Monocyte Chemotactic Factor in Rheumatoid Arthritis Synovial Tissue

PROBABLY A CROSS-LINKED DERIVATIVE OF S19 RIBOSOMAL PROTEIN*

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The extracts of rheumatoid arthritis-synovial lesions from seven patients possessed a strong chemotactic activity for monocytes and a negligible one for polymorphonuclear leukocytes. These results are consistent with a prominent histological feature of the synovial lesion, the mononuclear cell predominant infiltration. The major monocyte chemotactic factor in the synovial tissue extracts was purified to a single protein peak in reverse phase high performance liquid chromatography with a C4 column. NH2-terminal amino acid analysis of tissue extracts was purified to a single protein peak in reverse phase high performance liquid chromatography and PAGE. PAGE, polyacrylamide gel electrophoresis. 

The major monocyte chemotactic factor in the synovial lesion, the mononuclear cell predominant infiltration. The extracts of rheumatoid arthritis-synovial lesions from seven patients possessed a strong chemotactic activity for monocytes and a negligible one for polymorphonuclear leukocytes. These results are consistent with a prominent histological feature of the synovial lesion, the mononuclear cell predominant infiltration. The major monocyte chemotactic factor in the synovial tissue extracts was purified to a single protein peak in reverse phase high performance liquid chromatography with a C4 column. NH2-terminal amino acid analysis of tissue extracts was purified to a single protein peak in reverse phase high performance liquid chromatography and PAGE. PAGE, polyacrylamide gel electrophoresis.

Macrophages play important roles not only in the host defense but also in allergic reactions. In many types of immunologic inflammatory lesions including synovial tissue of rheumatoid arthritis (RA),1 macrophages are the predominant cells in the leukocyte infiltrate (1); however, in certain situations such as the reaction in synovial cavities of RA, macrophages are only a minor population, and polymorphonuclear leukocytes (PMNs) are most predominant (2, 3). The majority of the macrophages in the extravascular foci of inflammation are thought to be derived from blood monocytes, being attracted by chemotactic factors produced locally. A number of molecules possessing the chemoattracting capacity to monocytes are now known; for instance, complement C5a (4), a complement factor H derivative (5, 6), monocyte chemoattractant protein-1 (MCP-1) (7), tetranectin (8), and formyl-methionyl peptides (9, 10). However, many of them attract PMNs as well. To explain the different histopathological features regarding the macrophage infiltration in synovial tissues versus synovial cavities of RA as described above, we have been investigating chemical mediators that participate in the phenomena. We have already reported the presence of a strong inhibitory factor to the monocyte chemotaxis in RA-synovial fluid (11, 12), which seemed to be responsible for the PMN-predominant/monocyte-poor infiltration in RA-synovial cavities. Additionally, we have described the presence of a monocyte chemotactic factor in human serum (13) as well as in guinea pig serum (14), which is generated concurrently with plasma clotting. This factor does not attract PMNs. The monocyte-specific chemotactic factor is initially present as a precursor molecule. It is converted to the active form by a transglutaminase-catalyzed reaction (13).

The initial purpose of the present project was to analyze and identify a monocyte chemotactic factor(s) that would play a major role in the monocyte predominant infiltration in the RA-synovial tissue. In this report, we describe a surprising result that the major monocyte specific chemotactic factor in RA-synovial tissue seems to be S19 ribosomal protein (RP S19) itself or its homologue cross-linked by the transglutaminase catalyzed reaction.

EXPERIMENTAL PROCEDURES

Materials and Reference Compounds—Hank’s balanced salt solution (HBSS) and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. (Tokyo, J. Japan). Bovine serum albumin, cytochrome-C, ovalbumin, bovine γ-globulin, zymozan, dns-cadaverine (lysine doner) and p-chloromercuriphenyl sulfonic acid were purchased from Sigma. Purified human factor XIII was a gift from the Institute of the Chemotherapeutic Co. (Tokyo, J. Japan). Toyopearl HW-55S and CM-Toyopearl 650 M were obtained from Tosoh Company (Tokyo, J. Japan). DEAE-Sephadex A-50, protein G-Sepharose 4 Fast Flow, a Superdex 200 HR 10/30 column, CNBr-activated Sepharose 4B beads, and ampholine were purchased from Pharmacia Biotech Inc. Mouse anti-human MCP-1 monoclonal antibody was a gift from Dr. Yoshimura (National Cancer Institute). Anti-human complement C5 sheep IgG antibody was a product of Birmingham Research Institute (Birmingham, UK). A multiwell chamber for chemotaxis assay was obtained from Neuro Probe (Bethesda, MD). Nuclope filters were purchased from Nuclope (Pleasanton, CA). Elastatin, leupeptin, E-64, phosphoramidon, dipyrole fluorophosphate, pepstatin, and N-ethylmaleimide were purchased from Peptide Institute (Osaka, J. Japan). α-Phe-Pro-Arg-chloromethyl ketone was purchased from Calbiochem (La J. Jila, CA). So-
dium deoxycholate was purchased from Difco. 6-Amino-n-caproic acid and all other chemicals were obtained from Wako Pure Chemicals (Osaka, J. apan). Zymosan-activated human plasma (ZAP) was prepared according to the method of Fernandez (4) with a modification as described previously (11). IgG antibodies against human complement C4 or C5 and cytokine MCP-1 were coupled to CNBr-activated Sepharose 4B, respectively, by the method of Porach and Axen (15).

Preparation of RA-synovial Tissue Extract—RA-synovial tissues were surgically removed from the knee joint of RA patients. They were immediately frozen and weighed (g) and then kept at −80°C until use. They were sliced at 50 μm thick using a cryomicrotome and extracted for 4 h at 4°C with phosphate-buffered saline (PBS), pH 7.2, containing 0.1% n-propanol, 0.1 mM EDTA, 5 mM 0.1 mM p-amidinophenylmethanesulfonyl fluoride hydrochloride (p-APMSF), 0.1 mM elastatinal, 0.1 mM chymostatin, 0.1 mM leupeptin, 0.1 mM E-64, 0.1 mM phosphoramidom, and 0.1 mM bestatin at a ratio of 250 ml of the PBS solution to 100 g of tissue. After centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant was used as RA-synovial tissue extract.

Assay of Transglutaminase Activity—The transglutaminase activity of factor XIIIa was measured by a modified method of the Loran et al. (16) using an assay kit, Iatro-F L XIIIII (Iatron Laboratories, Tokyo, J. apan). One unit was defined as the activity contained in one ml of normal human plasma as factor XIII.

Chemotaxis Assays—Monocytes and PMNs were isolated from heparinized human venous blood of healthy donors according to the method of Falk et al. (11, 12). The monocytes and PMNs were suspended at a concentration of 1 × 10^7 cells/ml in HBSS containing 0.5% bovine serum albumin or in RPMI 1640, pH 7.2, for the morphologic polarization assay or for the multiwell chamber assay. The morphologic polarization assay was performed according to the method of Cianciolo and Snyderman (17) as described previously (11, 12). Unless otherwise specified, the assay samples were diluted at least 10-fold with HBSS prior to the assay. As a positive control and a negative one, 1% (v/v) ZAP, and HBSS were used as chemoattractants, respectively. The activity of the samples was initially calculated as the percentage of monocytes or PMNs with polarized morphology composed with the total numbers of cells counted. The ratio of polarized cells in percent was proportional to the logtransiment change of conformation of monocytes or PMNs.

Preparation of Ribosomal Protein Fractions—The total number of nucleotides that had migrated beyond the lower membrane was counted in five high power fields. As a positive control and a negative one, 1% (v/v) ZAP, and HBSS were used as chemoattractants, respectively. The activity of the samples was initially calculated as the percentage of monocytes or PMNs with polarized morphology composed with the total numbers of cells counted. The ratio of polarized cells in percent was proportional to the logarithmic change of conformation of monocytes or PMNs.

Tissue Extract—Brief Analysis of Monocyte Migration Factors in RA-synovial Tissue Extract—We examined migration attracting potencies of the RA-synovial tissue extract to monocytes and PMNs using the morphologic polarization assay. RA-synovial tissues obtained from seven RA patients were individually extracted, and the seven RA-synovial extracts were assayed separately. The results are shown in Fig. 1 as the mean values. The RA-synovial extracts possessed the polarization activity for monocytes but not for PMNs. Since the differences among the samples were very small in regard to the polarization activities, a mixture of the extracts obtained from the seven RA patients was used in the following experiments.

Brief Analysis of Monocyte Migration Factors in RA-synovial Tissue Extract—Molecular size analysis of monocyte migration factors in the RA-synovial tissue extract by gel permeation protein fractions were used in the present experiments.

Preparation of Recombinant RP S19-Maltose Binding Protein Fusion Protein—DNA was prepared from HepG2 cell mRNA by the common method using reverse transcriptase oligo(dT) and random primer (Takara). Two primers consisting of nucleotides based on cDNA sequences of human RP S19 were obtained from EMBL sequence data base; (i) a sense primer consisting of nucleotides 1–30, namely 5′-ATGCCCTGGAGTTACTGTAAA-3′ and (ii) an antisense primer consisting of nucleotides 423–453 in which G446 and T448 were changed to C and A, respectively, to construct the HindIII restriction site, namely 5′-TGGTCTGAGATCTCGAAACGAC-3′. Using these primers and HepG2 DNA, nucleotides coding the full length of the human RP S19 were prepared by the polymerase chain reaction with PuF DNA polymerase (Stratagene) (26, 27). The RP S19 nucleotides were processed with HindIII and were ligated to pMAL-c2 vector, which had been processed with XmnI and HindIII. The constructs were transfected into the competent Escherichia coli, JM109 cells by the heat shock method, and positive transformants were cloned. One of the positive clones was cultured, and the correct insertion of RP S19 was confirmed by making the restriction map. The cloned JM109 E. coli cells were cultured in Rich medium containing glucose (2 gliter) and ampicillin (100 gliter) and stimulated to synthesize the recombinant protein by adding isopropyl-D-1β-D-galactopyranoside for 2 h at 37°C. After washing by centrifugation for 20 min at 2,500 rpm, the JM109 cells were resuspended in a buffer (10 mM Tris-HCl containing 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 1 mM dithiothreitol), which could then be used for amylase resin column chromatography. The JM109 cells were broken by sonication, and periplasmic products were separated by centrifugation at 6,400 rpm for 30 min into the supernatant. This supernatant was applied to the amylose resin column. After extensive washing of the column, the recombinant fusion protein, MBP-RP S19 was eluted with 10 mM maltose in the same buffer. MBP-RP S19 was further purified by the Superdex 200 HR 10/30 column equilibrated with 10 mM Tris-HCl containing 200 mM NaCl, pH 7.5. The major fraction with an apparent molecular size of 60 kDa was used in the following studies.

RESULTS

Monocyte-directed Migration Activity of RA-synovial Tissue Extract—We examined migration attracting potencies of the RA-synovial tissue extract to monocytes and PMNs using the morphologic polarization assay. RA-synovial tissues obtained from seven RA patients were individually extracted, and the seven RA-synovial extracts were assayed separately. The results are shown in Fig. 1 as the mean values. The RA-synovial extracts possessed the polarization activity for monocytes but not for PMNs. Since the differences among the samples were very small in regard to the polarization activities, a mixture of the extracts obtained from the seven RA patients was used in the following experiments.

FIG. 1. Polarization inducing activity of RA-synovial tissue extract to monocytes and PMNs. Monocytes or PMNs were attracted with various concentrations of seven individual RA-synovial tissue extracts. The concentration on the horizontal axis means the final concentration of the samples. The lines with the closed circles or with the open circles denote the polarization responses of monocytes or PMNs, respectively. Values are mean ± S.D. (Three experiments were carried out for each extract).
activity in the minor peak fraction was partially absorbed either by the anti-MCP-1 antibody beads or by the anti-complement C5 antibody beads in a batch-wise method for 30 min at 20°C (data not shown).

Separation and Characterization of the Major Monocyte Migration Factor in RA-synovial Tissue Extract—The RA-synovial tissue extract (385 ml) was dialyzed against 10 mM phosphate buffer containing 40 mM NaCl, and 1 mM EDTA (pH 7.5) and subjected to anion-exchange column chromatography with DEAE-Sephadex A-50 (Ø, 25 × 390 mm; bed volume, 190 ml) equilibrated with the same buffer, at a flow rate of 12 ml/cm²-h at 4°C. The majority (about 80%) of the monocyte polarization activity passed through the column (data not shown). The active breakthrough fraction was dialyzed and applied to a cation exchange column with CM-Toyopearl 650W. As shown in Fig. 2b, the monocyte polarization activity totally bound to the column and was eluted with 140 mM NaCl in the gradient elution. The active fractions were pooled (280 ml) and applied to a protein G-Sepharose 4 Fast Flow column equilibrated with 10 mM phosphonate buffer containing 200 mM NaCl (pH 6.0) at a flow rate of 9 ml/h at 4°C. The whole activity passed through the column (data not shown). An aliquot of the breakthrough fraction of the protein G-Sepharose column chromatography was applied to the Superdex 200 HR 10/30 gel filtration column. As shown in Fig. 2c, the monocyte polarization activity was eluted as a single peak in a fraction, which corresponded to a molecular mass of 45 kDa. Using the active fraction of this gel permeation HPLC, the isoelectric point of the monocyte migration factor was examined. The monocyte polarization activity was focused at pH 8.1 (data not shown). Promotions of monocyte migration are classified into two categories, chemotaxis and chemokinesis. The effect of the monocyte migration factor was studied in this regard by the checkerboard analysis with the multiwell chamber. As shown in Table I, the monocyte migration factor was only effective when the concentration of the molecule was higher in the lower well than in the upper well, where the indicator cells were present. These results indicate that the effect of the monocyte migration factor was chemotaxis but not chemokinesis.

Purification of the Monocyte Chemotactic Factor—The remainder of the breakthrough fraction in the protein G column chromatography was applied to an anti-human complement C5 antibody column. The monocyte polarization activity was absorbed by the column except for a minor fraction (about 5% of the activity applied). After washing the column, the chemotactic factor was eluted with 0.1% trifluoroacetic acid. The active fraction eluted from the anti-C5 antibody column was pooled and analyzed by a reverse phase HPLC with the C4 column. A sharp single peak of absorbance at 235 nm associated with the monocyte chemotactic activity was eluted at an acetonitrile concentration of 47%. The molecules in the breakthrough possessing the absorbance at 235 nm had neither protein nature in the spectrum analysis nor chemotactic capacity. In the purification process, 5 µg of protein was obtained from 380 ml of the RA-synovial tissue extract.

NH₂₇-terminal Amino Acid Analyses—The protein eluted from the C4 column was analyzed by the protein sequencer. As shown in Fig. 3, the 20 NH₂-terminal amino acids of the synovial chemotactic factor were completely overlapped with those of human RP S19.

SDS-PAGE Analysis—As shown in Fig. 4, the peak fraction of the reverse phase HPLC demonstrated two silver staining bands in SDS-PAGE. The major band possessed the apparent molecular mass of 34 kDa and the minor one of 68 kDa. These molecular sizes are double and quadruple of the size of RP S19 (16 kDa).
the presence of 5 mM CaCl₂ for 20 min at 37°C. After this concentration, the 60 S ribosomal protein fraction nor the 60 S one elicited monocyte chemotaxis in the multiwell chemotaxis assay. The ribosomal protein subunits were prepared from bovine liver. Neither of the 40 S ribosome subunit. Ribosomal protein fractions of 40 and 60 S  

were treated with factor XIIIa (at a final concentration, 1 unit/ml) for 90 min at 37°C. In contrast to this, the 60 S ribosomal protein fraction was capable of attracting monocyte chemotaxis. In the absence of factor XIIIa, a significant amount of the monocyte chemotactic activity was observed after the incubation, although before the incubation, only negligible activity was observed. In SDS-PAGE analysis (Fig. 5b), with factor XIIIa, a band with an apparent molecular size 120 kDa newly appeared. Since the apparent molecular size of this band is double of MBP-RP S19 (60 kDa), the new band is thought to be a dimer of MBP-RP S19. The same experiments were carried out using simple MBP instead of MBP-RP S19. The same experiment was performed except for the presence of dns-cadaverine (lysine donor) and for a shorter incubation period (20 min). As shown in Fig. 6, the generation of the monocyte chemotactic activity significantly decreased as the dns-cadaverine concentration increased.  

**DISCUSSION**

Reflecting the histological picture of RA-synovial tissue, the chemotactic potency of all of the RA-synovial tissue extracts obtained from seven patients was much more potent for monocytes than for PMNs. As expected from previous immunohistochemical studies (1), multiple molecules that possess chemotactic capacity to monocytes were indeed present in the extract of the RA-synovial tissues including MCP-1 and C5a or its derivative (data not shown). However, the major chemotactic factor in the extract was not these previously known factors. The major factor seems to be a homodimer (to some extent a homotetramer) of MBP-RPS19. The same experiments were carried out using 48-well chemotaxis chamber. Vertical axis denotes the number of migrated monocytes beyond the lower membrane. Values are mean ± S.D. (Three experiments were carried out for each examination). b, samples (lane 1, 0 min; lane 2, 90 min) were electrophoresed in a 15% polyacrylamide gel, and stained by Coomassie Blue. The positions corresponding to factor XIIIa (80 kDa) and MBP-RP S19 (60 kDa) are shown at the right side.

**Fig. 4.** SDS-PAGE of the purified monocyte chemotactic factor. Approximately 30 ng of protein of the reverse phase HPLC fraction was electrophoresed in a 15% polyacrylamide gel and visualized by silver staining. The positions corresponding to three marker proteins are shown at the left side (67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase, respectively).

**Table I**

| In upper well | In lower well |
|---------------|--------------|
| H             | H            |
| 0             | 3.4 x 10⁻¹³  |
| 3.4 x 10⁻¹³   | 3.4 x 10⁻¹²  |
| 3.4 x 10⁻¹¹   | 3.4 x 10⁻¹⁰  |

**NH₂-terminal amino acid sequence**

| The synovial factor | PGVTKDVQGQVFRRALAF |
|---------------------|---------------------|
| Human S19 ribosomal protein | MGPVTKDVQGQVFRRALAF |

**Fig. 3.** NH₂-terminal amino acid sequence of the monocyte chemotactic factor. The chemotactic factor eluted from the C4 column was analyzed by the protein sequence for the initial twenty amino acid residues. Human RP S19 was picked up by the homology search using EMBL nucleic acid sequence data base. Amino acid sequence of the two proteins is comparatively shown.

**Fig. 5.** Factor XIIIa-mediated monocyte chemotactic activity of RP S19. MBP-RP S19 (final concentration 1 x 10⁻⁸M) was incubated with factor XIIIa (final concentration, 1 unit/ml) and 5 mM CaCl₂ at 37°C for 90 min. a, hatched columns denote monocyte chemotactic activity by chemotaxis assay using 48-well chemotaxis chamber. Vertical axis denotes the number of migrated monocytes beyond the lower membrane. Values are mean ± S.D. (Three experiments were carried out for each examination). b, samples (lane 1, 0 min; lane 2, 90 min) were electrophoresed in a 15% polyacrylamide gel, and stained by Coomassie Blue. The positions corresponding to factor XIIIa (80 kDa) and MBP-RP S19 (60 kDa) are shown at the right side.

Generation of Monocyte Chemotactic Activity in Ribosomal Protein Fraction—It is known that RP S19 is a component of 40 S ribosome subunit. Ribosomal protein fractions of 40 and 60 S subunits were prepared from bovine liver. Neither of the 40 S ribosomal protein fraction nor the 60 S one elicited monocyte chemotaxis in the multiwell chemotaxis assay. These ribosomal protein fractions were treated with factor XIIIa (at a final concentration 1 unit/ml) or the same volume of PBS, pH 7.2, in the presence of 5 mM CaCl₂ for 90 min at 37°C. After this treatment, the 40 S ribosomal protein fraction was capable of attracting monocyte chemotaxis. In contrast to this, the 60 S fraction exhibited a negligible activity even after the treatment with the transglutaminase (data not shown).

Potential Monocyte Chemotactic Activity in Ribosomal Protein Fraction—The purified MBP-RP S19 was incubated with factor XIIIa (final concentration, 1 unit/ml) and 5 mM CaCl₂ at 37°C for 90 min (Fig. 5a), a significant amount of the monocyte chemotactic activity was observed after the incubation, although before the incubation, only negligible activity was observed. In SDS-PAGE analysis (Fig. 5b), with factor XIIIa, a band (60 kDa) which would contain RP S19 appeared. Since the apparent molecular size of this band is double of MBP-RP S19 (60 kDa), the new band is thought to be a dimer of MBP-RP S19. The same experiments were carried out using simple MBP instead of MBP-RP S19. The same experiment was performed except for the presence of dns-cadaverine (lysine donor) and for a shorter incubation period (20 min). As shown in Fig. 6, the generation of the monocyte chemotactic activity significantly decreased as the dns-cadaverine concentration increased.
S19, exhibited monocyte chemotactic activity after the treatment with factor XIIIa. 4) The apparent molecular mass (34 and 68 kDa) of the monocyte chemotactic factor in the SDS-PAGE analysis (Fig. 4) were double and quadruple of that (16 kDa) of RP S19. (v) RP S19 possesses many glutamine (eight) and lysine (15) residues, which could be substrate residues of transglutaminase.

The monocyte chemotactic factor bound to the anti-C5 antibody beads. In the amino acid sequence of RP S19, no homologous portion to the sequence of C5 is present. Despite a lack of evidence, our speculation is that a tridimensional structure similar to C5a (C5-derived leukocyte chemotactic peptide) might appear on the dimer and oligomer molecules of RP S19 as a consequence of the transglutaminase-catalyzed intermolecular cross-linking.

We have previously reported the presence of a precursor molecule of monocyte chemotactic factor in plasma, which is converted to the active molecule by the transglutaminase-catalyzed reaction during plasma clotting (13). The relationship between the monocyte chemotactic factor in serum and RA-synovial tissue extract has not been fully elucidated. However, in consideration of their molecular mass and isoelectric point, the presence of a positive feedback circuit causing the production large amounts of the monocyte chemotactic factor in plasma. However, we cannot be sure whether factor XIIIa or cellular transglutaminase is the transglutaminase in the monocyte chemotactic factor formation in RA-synovial lesion. Recently, production large amounts of the monocyte-derived antigen-presenting cells was reported (28), and indeed the transglutaminase antigen is used for immunohistochemical identification of these cells (29). However, the presence of the monocyte-derived antigen-presenting cells in RA-synovial lesion has been well documented (1). Therefore, it is possible that the catalyst to construct the monocyte chemotactic factor in RA-synovial tissue is at least in part the cellular transglutaminase liberated from the blood monocyte-derived cells. Therefore, one could speculate the presence of a positive feedback circuit causing the monocyte/macrophage predominant infiltration via generation of the monocyte chemotactic factor by macrophage-derived transglutaminase in RA-synovial tissue lesions.

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