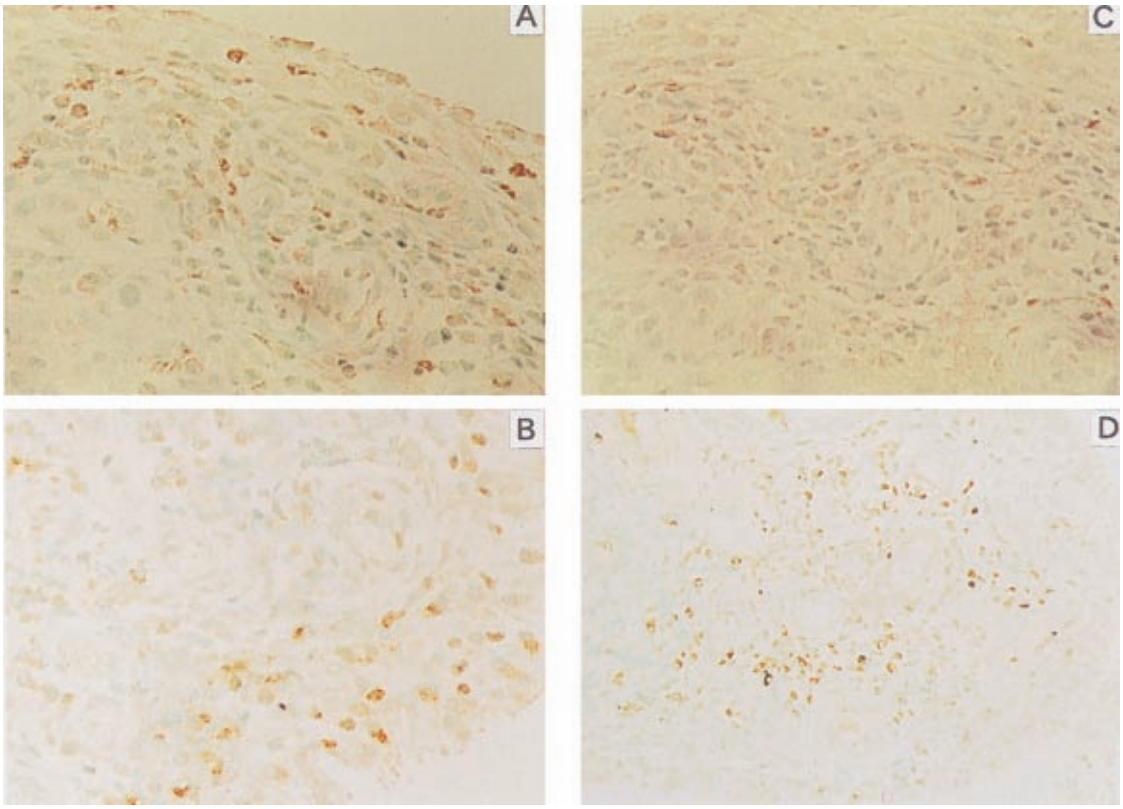

Fig. 2. Immunohistochemical demonstration of IL-8 (A, B) and MCAF (C, D) expression in synovial tissue of RA patient.
(A) early synovitis: Immunoreactive IL-8 was observed on synovial lining cells. (B) active synovitis: Immunoreactive IL-8 was observed on synovial lining cells, monocytes, fibroblasts, and endothelial cells. (C) early synovitis: Immunoreactive MCAF was scarecely observed in synovial tissue. (D) active synovitis: Immunoreactive MCAF was observed on synovial lining cells, monocytes, fibroblasts, and endothelial cells. (A, B, C, original magnification ×400; D, original magnification ×200)

Expression of IL-8 and MCAF mRNA on synovial tissues

IL-8 mRNA was present mainly in monocytes and synovial lining cells but rarely in fibroblasts or other types of cells in early synovitis (Table 1, Fig. 3A). On the other hand, presence of IL-8 mRNA was markedly increased in a variety of cells in active synovitis. Namely, an ISH study revealed that IL-8 mRNA was present in synovial lining cells, migrated monocytes, subliming fibroblastoid cells, endothelial cells, and migrated neutrophils in the synovial tissues but not in lymphocytes (Table 1, Fig. 3B). IL-8 mRNA was, however, undetectable by using the sense probe (data not shown). In fibrotic synovitis, IL-8 mRNA was detected slightly in each component of RA joints (Fig. 3C). The expression and the distribution pattern of the cells for IL-8 mRNA were similar with those of IL-8 antigenic in all samples and tended to be well correlated with the histological activity (Table 1, Fig. 3). We could scarcely detect IL-8 mRNA in OA synovial tissues or in normal synovium. The presence of MCAF and MCAF mRNA were

TABLE 1. *Expression of antigenic IL-8 and IL-8 mRNA in synovial cells*

Cases		Stage	Synovial cells					
			SLC	Mϕ	Fibroblast	Vessel	PMN	Ly
Group A	1	I	++/++[a]	++/++[a]	−/−[a]	−/−[a]	+/+[a]	−/−[a]
	2	I	++/+++	+/+	−/−	−/+	++/++	−/−
	3	I	++/++	++/++	−/−	−/−	−/−	−/−
	4	I	+++/+++	+/+	−/−	−/−	−/−	−/−
	5	I	++/++	+/+	−/−	−/−	++/++	−/−
	6	I	+++/+++	−/−	−/−	−/−	−/+	−/−
Group B	1	IV	+++/+++	++/+++	+/+	+/+	+++/+++	−/−
	2	II	+++/+++	+++/+++	++/++	++/++	+++/+++	−/−
	3	IV	+++/+++	++/+++	+/+	+/+	+++/+++	−/−
	4	IV	++/++	+++/++	++/++	++/+	++/++	−/−
	5	III	+++/+++	+++/+++	++/++	++/++	+++/+++	−/−
	6	IV	++/++	++/++	+/+	−/−	−/−	−/−
	7	IV	++/++	++/++	++/++	−/−	−/+	−/−
	8	III	+++/++	++/++	+/++	−/−	−/+	−/−
	9	III	++/++	++/++	+/+	+/+	++/++	−/−
	10	III	++/++	++/++	++/++	+/+	++/++	−/−
	11	IV	++/++	++/++	++/++	+/++	++/++	−/−
	12	III	++/++	+++/++	−/−	−/−	−/−	−/−
Group C	1	IV	+/+	++/+	+/+	+/−	+/+	−/−
	2	IV	−/−	++/++	+/+	−/−	+/+	−/−
	3	IV	+/+	++/+	+/+	−/−	−/+	−/−
	4	IV	+/+	+/+	−/−	+/−	−/−	−/−
	5	III	+/+	+/+	+/+	−/−	−/−	−/−
	6	IV	+/−	+/+	+/+	−/−	−/−	−/−
Group D	1		−/+	−/−	−/−	−/−	−/−	−/−
	2		−/−	−/−	+/−	−/−	+/−	−/−
	3		−/−	+/−	−/−	+/−	−/−	−/−
	4		n.d./−	n.d./−	n.d./+	n.d./−	n.d./−	n.d./−
	5		−/−	−/−	+/+	−/−	−/−	−/−
	6		−/n.d.	+/n.d.	+/n.d.	−/n.d.	−/n.d.	−/n.d.

Group A, B and C denote patients with RA showing early synovitis, active synovitis and fibrotic synovitis.

Group D denotes patients with osteoarthritis.

The expression of protein or mRNA on the cell types of synovium was graded as- (positive cells < 0, 1+ [0 < positive cells < 25%], 2+ [25% < positive cells < 50%], 3+ [50% < positive cells]).

n.d., not done.

[a]antigenic IL-8/IL-8 mRNA

SLC, synovial lining cell; Mϕ, macrophage; PMN, polymorphonuclear cell; Ly, lymphocyte.

TABLE 2. *Expression of antigenic MCAF and MCAF mRNA in synovial cells*

Cases		Stage	Synovial cells					
			SLC	Mϕ	Fibroblast	Vessel	PMN	Ly
Group A	1	I	−/−[a]	++/++[a]	−/−[a]	−/−[a]	−/−[a]	−/−[a]
	2	I	−/−	+/+	−/−	−/−	−/−	−/−
	3	I	+/+	+/++	+/+	−/+	−/−	−/−
	4	I	−/−	++/++	−/−	−/−	−/−	−/−
	5	I	−/−	+/+	−/−	−/−	−/−	−/−
	6	I	−/−	+/+	−/−	−/−	−/−	−/−
Group B	1	IV	++/++	+++/+++	+/+	+/+	−/−	−/−
	2	II	++/++	+++/+++	+/+	+/+	−/−	−/−
	3	IV	++/++	++/++	++/++	+/+	−/−	−/−
	4	IV	++/++	+++/++	++/+	+/+	−/−	−/−
	5	III	++/++	+++/++	++/+	+/+	−/−	−/−
	6	IV	+/+	++/+++	+/+	+/+	−/−	−/−
	7	IV	+/+	++/++	+/+	+/+	−/−	−/−
	8	III	+/++	+/++	+/+	+/+	−/−	−/−
	9	III	−/−	++/+	+/+	−/−	−/−	−/−
	10	III	++/++	++/++	+/+	−/−	−/−	−/−
	11	IV	+/+	++/+	+/+	+/+	−/−	−/−
	12	III	+/+	++/+	++/+	+/+	−/−	−/−
Group C	1	IV	−/−	−/−	+/−	−/−	−/−	−/−
	2	IV	−/−	+/+	−/−	−/−	−/−	−/−
	3	IV	−/−	+/+	−/−	−/−	−/−	−/−
	4	IV	+/+	++/+	+/+	−/−	−/−	−/−
	5	III	−/−	+/+	+/+	−/−	−/−	−/−
	6	IV	−/−	−/−	−/−	−/−	−/−	−/−
Group D	1		−/−	−/−	−/−	−/−	−/−	−/−
	2		−/−	+/−	−/−	−/−	−/−	−/−
	3		−/n.d.	+/n.d.	−/n.d.	−/n.d.	−/n.d.	−/n.d.
	4		n.d./−	n.d./−	n.d./−	n.d./−	n.d./−	n.d./−
	5		−/−	−/−	−/−	−/−	−/−	−/−
	6		−/n.d.	+/n.d.	+/n.d.	−/n.d.	−/n.d.	−/n.d.

Group A, B and C denote patients with RA showing early synovitis, active synovitis and fibrotic synovitis.

Group D denotes patients with osteoarthritis.

The expression of protein or mRNA on the cell types of synovium was graded as (positive cells < 0, 1+ [0 < positive cells < 25%], 2+ [25% < positive cells < 50%], 3+ [50% < positive cells]).

n.d., not done.

[a]antigenic MCAF/MCAF mRNA.

SLC, synovial lining cell; Mϕ, macrophage; PMN, polymorphonuclear cell; Ly, lymphocyte.

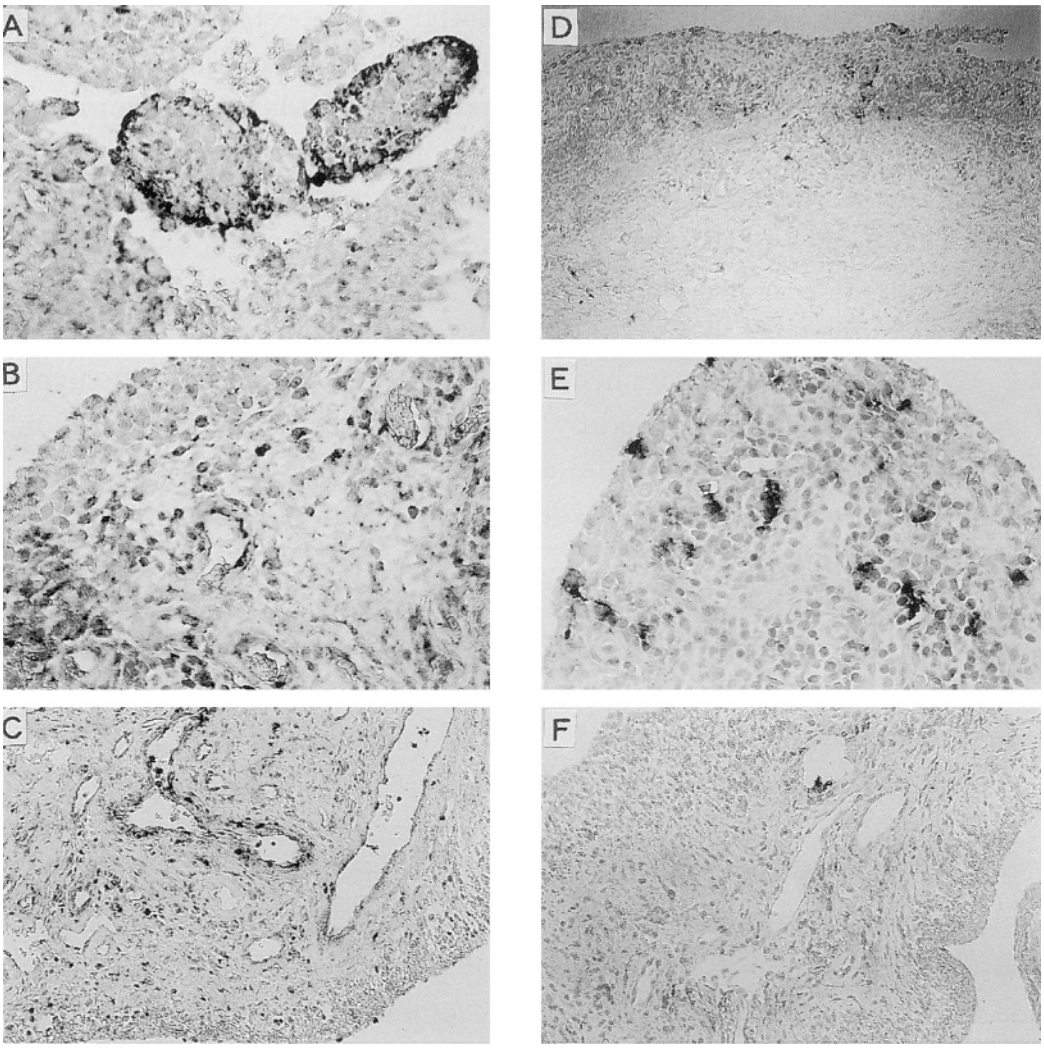

Fig. 3. Expression of IL-8 and MCAF genes.
A, B and C show IL-8 mRNA in RA synovium at an early synovitis, an active synovitis and a fibrotic synovitis. D, E and F show MCAF mRNA in RA synovium at an early synovitis, an active synovitis and a fibrotic synovitis. (A, B, C, E, F, original magnification ×400; D, original magnification ×200)

also tested in each specimen from synovium. Results revealed that the existence of MCAF mRNA was rare in most cells except monocyte at an early stage (Table 2, Fig. 3D). Most cells composed of synovial tissues, however, began to produce MCAF in active synovitis (Fig. 3E). On the other hand, MCAF mRNA as well as IL-8 were not existent in OA synovial tissues.

Discussion

In the present study, we should the participational IL-8 in RA synovium and MCAF in RA synovium, where the samples are classified into three groups based mainly on their histopathological findings in the joints (Yates and Scott 1975). The histological findings of rheumatoid synovium are characterized by extensive invasion of proliferation of inflammatory cells which are responsible for the process of cytokine production (Krane and Simon 1986; Harris 1990). The

resulting destruction of joints is mainly mediated by proteolytic enzymes which are released from monocytes/macrophages, fibroblasts, and neutrophils (Harris 1990). It has been well documented that neutrophils and macrophages are activated by IL-8 or MCAF, and our data confirm that the levels of IL-8 and MCAF in synovial fluid are markedly high, but those in sera are low (Koch et al. 1991, 1992; Akahoshi et al. 1993), although it was shown the expression of IL-8 mRNA was elevated in peripheral blood from patients with RA (Koops et al. 1997). These indicate IL-8 and/or MCAF might be derived from synovial cells in RA. By using immunohistochemistry and ISH techniques, we suggest here that IL-8 and MCAF are produced by synovial lining cells, migrated monocytes, sublining fibroblasts, and endothelial cells, in the synovium of RA joints. IL-8- and MCAF-producing cells are regarded as the same type of cells as shown by results in histological studies.

It has been demonstrated that both IL-8 and MCAF play an important role in the promotion of inflammation, and that the prolonged inflammation in RA is required for the continuous activation of the cells mentioned above. Our results may explain at least in part the mechanism associated with the extensive cell infiltration or activation of the inflammatory cells in an active phase of RA.

We also analyzed the distribution patterns of IL-8 and MCAF mRNA at an early stage of the disease, when an increased number of synovial lining cells is observed along with perivascular infiltration with lymphocytes. Extensive data has indicated that inflammation occurring at the initial stage may be attributable to tissue-infiltrating CD4(+) T cells. The cytokine from T cell origin such as IL-2 or IFN-γ are, however, present at low concentrations in RA joints (Firestein and Zvaifler 1990) or rather, IL-1β, TNF-α and other cytokines secreted from macrophages or other cells may account for the progression of the inflammation in the joints (Cutolo et al. 1993). It may be noted here that IL-8 was restrictedly positive in synovial lining cells except a small number of macrophages, indicating that IL-8, a chemokine, participates in the inflammatory process at an early stage of RA, when monocyte/macrophages are not yet prominent in the tissues. These findings are compatible with the findings in experimental arthritis induced by LPS and IL-1β (Akahoshi et al. 1994), indicating that synovial lining cells may be a target for the stimulation with some agents associated with RA in an early stage and the resulting stimulus with IL-1β or other cytokines induce the production of IL-8 or MCAF from SLE or macrophages (Hachicha et al. 1993; Rathanaswami et al. 1993). Thus, local production of IL-8 in SLC can evoke inflammation causing pathologic and clinical manifestation in an early stage of RA. MCAF may then enhance synergenically with IL-8 and maintain the inflammation at an active phase.

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