Urocortin in the synovial tissue of patients with rheumatoid arthritis

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ABSTRACT

Urocortin is a newly identified member of the corticotropin-releasing factor (CRF) neuropeptide family, and is known to be involved in the modulation of the inflammatory process. We examined the expression of urocortin, CRF and their receptors (CRF receptor; CRF-R) in the synovial tissue of patients with rheumatoid arthritis (RA) in order to study the possible biological roles of urocortin. Synovial tissues/fluids were obtained from 38 patients with RA, nine patients with osteoarthritis and four with trauma. We studied the concentration of urocortin in the synovial fluid using RIA, and the expression of urocortin in synovial tissue using immunohistochemistry, mRNA in situ hybridization and reverse transcriptase–PCR (RT-PCR). In addition, we examined the immunolocalization of CRF and the expression of CRF-R1, -R2-a and -R2-b mRNAs utilizing RT-PCR in these synovial tissues. Urocortin concentrations in synovial fluid were higher in RA patients (79.8±154 pg/ml) than in control patients (12.3±4.8 pg/ml; P≤0.05). Urocortin immunoreactivity and mRNA signals were both detected in synovial cells, lymphocytes, fibroblasts and macrophages. The number of urocortin-positive cells in the synovium was significantly higher in RA (73.1±32.1 cells per high-power field) than in control (18.4±10.4 cells per high-power field) patients. In addition, both urocortin immunoreactivity and mRNA signals in the synovium reached maximum levels in the active stage of RA inflammation. Moreover, the number of immunoreactive urocortin-positive cells was significantly correlated with the urocortin concentration in synovial fluid (r=0.705; P<0.001) and with histologically defined local inflammatory activity (r=0.641; P<0.001). The distribution and number of immunoreactive CRF-positive cells in synovial tissue were similar to those of urocortin-positive cells (r=0.701; P<0.001). Urocortin, CRF-R1 and CRF-R2-a mRNAs detected by RT-PCR were expressed in the synovium of 10/10, 10/10 and 2/10 RA patients respectively, but CRF-R2-b was not expressed. Urocortin was actively synthesized in the synovium of RA patients. The present study suggests that urocortin may play an important role as an autocrine and/or paracrine regulator of synovial inflammation in RA.

Key words: corticotropin-releasing factor, corticotropin-releasing factor receptor, rheumatoid arthritis, synovial fluid, synovium, urocortin.

Abbreviations: CRF, corticotropin-releasing factor; CRF-R, CRF receptor; EMA, epithelial membrane antigen; 4HP, prolyl 4-hydroxylase; HPF, high-power field; LCA, leucocyte common antigen; OA, osteoarthritis; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase–PCR.

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INTRODUCTION

Various neuropeptides, especially those involved in the hypothalamic/pituitary/adrenal axis, play important roles in animal models of autoimmunity or human autoimmune diseases [1,2]. Co-ordination of both the immune and neuroendocrine systems is considered to contribute to the maintenance of the intricate homoeostasis of inflammatory stress. In the activated immune system, tumour necrosis factor α, interleukin-1 and interleukin-6 have all been demonstrated to stimulate the production of corticotropin-releasing factor (CRF) in the hypothalamus [1,2]. CRF is a major regulator of the hypothalamic/pituitary/adrenal axis. Its release leads to the pituitary production of corticotropin (‘ACTH’), resulting in glucocorticoid secretion by the adrenal cortex. CRF has also been shown to be present at various peripheral sites, where it may act as a ‘pro-inflammatory peptide’ [3]. Karalis et al. [4] demonstrated that the inflammatory response could be markedly inhibited by the administration of anti-CRF antibodies. However, the source of peripheral CRF has remained obscure. Human lymphocytes were shown to produce immunoreactive CRF, but the data on CRF mRNA expression in these cells are equivocal. Bamberger et al. [5] investigated the expression of CRF and urocortin in human lymphocytes, and demonstrated that human lymphocytes produce urocortin, but not CRF.

Urocortin is a newly identified peptide of the CRF neuropeptide family [6]. This peptide has 43% amino acid sequence identity with human CRF, and has been demonstrated to bind to both CRF receptor 1 (CRF-R1) and CRF-R2, and to CRF-binding protein [7–10]. Urocortin has been shown to be involved in the modulation of various biological activities in experimental animal models [11–17]. In humans, the presence of urocortin immunoreactivity and urocortin mRNA has been demonstrated in the placenta [18], pituitary gland and its neoplasms [19], pons, medulla oblongata, cerebellum [19], and lymphocytes [5]. However, in humans, the involvement of urocortin in inflammation, including inflammatory disorders, has not been examined. Rheumatoid arthritis (RA) is one of the most common autoimmune diseases.

We first studied urocortin concentrations in the synovial fluid of patients with RA, osteoarthritis (OA) or trauma or joint injury. We then characterized urocortin expression in the synovial tissue of RA patients using immunohistochemistry, mRNA in situ hybridization and reverse transcriptase-PCR (RT-PCR), in order to study its possible biological significance in the inflammatory processes of RA. We also examined immunoreactive CRF in synovial tissue. In addition, we examined the expression of CRF-R1, -R2-α and -R2-β, to which urocortin is known to bind, in synovial tissue specimens obtained from patients with RA, in order to clarify whether or not locally produced urocortin exerts its effects in situ.

In the present study, we examined the expression of urocortin, CRF and its receptors, to elucidate the possible biological roles of urocortin in the synovial tissue of patients diagnosed with RA.

MATERIALS AND METHODS

Subjects and materials

All tissue specimens utilized in this study were obtained from hospitals affiliated with Tohoku University Hospital, Sendai, or Iwate Medical University Hospital, Morioka, Japan, between 1990 and 1998. Characteristics

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical features of the patients studied</th>
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<tr>
<td></td>
<td>Trauma</td>
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<td>n</td>
<td>4</td>
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<tr>
<td>Age (years)</td>
<td>38.0 ± 19.9</td>
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<tr>
<td>Sex (male/female)</td>
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<tr>
<td>Disease duration (years)</td>
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<tr>
<td>ESR (mm/h)</td>
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<tr>
<td>CRP (mg/dl)</td>
<td>0.0 ± 0.10</td>
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<tr>
<td>Inflammatory score</td>
<td>6.0 ± 4.2</td>
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<tr>
<td>Urocortin in synovial fluid (pg/ml)</td>
<td>&lt; 0.2</td>
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<tr>
<td>Urocortin-positive cells (no./HPF)</td>
<td>0.488 ± 0.159</td>
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<tr>
<td>CRF-positive cells (no./HPF)</td>
<td>0.5 ± 1.5</td>
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<td>Medication (no. of cases)</td>
<td>NSAIDs (1)</td>
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of the patients from whom tissues were obtained are shown in Table 1. Informed consent was obtained from each patient. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and was approved by the ethics committees of the hospitals involved.

A total of 38 patients (31 women; seven men), who met the American College of Rheumatology criteria for RA [19a], were examined. Patients with OA (nine women) or trauma (three women; one man) without inflammation were used as controls. We selected control patients that showed no significant elevation in the value of the erythrocyte sedimentation rate or C-reactive protein, and histologically showed little lymphocyte or neutrophil infiltration. All the subjects, including the patients with RA, did not have any endocrinological or neurological disorders clinically, and they did not have prior histories of glucocorticoid treatment. Following patient consent, synovial fluid samples were aspirated from 31 RA (26 women; five men), seven OA (seven women) and four trauma (three women; one man) patients. Synovial fluid specimens were centrifuged immediately to remove cells and debris, and stored at $-80^\circ$C until further analysis.

Synovial tissues used in this present study were obtained during total knee replacement surgery or arthroscopical synovectomy. Following the removal of these tissues, they were dissected into pieces of 3–5 mm$^3$. Of samples from the 38 RA and nine OA cases, specimens from 10 RA and two OA cases were available for RT-PCR analysis. These 12 specimens were immediately frozen in dry ice and stored at $-80^\circ$C until further use. Tissue specimens from 38 cases (30 RA, six OA and two trauma) were available for immunohistochemistry and mRNA in situ hybridization analysis. These 38 specimens were fixed in 4% (w/v) paraformaldehyde dissolved in PBS for 2 h at room temperature, and embedded in paraffin. Sections (2 $\mu$m thickness) were cut and mounted on poly(L-lysine)- or silane-coated glass slides. In each case, serial tissue sections were stained with haematoxylin and eosin in order to classify the histological stage of RA synovial inflammation, and were processed for immunohistochemistry and mRNA in situ hybridization.

**Histological classification of synovial inflammation in RA by haematoxylin/eosin staining**

Sections were stained with haematoxylin/eosin. The degree of synovial inflammation was evaluated in 30 RA cases according to Rooney’s histological index [20]. Sections were randomized and coded in at least 20 different microscopic fields, and six histological features were scored separately on a scale of 0–10. The scoring technique used for all six features is summarized below.

**Synovial hyperplasia**

A normal synoviocyte monolayer was given a zero score. As the synoviocyte lining layer increased in depth, the score increased, to a maximum value of 10.

**Fibrosis**

The degree of fibrosis was estimated as the amount of fibrous tissue that had replaced the normal loose connective tissue present beneath the synovial lining layer. Sections containing <10% fibrous tissue were considered normal and graded with a score of zero. As the percentage of fibrosis increased, the score increased, to a maximum value of 10.

**Proliferating blood vessels**

The presence of less than three blood vessels per high-power field (HPF) was considered normal, and was given a score of zero. The score was increased as the number of vessels increased.

**Perivascular infiltrates of lymphocytes**

Perivascular infiltrates were characterized as aggregates of lymphocytes that were contiguous with the vessel wall and were no more than 10 cells in diameter. When no vessels were involved, the grade was zero. As the percentage of vessels surrounded by lymphocytes increased, the score obtained increased, to a maximum grade of 10.

**Focal aggregates of lymphocytes**

This feature assessed aggregates of lymphocytes that were not intimately related to a synovial vessel, or those in which the perivascular cuff of lymphocytes exceeded 10 cells in diameter. Scoring of focal aggregates was determined by their size. The absence of focal aggregates was scored as zero. As the cell numbers in the diameter of the focal aggregates increased, the score increased, to a maximum value of 10.

**Diffuse infiltrates of lymphocytes**

Those lymphocytes that did not fall into either of the above categories of perivascular or focal aggregates were considered as diffuse infiltrates. An estimate was made of the percentage of cells per HPF that were lymphocytes, and an increasing percentage resulted in a higher score.

**Preparation of urocortin antiserum and CRF antiserum**

A specific antiserum against urocortin, which did not cross-react with other CRF-related peptides, was raised...
in a rabbit immunized with a peptide corresponding to amino acid residues 21–35 of human urocortin. Methods of immunization and characterization of the antiserum have been reported previously [19,21–23]. A specific antiserum against CRF was raised in a rabbit immunized with human CRF. Methods of immunization and characterization of the antiserum have been reported previously [24,25]. This antiserum showed no significant cross-reaction (< 0.001 %) with human urocortin in RIA [25].

**RIA for urocortin**

Synovial fluid samples were frozen at −80 °C until determination of urocortin. The sample was heated in boiling water for 5 min to denature proteolytic enzymes, and then diluted with ice-cold RIA buffer (63 mM Na₂HPO₄, 12.7 mM EDTA·Na₂H₂O, 0.05 % NaN₃ and 500 kallikrein-inhibitory units/ml aprotinin, pH 7.4).

Urocortin RIA was carried out according to the method of Oki et al. [21,22]. Briefly, the synthetic peptide [Tyr¹⁹]urocortin-(19–37) was used as the ¹²⁵I-labelled tracer. The standard peptide used in the RIA was human urocortin-(1–40). Final dilution of the urocortin antiserum was 1:75 000. The intra- and inter-assay coefficients of variation were 6 % and 11 % respectively. The smallest detectable immunoreactive urocortin concentration was 0.4 fmol/tube. The assay did not cross-react with rat/human CRF, ovine CRF, urotensin I, sauvagine or corticotropin.

**Immunohistochemistry**

We examined the immunolocalization of urocortin and CRF in synovial tissue, and compared the distribution of urocortin-positive cells with that of CRF-positive cells in the same fields of the serial mirror-image tissue sections. Details of immunohistochemical procedures have been reported previously [19,23,26,27]. Immunostaining of paraffin-embedded sections was performed by the modified ABC method using an avidin–biotin complex (ABC) kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.). After removal of paraffin, to retrieve urocortin or CRF antigenicity, the sections were pretreated by boiling water for 5 min to denature proteolytic enzymes, and sections were covered with the first antibody (anti-urocortin or anti-CRF polyclonal antibody; rabbit) and incubated overnight (12–18 h) at 4 °C. The optimal antibody dilutions were 1:2500 and 1:2000 respectively. Following washing in PBS, sections were covered with the second antibody [biotinylated anti-(rabbit IgG); goat] and incubated for 30 min at room temperature. After washing in PBS, sections were covered with avidin–biotin–antiperoxidase complex (ABC kit; Vector Laboratories) for 30 min at room temperature and then rinsed in PBS. Antigenic sites were demonstrated by treating the sections with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 7 min. The sections were then counterstained with Methyl Green, dehydrated in ethanol, cleared in xylene and mounted. As a negative control, preabsorbed antiserum or non-immune rabbit IgG was used instead of primary antibodies for urocortin or CRF. For absorption tests of urocortin immunoreactivity, an antibody/antigen mixture containing equal volumes of the optically diluted antiserum against urocortin peptide solution (20 μmol/l final concentration) was incubated at 4 °C overnight. After centrifugation (14000 g; 30 min), the supernatants were used as preabsorbed antibody [19].

**Comparative study of immunoreactivity of urocortin and other markers in serial mirror-image tissue sections**

Four pairs of mirror-image sections were treated individually with urocortin antiserum and leucocyte common antigen (LCA) as markers for leucocytes. In addition, epithelial membrane antigen (EMA), CD68 and prolyl 4-hydroxylase (4HP) were used as markers for synovial cells, macrophages and fibroblasts respectively. Synovial tissue is of mesenchymal origin, but a specific marker has not been established at this juncture. Synovial cells are classified into several types morphologically: macrophage-like cells, fibroblast-like cells and intermediate-type cells. In the synovial tissue of RA patients, synovial cells demonstrate more varied morphological features than do those of normal synovium. EMA is a marker of epithelial cells, and normal synovial cells are immunohistochemically negative for EMA. EMA is also considered as a marker of synovial sarcoma. Despite its non-neoplastic nature, RA synovium is well known to express p53, c-fos and other markers associated with various neoplasms. Therefore we employed EMA as a mesenchymal neoplastic marker. The number of immunopositive cells was determined in these serial mirror-image tissue sections. Double immunostaining is required to determine the characteristics of urocortin-positive cells, because their cellular characteristics cannot necessarily be determined by morphological observation alone. Fluorescent double immunostaining is suitable for the confirmation of double-positive cells, but the fluorescent immunostain is associated with various technical difficulties. We compared the distribution of the urocortin-positive cells with those of LCA-, EMA-, CD68- and 4HP-positive cells in the same fields of the
complete mirror-image section, from photographs. In order to examine which urocortin-positive cells were correlated with urocortin levels in the synovial fluid, and to determine the characteristics of urocortin-positive cells, we counted the numbers of all types of urocortin-positive cells in the synovial tissues separately, from photographs. The correlation between the concentration of urocortin in the synovial fluid and the number of urocortin-positive cells was then examined.

**Preparation of cDNA probes for mRNA in situ hybridization**

The sequence of the 28-base urocortin antisense oligonucleotide probe used for mRNA in situ hybridization analysis was 5′-TCCGCAAGGAGTGAAGGTGAGTCAT-3′ (positions 305–332). A sense oligonucleotide probe (5′-ATTGACCTACACCTTCA-CCTGCTGCGGA-3′) was used as a negative control. The probes were synthesized with a 3′-biotinylated tail (Brigati tail: 5′-probe-biotin-biotin-biotin-TAG-TAG-biotin-biotin-biotin-3′) [19,29].

**mRNA in situ hybridization**

mRNA in situ hybridization was performed by utilizing a manual capillary actions system (MicroProbe™ Staining System; Fisher Scientific, Pittsburgh, PA, U.S.A.) [30] with a modification of previously reported methods [19,26]. Paraffin was removed rapidly from tissue sections (2 μm; applied to Probe On Plus slides; Fisher Scientific), which were then cleared with alcohol, rehydrated with a Tris-based buffer, pH 7.4 (Universal Buffer; Research Genetics) [30] and digested with pepsin (2.5 mg/ml; Research Genetics, Huntsville, AL, U.S.A.), and digested with RNase H reverse transcriptase (Super Script™ II RNase H reverse transcriptase; Life Technologies). The reaction mixture was then heated at 70 °C for 15 min, and immediately chilled on ice.

The reverse transcriptase products (urocortin and β-actin, 2 μl; CRF-R1, -R2-α and -R2-β, 5 μl) were subjected to PCR in a reaction mixture (20 μl) containing 10 mmol/l Tris HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l MgCl₂, 0.001 % (w/v) gelatin, 4 mmol of deoxy-NTPs, 5 pmol of primers and 0.5 unit of Taq DNA polymerase (Pharmacia, Piscataway, NJ, U.S.A.). For urocortin, the sense primer was 5′-CAGGGCGACGGCCGCGG-3′ and the antisense primer was 5′-CTTGCACCCAGG-GTGCAAT-3′. These oligonucleotide primers were designed based on the report by Petraglia et al. [18]. The urocortin PCR product corresponds to bases 2809–2954 of the sequence published by Donaldson et al. [7] and Zhao et al. [31] (GenBank accession number AF 038633). A human full-term placenta in which urocortin mRNA was detected was included as a positive control. To check for the presence of CRF-R mRNAs in tissues from RA patients, we used the following primer sets: CRF-R1 [8,32], 5′-CAAAAAATGGCTACCGGGAG-3′ (sense) and 5′-ACACCCAGCAATGCGA-3′ (antisense); CRF-R2-α [10], 5′-GACCGCGACTGTGCTCCAGACA-G-3′ (sense) and 5′-GAATCGGGGCGTGTTGCTT-3′ (antisense); CRF-R2-β [33], 5′-CCCTCAACCACCTCTCAGTCC-3′ (sense) and 5′-CAGTCCTAGTCTCTGTTGC-3′ (antisense). These CRF-R oligonucleotide primers were designed by K. Totsune. The primers for β-actin [34] [5′-GATTCTATATGTGCGGAGGGAG-3′ (sense) and 5′-CCATCTCTCTCAGTCC-3′ (antisense)] were used as an internal control. Human pituitary gland, hypothalamus and left atrium were used as positive controls for CRF-R1, CRF-R2-α and CRF-R2-β respectively. The RT product was preheated at 94 °C for 2 min, followed by standard PCR denaturation, annealing and extension steps, which were carried out at 94 °C for 15 s, at 62 °C (urocortin), 66 °C (CRF-R1, -R2-α and -R2-β) or 61 °C (β-actin) for 30 s, and at 72 °C for 1 min respectively. The RT product was amplified utilizing PCR for 35 (urocortin), 37 (CRF-R1, -R2-α and -R2-β) or 30 (β-actin) cycles. Amplification products were subjected to electrophoresis in a 5% (w/v) polyacrylamide gel, stained with ethidium bromide and viewed in a UV box. The negative control contained all reagents, except that 4 μl of water was added instead of the RT reaction product.

**RT-PCR procedures**

Total RNA was extracted from tissues by the guanidine isothiocyanate/cesium chloride method. Total RNA (4 μg) was denatured at 70 °C for 10 min and then reverse-transcribed at 42 °C for 50 min in a total volume of 20 μl of reaction buffer (Life Technologies, Grand Island, NY, U.S.A.) containing 0.5 μg of oligo(dT)₁₂₋₁₈ (Pharmacia, Uppsala, Sweden), 10 nmol of deoxy-NTPs and 200 units of reverse transcriptase (Super Script™ II RNase H reverse transcriptase; Life Technologies). The reaction mixture was then heated at 70 °C for 15 min, and immediately chilled on ice.

The reverse transcriptase products (urocortin and β-actin, 2 μl; CRF-R1, -R2-α and -R2-β, 5 μl) were subjected to PCR in a reaction mixture (20 μl) containing 10 mmol/l Tris HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l MgCl₂, 0.001 % (w/v) gelatin, 4 mmol of deoxy-NTPs, 5 pmol of primers and 0.5 unit of Taq DNA polymerase (Pharmacia, Piscataway, NJ, U.S.A.). For urocortin, the sense primer was 5′-CAGGGCGACGGCCGCGG-3′ and the antisense primer was 5′-CTTGCACCCAGG-GTGCAAT-3′. These oligonucleotide primers were designed based on the report by Petraglia et al. [18]. The urocortin PCR product corresponds to bases 2809–2954 of the sequence published by Donaldson et al. [7] and Zhao et al. [31] (GenBank accession number AF 038633). A human full-term placenta in which urocortin mRNA was detected was included as a positive control. To check for the presence of CRF-R mRNAs in tissues from RA patients, we used the following primer sets: CRF-R1 [8,32], 5′-CAAAAAATGGCTACCGGGAG-3′ (sense) and 5′-ACACCCAGCAATGCGA-3′ (antisense); CRF-R2-α [10], 5′-GACCGCGACTGTGCTCCAGACA-G-3′ (sense) and 5′-GAATCGGGGCGTGTTGCTT-3′ (antisense); CRF-R2-β [33], 5′-CCCTCAACCACCTCTCAGTCC-3′ (sense) and 5′-CAGTCCTAGTCTCTGTTGC-3′ (antisense). These CRF-R oligonucleotide primers were designed by K. Totsune. The primers for β-actin [34] [5′-GATTCTATATGTGCGGAGGGAG-3′ (sense) and 5′-CCATCTCTCTCAGTCC-3′ (antisense)] were used as an internal control. Human pituitary gland, hypothalamus and left atrium were used as positive controls for CRF-R1, CRF-R2-α and CRF-R2-β respectively. The RT product was preheated at 94 °C for 2 min, followed by standard PCR denaturation, annealing and extension steps, which were carried out at 94 °C for 15 s, at 62 °C (urocortin), 66 °C (CRF-R1, -R2-α and -R2-β) or 61 °C (β-actin) for 30 s, and at 72 °C for 1 min respectively. The RT product was amplified utilizing PCR for 35 (urocortin), 37 (CRF-R1, -R2-α and -R2-β) or 30 (β-actin) cycles. Amplification products were subjected to electrophoresis in a 5% (w/v) polyacrylamide gel, stained with ethidium bromide and viewed in a UV box. The negative control contained all reagents, except that 4 μl of water was added instead of the RT reaction product.

**Quantitative analysis of urocortin- or CRF-positive cells**

In all specimens that were processed for immunohistochemistry or in situ hybridization, cells positive for
urocortin or CRF were examined in at least 20 different microscopic fields chosen randomly from each section. The numbers of urocortin- or CRF-positive cells were counted from photographs. All microscopic images were measured at a magnification of ×400 (HPF). The correlations between the degree of synovial inflammation and the number of positive cells for urocortin or CRF were examined.

Statistical analysis
Data are presented as means±S.D., and were analysed using the non-parametric Mann–Whitney U test, Student’s paired t test and Pearson correlation coefficients. P values of < 0.05 were considered significant.

RESULTS

Urocortin concentration in synovial fluid
The concentrations of immunoreactive urocortin in the synovial fluid were approx. 10–100 times higher in patients diagnosed with RA (79.7 ± 154 pg/ml) than in non-inflamed control patients (12.3 ± 4.8 pg/ml; P < 0.05) (Figure 1). The urocortin concentration in synovial fluid varied according to the degree of synovial inflammatory activities, as scored using Rooney’s histological index. The concentration of urocortin in the synovial fluid was significantly correlated with the degree of inflammation of the synovium (r = 0.57; P < 0.001) (Figure 2).

Urocortin immunoreactivity in synovial tissue
Urocortin-immunoreactive cells were observed in all cases examined. Urocortin immunoreactivity was completely abolished by urocortin antiserum preabsorbed with the antigen or a peptide corresponding to amino acid residues 21–35 of human urocortin [5]. In all RA cases, prominent urocortin immunoreactivity was detected in the synovial cells, lymphocytes, macrophages, fibroblast-like cells and endothelial cells. Urocortin-positive synovial cells were present mainly in the synovial lining layer, especially for RA cases (Figures 3a and 3b). The number of urocortin-positive cells in synovia from RA patients (73.1 ± 32.1 per HPF) was significantly greater than that in non-inflamed control synovia. In addition, the urocortin concentration in the synovial fluid was significantly correlated with the number of urocortin-positive cells in the synovium (r = 0.705; P < 0.001) (Figure 4). The numbers of urocortin-positive synovial cells, leucocytes, macrophages, blood vessels and fibroblasts were significantly correlated with urocortin levels in the synovial fluid (synovial cells, r = 0.767; leucocytes, r = 0.6956; macro-
Urocortin in rheumatoid arthritis patients

Figure 3  Cells positive for urocortin immunoreactivity

(a, b) Urocortin-positive cells present in synovial tissue from trauma (a) and RA (b) patients, with a dominant distribution in the RA cases. (c) The number of urocortin-positive cells was significantly higher in RA cases (73.1 ± 32.1 per HPF) than in non-inflamed control cases (18.4 ± 10.4 per HPF) (P < 0.001). Original magnification × 50. The symbols with error bars denote means ± S.D.

Correlation of the distribution of urocortin-positive cells with those of LCA-, EMA-, CD68- and 4HP-positive cells

We examined the correlations between the distribution of urocortin-positive cells and those of cells positive for LCA (lymphocytes), EMA (synovial cells), CD68 (macrophages) or 4HP (fibroblasts) in the same fields of the serial mirror-image tissue sections. The distribution of urocortin-positive cells (Figures 5a–5d) overlapped with those of cells positive for LCA (Figure 5a), EMA (Figure 5b), CD68 (Figure 5c) and 4HP (Figure 5d) in synovial tissue from patients with RA.

Urocortin mRNA localization in synovial tissue

Urocortin mRNA hybridization signals appearing red as a result of the Fast Red salt reaction were detected in the cytoplasm of synovial cells (Figure 6a), lymphocytes (Figure 6b), endothelial cells, macrophages (Figure 6c) and fibroblast-like cells (Figure 6d). In negative controls, using the sense oligonucleotide probe, no significant accumulation of urocortin mRNA hybridization signals was detected. The distribution of urocortin mRNA hybridization signals was comparable with that of urocortin immunoreactivity. The positive reaction of urocortin mRNA hybridization signals in RA synovia was significantly greater than that in non-inflamed control synovia (OA or traumatic injury cases) (Figures 7a and 7b), and the number of positive cells increased in proportion to the progression of synovial inflammation.

Figure 4  Correlations between the concentration of urocortin in synovial fluid and the number of urocortin-positive cells in synovial tissue

The concentration of urocortin in the synovial fluid (SF) was significantly correlated with the number of urocortin-positive cells (r = 0.705; P < 0.001).

CRF immunoreactivity in synovial tissue

CRF-immunoreactive cells were detected in all cases examined. In all RA cases, marked CRF immunoreactivity was detected in the synovial cells, lymphocytes, macrophages, fibroblast-like cells and endothelial cells. The distribution of immunoreactive CRF was similar to that of urocortin in the same fields of the serial mirror-image tissue sections. CRF-positive synovial cells were present mainly in the synovial lining layer, especially in RA cases. The number of CRF-positive cells in RA synovia (128.2 ± 46.0 per HPF) was significantly greater than in non-inflamed control synovia (OA or traumatic injury cases) (32.1 ± 25.5 per HPF), and the number of CRF-positive cells increased in proportion to the progression of synovial inflammation.
of CRF-positive cells increased in parallel with the progression of inflammation of the synovium ($r = 0.671; P < 0.001$). The relative immunointensity of CRF-positive cells in RA synovia was greater than that in non-inflamed control synovia. In addition, the number of CRF-immunoreactive cells was significantly correlated with that of urocortin-immunoreactive cells ($r = 0.701; P < 0.001$) (Figure 8).
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Figure 6  Urocortin gene expression in synovial tissue
Urocortin mRNA hybridization signals (appearing red) were detected in the cytoplasm of (a) synovial cells, (b) lymphocytes, (c) macrophages and (d) fibroblast-like cells. The distribution of urocortin mRNA hybridization signals was comparable with that of urocortin immunoreactivity. Original magnification × 66.

Figure 7  Cells showing urocortin mRNA hybridization signals
Urocortin mRNA hybridization signals (appearing red) were present in synovial tissue, with a dominant distribution in samples from RA cases (b). In samples from non-inflamed control cases (OA cases or traumatic injury), small amounts of urocortin-positive cells were seen (a). Original magnification × 66.

RT-PCR of urocortin and CRF-Rs in synovial tissue
The RT-PCR results demonstrated the expression of urocortin, CRF-Rs (CRF-R1, -R2-α and -R2-β) and β-actin mRNAs in 12 synovial samples (10 from RA and two from OA patients) and in positive control tissues (urocortin and β-actin, human full-term placenta; CRF-R1, human pituitary gland; CRF-R2-α, human hypothalamus; CRF-R2-β, human left atrium) (Figure 9). A 146 bp DNA band, which corresponds to the expected length for urocortin, and a 475 bp DNA band, corresponding to the expected length for CRF-R1, were amplified in all the specimens examined. However, a 233 bp DNA band, corresponding to the expected length for CRF-R2-α, was only amplified in three cases (RA2,
DISCUSSION

We have demonstrated that urocortin was present in the synovial tissues and fluids of patients with RA. In these patients, both the urocortin concentration in the synovial fluid and the number of urocortin-positive cells in synovial tissues were higher than in controls, which included patients with OA and trauma. The distribution of urocortin-positive cells, as demonstrated by immunohistochemistry or in situ hybridization, overlapped with those of cells positive for LCA, EMA, CD68 and 4HP, which suggests that synovial lining cells, lymphocytes, macrophages and fibroblasts can all produce urocortin peptide. There was a positive correlation between the concentration of urocortin peptide in the synovial fluid and the degree of synovial inflammation in RA cases. In addition, there was a positive correlation between the concentration of urocortin in the synovial fluid and the number of urocortin-positive cells in synovial tissues. Furthermore, the number of urocortin-positive cells in samples of synovial cells, leucocytes, macrophages, blood vessels and fibroblasts was significantly correlated with urocortin levels in the synovial fluid. These findings suggest that the increase in the number of these urocortin-positive cells may result in an elevation of the concentration of urocortin in synovial fluid in inflammatory states. Therefore the degree of urocortin expression in the synovium of patients with RA may reflect that of inflammatory changes in the synovium.

The possibility of an influence of non-specific factors and enzymes cannot be excluded completely, because we did not carry out HPLC. Moreover, in RA, rheumatoid factor in the synovial fluid may interfere with immunoassays. Strictly, further studies, such as those involving HPLC, are required to confirm the accuracy of our RIA method. However, since synovial fluid samples were heated and diluted with buffer containing EDTA and aprotinin to inhibit the activity of proteolytic enzymes, it is likely that the influence of these factors was negligible. Previously we examined the effects of rheumatoid factor on ELISA of several cytokines and proteinases. Addition of rheumatoid factor to the samples (synovial fluid and serum) had no effect on the results (not shown). In RA synovium, the total number and the percentage of urocortin-positive cells both increased. Therefore the increase in the number of positive cells is considered to reflect both an increase in total cellularity of the synovium and up-regulation of urocortin expression in the RA synovium. As reported in many previous studies, the production of various enzymes or cytokines is considered to represent up-regulation in RA synovial tissue.

CRF is well known to indirectly suppress the immune system, via the secretion of pituitary corticotropin and anti-inflammatory adrenal corticosteroids [35]. Several reports have indicated possible abnormalities of the
hypothalamic/pituitary/adrenal axis in patients with RA. For instance, Baerwald et al. [36] demonstrated that patients with RA have an impaired CRF response to stress. This may possibly be due to polymorphisms in the 5' regulatory region of the CRF gene, which can result in decreased CRF responsiveness. In addition, during the process of inflammatory stress, various cytokines stimulate CRF secretion at local inflammatory sites, such as in the arthritic joint, but its action appears to be pro-inflammatory. Nishioka et al. [37] reported that concentrations of CRF and interleukin-6 in synovial fluid were higher in RA patients than in OA patients, but that plasma corticotropin and CRF levels were significantly lower in RA patients than in OA patients. This may suggest that the secretion of CRF in synovial fluid is regulated differently from plasma CRF secretion, since CRF levels in the synovial fluid and plasma of RA patients showed opposite trends. Karalis et al. [4] also demonstrated the presence of CRF-like immunoactivity at sites of chemically induced inflammation. In addition, it was shown that local administration of anti-CRF antibodies or of a CRF-R antagonist markedly reduced the degree of edema in inflamed skin, suggestive of a possible pro-inflammatory role of CRF [4,38]. Increased levels of immunoreactive CRF have been found previously in the synovial fluid of patients with RA [39]. Crofford et al. [39] reported that CRF was detected in the synovial fluid and tissues of RA patients, and that the concentration and the number of positive cells were higher in RA than in OA patients. In addition, they reported that positive cells were localized to synovial lining cells, blood vessels and mononuclear inflammatory cells [39]. In our study, the distribution of immunoreactive CRF in RA synovium was similar to the previously published data [39]. Our present findings of urocortin expression in the synovium of RA patients are similar to those of CRF expression in RA synovium in the same fields of the serial mirror-images of tissue sections. Urocortin is similar to CRF not only in its structure but also in its distribution in RA synovial tissue. In addition, the number of urocortin-immunoreactive cells was significantly correlated with that of CRF-immunoreactive cells, which indicates that not only CRF, but also urocortin, is involved in the inflammatory processes of RA.

CRF-Rs have been detected in peripheral sites of the immune system [40] and immune cells [41], and CRF was actually demonstrated to promote several immune functions in vitro. Our present RT-PCR study in the synovium also demonstrated the expression of urocortin and its receptors CRF-R1 and CRF-R2-α in synovial tissues of patients with RA or OA. Urocortin and CRF-R1 mRNAs were detected in all samples examined in the present study, but CRF-R2-α mRNA was detected in only three samples (two from RA patients and one from OA patients) of the 12 cases examined. Although the number of cases was limited in our study, the patients in which CRF-R2-α mRNA was detected all demonstrated active inflammation of the synovium. Further studies, such as mRNA in situ hybridization of CRF-Rs, are required to clarify which cell types express their mRNAs. The absence of CRF-R2-β may be consistent with the fact that this CRF-R subtype is not expressed ubiquitously; it is expressed almost exclusively in the heart of rodents, and appears to be a minor isoform in both heart and skeletal muscle in humans [33].

Urocortin has been demonstrated to be a potent inhibitor of heat-induced oedema in a rat thermal injury model, possibly mediated by CRF-R2 [16]. Urocortin and CRF have also been demonstrated to inhibit the development of experimental autoimmune encephalomyelitis [14]. Therefore, in the synovial tissue of patients with RA, urocortin may exert its effects through binding to CRF-Rs, possibly as an inhibitor of extravasation and associated inflammatory processes, as reported previously in a rat thermal injury model [16]. Singh et al. [42] demonstrated that intradermal urocortin administration induced skin mast cell degranulation and increased vascular permeability; both actions appeared to be more potent than those shown previously for CRH [43]. These workers also reported that vascular permeability triggered by urocortin was partially reduced by a selective CRH-R1 antagonist and by a more selective CRF-R2 antagonist [42]. The effects of urocortin were inhibited to a greater extent by the CRF-R2 antagonist than by the CRF-R1 antagonist. These results demonstrated that urocortin stimulates both CRF-R1 and CRF-R2, but especially CRF-R2, following intradermal urocortin administration. However, in states of chronic inflammation such as RA, mast cells are not abundantly observed. Therefore the results reported by Singh et al. [42] may not be applicable to the inflammation seen in RA.

Both urocortin and CRF may play important roles as autocrine and/or paracrine regulators of synovial inflammation in patients diagnosed with RA. The presence of CRF-Rs in the synovium of arthritic patients may also suggest that CRF-R-selective ligands may be useful modulators of inflammation associated with RA. Further studies, including the localization of CRF-R1 and CRF-R2-α, are required in order to clarify the precise biological roles of this neuropeptide in the process of inflammation associated with RA.

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