

# Endorphin and Enkephalin Ameliorate Excessive Synovial Cell Functions in Patients with Rheumatoid Arthritis

YUKO TAKEBA, NOBORU SUZUKI, ATSUSHI KANEKO, TOMIAKI ASAI, and TSUYOSHI SAKANE

**ABSTRACT. Objective.** To determine whether endorphin (END) and enkephalin (ENK) modulate excessive synovial cell functions in patients with rheumatoid arthritis (RA).

**Methods.** Effects of leucine-enkephalin (leu-ENK), methionine-enkephalin (met-ENK), and  $\beta$ -endorphin (END) on proinflammatory cytokine and matrix metalloproteinase (MMP) production by RA synovial cells were analyzed by immunoblotting, and their mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) using limiting dilution of complementary DNA. Expression of opioid receptors on RA synovial cells was assessed by immunohistochemical staining, radioreceptor assay, and RT-PCR.

**Results.** Leu-ENK, met-ENK, and END inhibited tumor necrosis factor- $\alpha$  and interleukin 1 $\beta$  production at the level of mRNA expression. ENK and END inhibited MMP-9 production and its enzymatic activity by RA synovial cells. The  $\mu$ -subtype opioid receptor was expressed in the RA synovial lining and sublining cells. Radioreceptor assay suggested expression of high affinity receptor for END on RA synovial cells. The  $\mu$ -subtype opioid receptor-specific antagonist, naloxone, reversed the inhibitory effect of the opioid peptides. The opioid peptides inhibited nuclear translocation and phosphorylation of the transcription factor, cyclic AMP responsive element binding protein (CREB) in RA synovial cells.

**Conclusion.** Leu-ENK, met-ENK, and END inhibited excessive RA synovial cell functions *in vitro*. The opioid hormones may have not only antinociceptive action, but also antiinflammatory effects on synovitis itself in RA. (J Rheumatol 2001;28:2176–83)

*Key Indexing Terms:*

RHEUMATOID ARTHRITIS  
LEUCINE-ENKEPHALIN

SYNOVIAL CELLS  
 $\beta$ -ENDORPHIN

METHIONINE-ENKEPHALIN  
CREB

Rheumatoid arthritis (RA) is characterized by synovial membrane inflammation and chronic polyarticular destruction<sup>1-3</sup>. The synovial cells contribute to inflammatory responses by producing proinflammatory cytokines such as interleukin 1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), degrading enzymes such as matrix metalloproteinase (MMP), and prostaglandins, leading to tissue destruction<sup>4</sup>.

Clinical features and experimental observation suggest a role of neural mechanisms in the pathophysiology of RA<sup>5,6</sup>. In

particular, substance P has been assigned an important role in pathogenesis<sup>7</sup>.

Another class of neuropeptides that deserves attention in arthritis is the opioid peptides<sup>8</sup>. Opioid peptides are encoded by 3 genes, the proopiomelanocortin (POMC) gene, the preproenkephalin gene, and prodynorphin gene. They all give rise to precursor proteins that are processed in a tissue-specific manner into smaller biologically active peptides<sup>8-10</sup>. Opioid peptides contribute to control of pain transmission by interacting with opioid receptors within the nervous system<sup>9</sup>. Enkephalin (ENK), found in spinal interneurons and long tract neurons, acts mainly on  $\delta$  and  $\mu$ -opioid receptors and may be important in evoked pre and postsynaptic inhibition of the pain pathway<sup>10</sup>. Opioids of the POMC and proenkephalin families have been found in increased concentrations in inflamed joint tissues and fluids<sup>11</sup>. Endogenous opioids are produced by non-neural inflammatory cells<sup>12</sup> and interact with opioid receptors on peripheral sensory nerve terminals to inhibit inflammatory pain. The functional role of  $\beta$ -endorphin (END) released in the inflamed subcutaneous tissue is evident from the fact that injection of corticotropin releasing hormone (CRH) and IL-1 into the inflamed site produced analgesic effects that were inhibited by a locally applied anti-END antibody<sup>13</sup>. Indeed, intraarticular administration of opioid pep-

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From the Departments of Immunology and Medicine, St. Marianna University School of Medicine, Kawasaki, Kanagawa; and the Department of Orthopedic Surgery, Nagoya National Hospital, Nagoya, Aichi, Japan.

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Y. Takeba, DVM; N. Suzuki, MD, PhD; T. Sakane, MD, PhD; Departments of Immunology and Medicine, St. Marianna University School of Medicine; A. Kaneko, MD; T. Asai MD, Department of Orthopedic Surgery, Nagoya National Hospital.

Address reprint requests to Dr. N. Suzuki, Departments of Immunology and Medicine, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan.  
E-mail: n3suzuki@marianna-u.ac.jp

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tides is effective for controlling pain after knee surgery<sup>14</sup>; locally administered opioid agonists interact with opioid receptors that are present on peripheral sensory nerves and are apparently upregulated during the development of inflammation<sup>15-17</sup>. It has been reported that opioid peptides are found in resident immune cells within inflamed peripheral tissue<sup>12,17</sup>, consistent with studies showing that opioid peptides are produced within immune cells in culture<sup>12,17,18</sup>. Opioid-containing cells have been described in inflamed human synovial tissue<sup>15-17</sup>.

We examined the effects of ENK and END, both of which have been found to be increased in RA joints<sup>11</sup>, on several functions of synovial cells from patients with RA. We found that the opioid peptides modulate excessive RA synovial cell functions *in vitro*, and it is suggested that these peptides not only act in an antinociceptive manner but also ameliorate synovitis itself in RA.

## MATERIALS AND METHODS

**Patients.** Thirty-two patients with RA as defined by the revised criteria of the American College of Rheumatology<sup>19</sup> were recruited; 26 were women and 6 were men. Their mean  $\pm$  SD age was 57.0  $\pm$  11.3 yrs. All were or had been positive for rheumatoid factor.

Most of the patients were receiving nonsteroidal antiinflammatory and/or disease modifying antirheumatic drugs including methotrexate. None had been treated with high doses of corticosteroids, cytotoxic drugs, or immunosuppressants. Approval of the Human Studies Committee and individual informed consent from each patient were obtained. Synovial tissues were obtained from the joints of patients undergoing total knee replacement.

**Culture medium.** The culture medium used was Iscove's modified Dulbecco's medium (IMDM; Flow Laboratories, Rockville, MD, USA) containing penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml; Life Technologies, Tokyo, Japan), and 10% fetal calf serum (FCS; Life Technologies).

**Reagents.** The following reagents were used: leucine-enkephalin (leu-ENK) (Sigma, St. Louis, MO, USA), methionine-enkephalin (met-ENK) (Sigma), END (Sigma), and naloxone (Wako, Osaka, Japan). In some experiments, somatostatin (SOM; Sigma) and calcitonin gene related peptide (CGRP; Sigma) were included as positive and negative controls, respectively<sup>20,22</sup>. Reagents were initially diluted in phosphate buffered saline (PBS) to 10 mM, filter sterilized, and stored at  $-20^{\circ}\text{C}$  until use.

**Synovial cells.** Synovial tissue cells were isolated by enzymatic dispersion of synovial tissues obtained from patients with RA undergoing joint surgery. The tissues were minced into 2-3 mm pieces and digested with 1 mg/ml collagenase for 3-4 h at  $37^{\circ}\text{C}$  in serum-free IMDM, followed by trypsin treatment for 1 h. The resultant cell suspension was filtered to remove fragments of undigested synovium. Then the cells were cultured in the medium overnight<sup>20,22</sup>, and used as primary synovial cells; the major types of primary synovial cells are fibroblast-like synovial cells, macrophage-like synovial cells, and infiltrating lymphocytes.

**Proliferation assay.** Optimal culture conditions for proliferation of RA synovial cells were as determined<sup>20</sup>. Six replicate cultures of RA synovial cells were established in 96 well flat bottom microtiter plates (Costar, Cambridge, MA, USA) in a total volume of 200  $\mu$ l of IMDM with 10% FCS and incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air. Synovial cells were cultured with various concentrations of leu-ENK, met-ENK, and END for 72 h. Proliferation response was measured by the incorporation of [ $^3\text{H}$ ]-thymidine (1  $\mu$ Ci/well; Amersham International, Buckinghamshire, UK) during the last 8 h of the cultures.

**Limiting dilution reverse transcription-polymerase chain reaction (RT-PCR) analysis.** TNF- $\alpha$ , IL-1 $\beta$ , and MMP-9 mRNA expression of RA synovial cells

was estimated by RT-PCR<sup>20,22</sup>. Briefly, RA synovial cells were cultured for 16 h with opioid peptides. Total RNA was extracted from the RA synovial cells, then RNA concentration of the clinical samples was quantified using the GeneQuant RNA/DNA calculator (Pharmacia Biotech, Cambridge, UK), and complementary DNA (cDNA) was synthesized.  $\beta$ -actin primers were used to compare and monitor efficient cDNA synthesis between different samples<sup>20,22</sup>. For all reactions, temperature cycling was as follows: step 1,  $94^{\circ}\text{C}$  for 1 min; step 2,  $55^{\circ}\text{C}$  for 1 min; and step 3,  $72^{\circ}\text{C}$  for 2 min. Steps 1 through 3 were repeated 35 times, followed by  $72^{\circ}\text{C}$  for 10 min. We adapted RT-PCR analysis using limiting dilutions of cDNA to accurately compare the relative amounts of mRNA expressed in different samples, as reported<sup>20,22</sup>. cDNA of all the samples were diluted into 100  $\mu$ l. For one cDNA sample, we performed 3 PCR reactions that were exactly the same except for introduction of different amounts of cDNA into the reactions — one reaction contained 2  $\mu$ l of cDNA (2%), the second 5  $\mu$ l (5%), and the third 10  $\mu$ l (10%). After PCR amplification, 10  $\mu$ l each of the 3 amplified reactions were run in parallel in the gel. This showed that the PCR reaction ranges within the logarithmic phase of the PCR amplification.

mRNA expression of 3 subtypes of opioid receptor — referred to as  $\mu$ ,  $\delta$ , and  $\kappa$  — in RA synovial cells was studied using the RT-PCR method<sup>23-25</sup>. The reaction primers used are shown in Table 1.

**Immunoblotting analysis.** TNF- $\alpha$  and IL-1 $\beta$  protein production of RA synovial cells was estimated by immunoblotting analysis<sup>21</sup>, because we found that RA primary synovial cells spontaneously secreted large quantities of rheumatoid factors that interfered with the cytokine ELISA systems. Thus, it was not possible to accurately measure cytokine concentrations of culture supernatants of the primary synovial cells using ELISA.

Briefly, RA synovial cells were cultured for 24 h with opioid peptides. Equivalent amounts of culture supernatants were resolved by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocked with 5% bovine serum albumin (BSA). Blots were probed with anti-TNF- $\alpha$  and anti-IL-1 $\beta$  antibodies and biotin conjugated anti-mouse IgG antibody, followed by streptavidin-peroxidase and detection by chemiluminescence (Amersham International). The intensity of the detected bands was analyzed with gel plotting Image 1.55 software from the US National Institutes of Health<sup>21,22</sup>.

**Zymography.** The culture supernatants were resolved as zymograph samples in 0.05 M Tris-HCl, pH 7.4, 5 mM  $\text{CaCl}_2$ , 1% SDS, 5% glycerol, and applied

Table 1. Primers used in this study.

Primer	Amplified Products, Size (bp)	Sequence
$\beta$ -actin	314	sense; TCCTGTGGCATCCACGAAACT antisense; GAAGCATTTGCGGTGGACGAT
TNF- $\alpha$	481	sense; ATGAGCACTGAAAGCATGATC antisense; GGCGATGCGGCTGATGGT
IL-1 $\beta$	438	sense; GTACCTGAGCTCGCCAGTG antisense; TCCCTGGAGGTGGAGAGC
MMP-9	849	sense; CCCGGACCAAGGATACAG antisense; GGCTTTCTCTCGGTACTG
$\mu$ receptor	432	sense; GATCATGGCCCTCTACTCCATC antisense; GGAACCTTGCCTGTATTTTGT
$\delta$ receptor	716	sense; CAAGTACCTGATGGAGACGT antisense; CTGAAGCTGCTGGGGTCTGGGC
$\kappa$ receptor	724	sense; GTCTACTTGATGAATTCCTGG antisense; CTGCTAGTGCTCTGCCGCTC

IL: interleukin, TNF: tumor necrosis factor, MMP-9: matrix metalloproteinase 9.

on a 7.5% SDS-polyacrylamide gel containing 0.5% gelatin<sup>21</sup>. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h. The gelatinolytic reaction was induced by incubating the gels in the reaction buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 1 mM ZnCl<sub>2</sub>) at 37°C for an appropriate period of time (usually 24 h)<sup>21,22</sup>. Staining and destaining were carried out with Coomassie brilliant blue R-250 and 10% acetic acid/10% methanol, respectively.

**Radioreceptor assay.** Radioreceptor assay was performed as described<sup>22,26</sup>. Briefly, RA synovial cells were preincubated in incubation buffer at room temperature (50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, with 0.4% BSA). <sup>125</sup>I labeled human END (10<sup>-10</sup> M; NEN Life Science Products, Boston, MA, USA) were introduced into the cell cultures in the presence of increasing amounts (10<sup>-6</sup> to 10<sup>-10</sup> M) of END and other competing peptides including somatostatin and substance P. Specific binding was determined as the difference between the amounts of radiolabeled neuropeptide bound in the absence and in the presence of nonradiolabeled neuropeptide. The reaction was terminated by washing the cells twice in incubation buffer, and the cells were then lysed in 1 ml of 1% BSA-PBS-0.1% Triton X-100. Radioactivity in the cell lysate was quantified in a  $\gamma$  counting system. The dissociation constant (K<sub>d</sub>) and the number of binding sites (B<sub>max</sub>) of RA synovial cells were determined by Scatchard plot analysis<sup>22</sup>.

**Immunohistochemical staining.** Paraffin sections 3  $\mu$ m thick were cut, deparaffinized, and rinsed in wash buffer (0.05 M Tris-HCl, pH 7.6, 0.3 M NaCl, 0.1% Tween-20). All subsequent procedures were performed using a Dako labeled streptavidin-biotin (LSAB) kit (Dako, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked by incubating sections with 3% H<sub>2</sub>O<sub>2</sub> in water at room temperature for 10 min. Nonspecific binding sites were blocked with blocking buffer (serum-free protein in PBS with 0.015 M sodium azide) for 30 min. The samples were incubated with rabbit polyclonal anti- $\mu$ -subtype opioid receptor antibody (Chemicon, Temecula, CA, USA) overnight at 4°C. The reactivity was visualized with streptavidin-biotin complex and diaminobenzidine tetrahydrochloride-H<sub>2</sub>O<sub>2</sub> solution, and counterstained with hematoxylin.

**Immunocytochemical staining.** RA synovial cells were cultured in microchamber slides (Nalge Nunc, Naperville, IL, USA) and allowed to adhere overnight<sup>20,22</sup>. They were preincubated with opioid peptides for 1 h, followed by stimulation with TNF- $\alpha$  for 2 h. The samples were fixed with cold acetone for 15 min and then blocked with 2% skim milk for 30 min. The

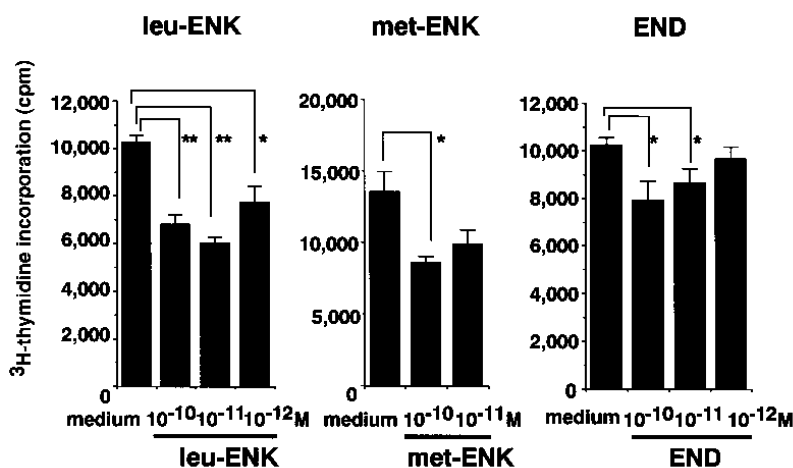
samples were incubated with rabbit polyclonal anti-CREB antibody (Upstate Biotechnology, New York, NY, USA) and anti-ser-133 phosphorylated CREB-specific antibody (BioLabs, Beverly, MA, USA) overnight at 4°C. All subsequent procedures were performed using the Dako LSAB alkaline phosphatase kit, as suggested by the manufacturer.

**Statistical analysis.** Results of irregular distribution in the experiments were compared by Mann-Whitney U test. A p value < 0.05 was judged to be statistically significant.

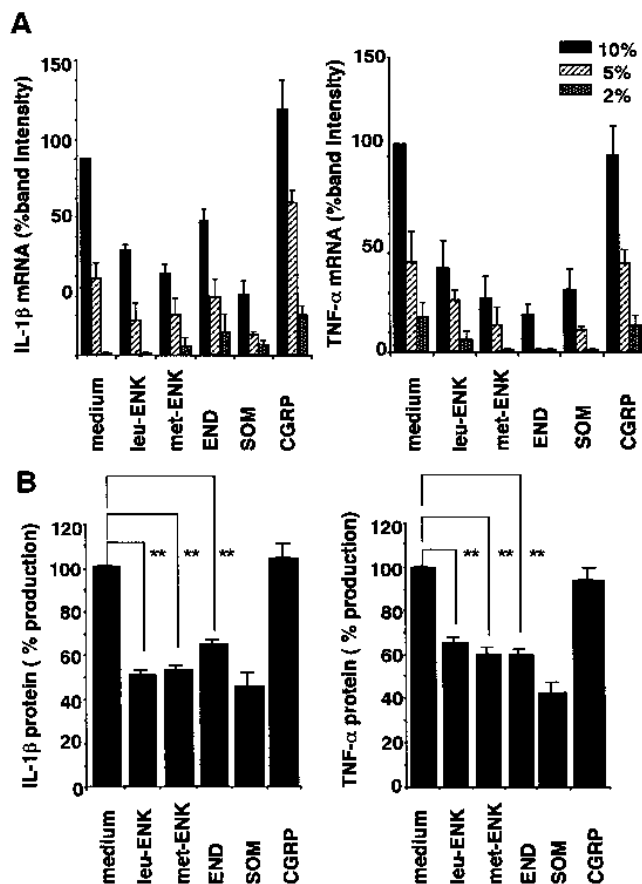
## RESULTS

**Effect of END and ENK on proliferation responses of RA synovial cells.** We first studied proliferation responses of RA primary synovial cells in the presence of various concentrations of leu-ENK, met-ENK, and END. We found that proliferation was inhibited by the addition of leu-ENK, met-ENK, and END into the cell cultures (Figure 1). Thus it is evident that the opioid peptides significantly inhibit proliferation of RA synovial cells *in vitro*. We also found that proliferation of fibroblast-like synovial cell lines established after several passages from RA synovial cells was not inhibited by the opioid peptides (data not shown), suggesting that proliferation of fibroblasts is rather refractory to the opioids.

**Effect of END and ENK on cytokine production of RA synovial cells.** We next studied effects of END and ENK on proinflammatory cytokine mRNA expression of RA synovial cells (Figure 2A). RA primary synovial cells were cultured in the presence or absence of leu-ENK, met-ENK, and END for 16 h. We used limiting dilution RT-PCR analysis to detect any change of mRNA expression. Proinflammatory cytokine protein secretion of RA primary synovial cells cultured for 24 h in the presence of the opioids was assessed by immunoblotting the culture supernatants (Figure 2B), because we found that RA primary synovial cells spontaneously secreted large



**Figure 1.** Effects of leu-ENK, met-ENK, and END on proliferative responses of RA synovial cells. RA synovial cells ( $2 \times 10^4$  cells) were cultured in 6 replicates with various concentrations of leu-ENK, met-ENK, and END for 3 days. Results shown here were mean + SEM of 6 independent experiments using primary synovial cells from 6 different patients. \*p < 0.05, \*\*p < 0.01, Mann-Whitney U test.



**Figure 2.** Effects of leu-ENK, met-ENK, and END on proinflammatory cytokine mRNA expression and cytokine protein production of RA synovial cells. RA synovial cells ( $1 \times 10^6$  cells) were cultured with leu-ENK, met-ENK, and END for 16 h (RT-PCR) and for 24 h (protein production). A. Cytokine mRNA expression was assessed by limiting dilution RT-PCR; 10%, 5%, and 2% represent amounts of total cDNA used for RT-PCR. cDNA was also subjected to PCR amplification for expression of  $\beta$ -actin gene. Relative band intensity (band intensity of IL-1 $\beta$  or TNF- $\alpha$  per that of  $\beta$ -actin) of the sample was assessed by NIH image software. Relative band intensity of the PCR using 10% of total cDNA from nonstimulated cultures (medium) was used as control (100% band intensity). Accordingly, relative band intensity of each reaction was expressed as percentage of band intensity. Results shown here were mean + SEM of 6 independent experiments using 6 synovial cells from 6 different patients. B. Spontaneous IL-1 $\beta$  and TNF- $\alpha$  protein production were analyzed by immunoblotting. Relative band intensity was similarly assessed. Relative band intensity of the nonstimulated cultures (medium) was used as control (100% activity). Accordingly, relative band intensity of the other cell cultures was expressed as percentage production. Results shown here were mean + SEM of 6 independent experiments using 6 synovial cells from 6 different patients. \*\* $p < 0.01$ , Mann-Whitney U test.

quantities of rheumatoid factors, and that the rheumatoid factors actually interfered with the cytokine-ELISA systems.

Primary synovial cells expressed mRNA of TNF- $\alpha$  and IL-1 $\beta$  spontaneously. Treatment with END and ENK ( $10^{-10}$  M) inhibited TNF- $\alpha$  and IL-1 $\beta$  mRNA expression of RA primary synovial cells. Treatment of the synovial cells with END and ENK reduced production of TNF- $\alpha$  and IL-1 $\beta$ . In contrast, END and ENK did not affect spontaneous IL-6 and IL-8

mRNA expression and protein production by the synovial cells (data not shown). Taken together, the results suggest that END and ENK reduce proinflammatory cytokine production of RA synovial cells.

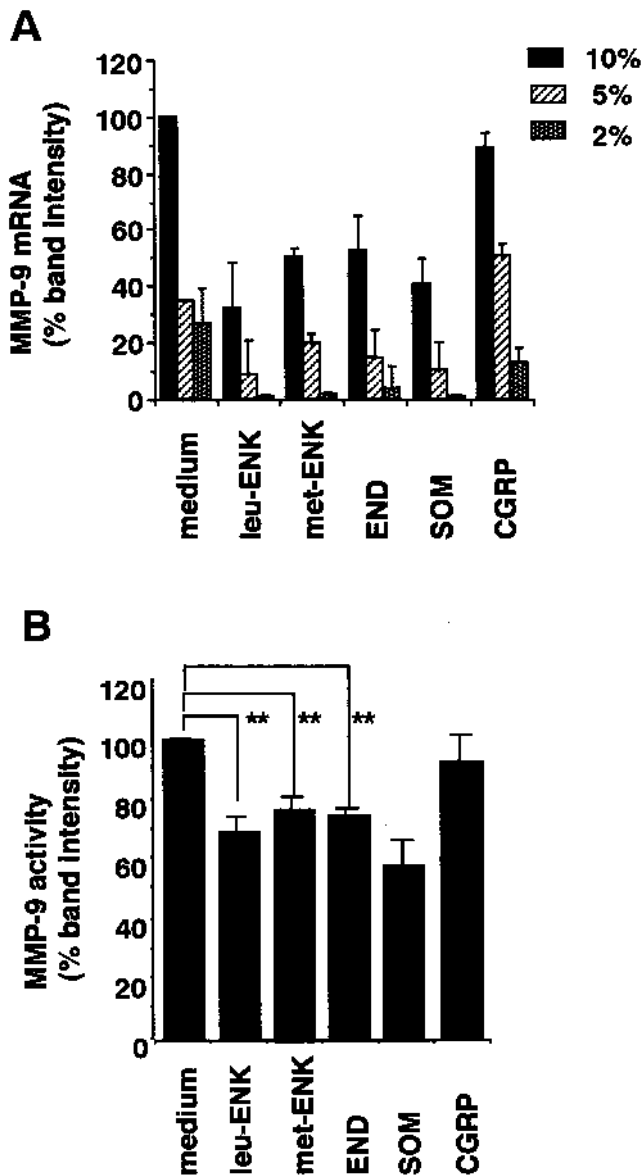
**Effect of END and ENK on MMP production by RA synovial cells.** We further tested whether END and ENK inhibit MMP production of RA synovial cells. As shown in Figure 3A, treatment with END and ENK significantly reduced MMP-9 mRNA expression of the RA synovial cells. MMP activity of the samples was studied using gelatin zymography (Figure 3B). The treatment reduced the MMP-9 enzymatic activity (gelatinase B activity, MW 84 kDa) of synovial cells. The opioid peptides did not elicit any effect on the activity of other MMP, such as MMP-1 and MMP-2.

**Characterization of opioid receptors expressed on RA synovial cells.** We investigated opioid receptor expression of RA synovial cells using RT-PCR. We found that  $\mu$ -subtype opioid receptor mRNA was expressed constitutively on synovial cells of all the patients with RA studied (Figure 4A).  $\delta$ -subtype receptor was expressed in 4 out of the 10 patients studied.  $\kappa$ -subtype receptor was not expressed in all the patients. In addition, we confirmed by immunohistochemical staining of RA synovial tissue that  $\mu$ -subtype receptor was actually detected in RA synovial lining and sublining cells (Figure 4B).

We further characterized the opioid receptor(s) on RA synovial cells using a radioreceptor assay, even though the primary RA synovial cells contained several cell types. To confirm the specificity of  $^{125}$ I-END binding to RA synovial cells, competitive inhibition studies were carried out using nonradiolabeled END, somatostatin, and substance P as inhibitors. Nonradiolabeled END, but not somatostatin or substance P, inhibited the  $^{125}$ I-END binding to RA synovial cells. Scatchard plot analysis (Figure 4C) suggested the presence of a single class of binding site<sup>22</sup>. END binding sites had a  $K_d$  of  $1.4 \times 10^{-9}$  M and  $B_{max}$  of  $5.5 \times 10^{-7}$  M ( $7.3 \times 10^4$  sites/cell).

We also studied effects of a specific  $\mu$ -opioid receptor antagonist, naloxone<sup>27</sup>, on the inhibitory effects of END and ENK on RA synovial cell function (Figure 4D). We found that naloxone reversed END and ENK induced reduction of TNF- $\alpha$  and IL-1 $\beta$  protein production by RA synovial cells.

**Effect of END and ENK on nuclear translocation of CREB in RA synovial cells.** It has been shown in other cell types that opioid peptides modulate cyclic AMP (cAMP) mediated responses and activation of nuclear transcription factor cAMP responsive element binding protein (CREB), followed by reduction of transcriptional activity of relevant genes<sup>28,29</sup>. To test whether END and ENK act on CREB in RA synovial cells, we examined nuclear translocation of CREB preexisting within the cytoplasm in response to TNF- $\alpha$  stimulation in RA primary synovial cells using immunocytochemical staining. As shown in Figure 5, CREB protein accumulated in nuclei when synovial cells were stimulated with TNF- $\alpha$ . This nuclear accumulation of CREB protein was inhibited by treat-



**Figure 3.** Effects of leu-ENK, met-ENK, and END on MMP-9 expression and enzymatic activity of RA synovial cells. RA synovial cells ( $1 \times 10^6$  cells) were incubated with leu-ENK, met-ENK, and END for 16 h (A) and 24 h (B). A. MMP-9 mRNA expression was assessed by limiting dilution RT-PCR. Relative band intensity (band intensity of MMP-9/ $\beta$ -actin) was calculated using NIH image software. Relative band intensity of the nonstimulated culture (medium) was used as control (100% intensity). Results shown were mean + SEM of 6 independent experiments. B. MMP-9 activity (gelatinase activity, MW 84 kDa) of culture supernatants of RA synovial cells was similarly estimated using gelatin zymography. Results shown were mean + SEM of 6 independent experiments. \*\* $p < 0.01$  by Mann-Whitney U test.

ment of the synovial cells with leu-ENK, met-ENK, and END  $10^{-10}$  M. This inhibition of nuclear translocation of CREB was confirmed when we employed anti-ser-133 phosphorylated CREB-specific antibody (Figure 5). Thus, it is evident that the opioid peptides inhibit nuclear translocation and phosphorylation of CREB transcription factor to exert their inhibitory effects.

## DISCUSSION

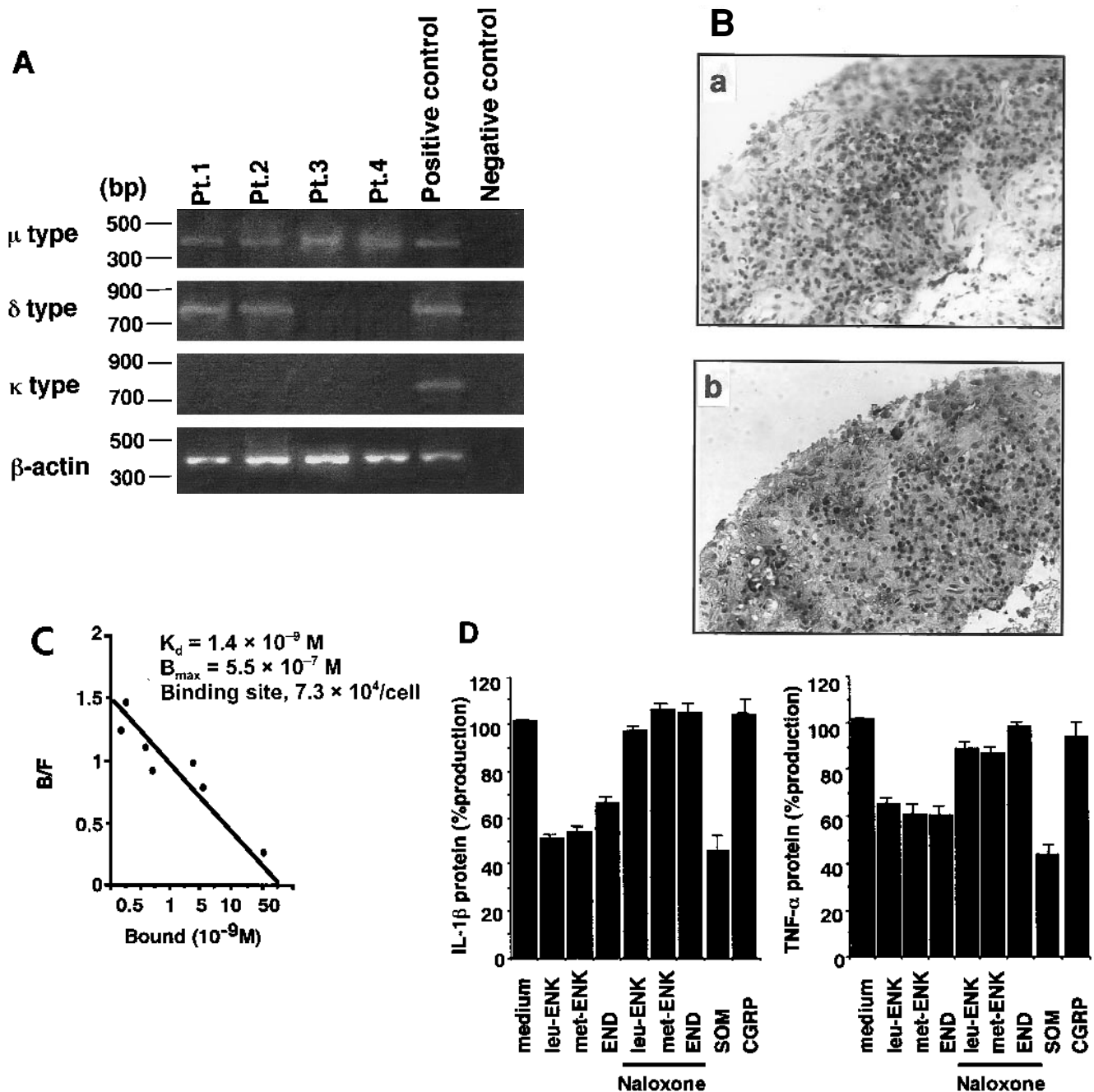
We found that ENK and END inhibit proliferation of and secretion of proinflammatory cytokines and MMP production by RA synovial cells. The  $\mu$ -type receptor antagonist naloxone reversed the antiinflammatory effects of the opioid peptides, confirming involvement of the  $\mu$ -type receptor for the inhibitory effects. Thus, the opioid peptides may exert not only antinociceptive action as reported<sup>14</sup>, but also antiinflammatory effects on synovitis of patients with RA. Our results favor the hypothesis that ENK and END inhibit the pathological responses of RA and have rather beneficial effects on RA pathophysiology.

With regard to proinflammatory cytokine and MMP production, we found that IL-1 $\beta$ , TNF- $\alpha$ , and MMP-9 production were modulated by ENK and END. However, IL-6, IL-8, MMP-1, and MMP-2 production was unaffected by the treatment (data not shown). We have previously shown that somatostatin, CGRP, and vasoactive intestinal peptide modulate production of the opioid refractory cytokines and MMP. It is thus suggested that each neuropeptide or opioid peptide plays different roles for regulating RA synovial cell functions, even though they sometimes share the same effect<sup>20,22</sup>.

We found that  $\mu$ -subtype receptor is constitutively expressed on and is functional in RA synovial cells. However, we do not know whether the  $\delta$ -subtype receptor, whose mRNA is expressed on synovial cells of a minority of patients with RA (4 out of 10 patients tested), is functional and is involved in the opioid mediated inhibitory effects. The  $\mu$ -type receptor selective antagonist naloxone completely reversed the inhibitory effects, suggesting that  $\mu$ -type receptor is responsible for the inhibitory effects of both ENK and END. It remains to be determined whether  $\delta$ -receptor is expressed and functionally involved in the opioid mediated alteration of synovial cell functions. With regard to  $\kappa$ -type receptor, whose natural ligand is dynorphin<sup>30</sup>, we did not detect expression of the  $\kappa$ -subtype receptor mRNA on RA synovial cells. We interpreted this as meaning that dynorphin did not play a major role in modulation of RA synovial cell functions in the joints.

It is interesting that opioid receptors have been found in activated lymphocytes and monocytes, thus it is likely that ENK and END released within the affected joints modulate functions of synovium infiltrating lymphocytes<sup>18</sup>. It has been reported that the effects of END on immune function are biphasic, with a reduction of responses at the higher concentrations and an increase at lower concentrations<sup>31</sup>. Thus, it is possible that ENK and END exert their inhibitory effects on synovial cells, synovium infiltrating lymphocytes, and nerve fibers innervating synovial tissue.

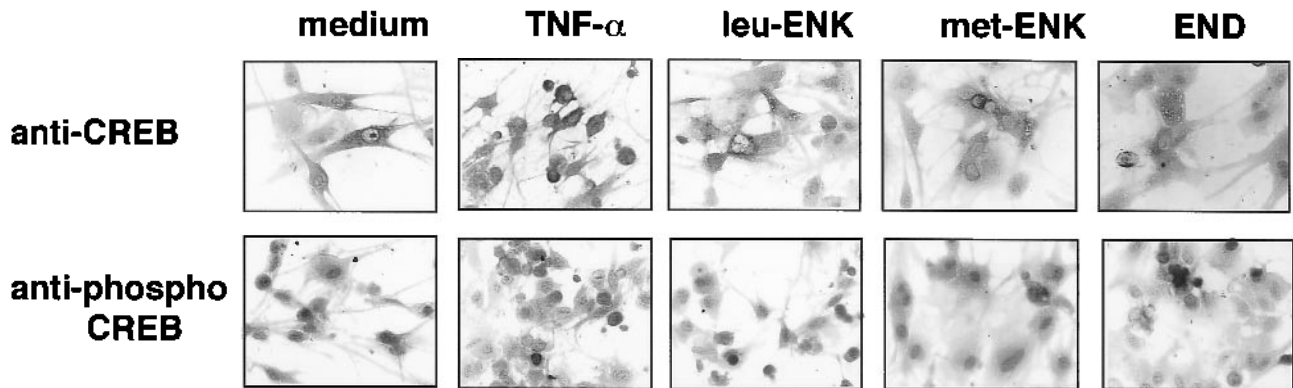
END is a prominent opioid peptide involved in pain<sup>15,17,32</sup>. In inflamed subcutaneous tissue, mRNA encoding POMC is upregulated and END is readily detectable within lymphocyte and monocytic cells<sup>12,33,34</sup>. END is mostly present in memory T cells<sup>35</sup>. We have confirmed that POMC and prepro-enkephalin, precursors of ENK and END, respectively, are



**Figure 4.** Analysis of opioid receptor expression on RA synovial cells. **A.** RA synovial cells ( $1 \times 10^6$  cells) were cultured for 16 h.  $\mu$ ,  $\delta$ , and  $\kappa$ -opioid receptor mRNA expression of RA synovial cells were studied by RT-PCR. Results shown are representative of 6 independent experiments with similar results. **B.**  $\mu$ -subtype opioid receptor expression of RA synovial tissue was examined by immunohistochemical staining. (a) Negative control staining; (b)  $\mu$ -subtype opioid receptor staining. **C.** RA primary synovial cells ( $5 \times 10^4$  cells) were precultured with various concentrations of nonradiolabeled END for 30 min, then  $10^{-10}$  M [ $^{125}$ I] human END was introduced into the cell cultures. Cells were incubated for 120 min at room temperature. Dissociation constant ( $K_d$ ) and number of binding sites ( $B_{max}$ ) for cells were determined by Scatchard plot analysis<sup>22</sup>. Results shown are representative of 5 independent experiments with similar results. **D.** RA synovial cells ( $2.5 \times 10^4$  cells) were preincubated with  $\mu$ -subtype opioid receptor antagonist (naloxone) for 60 min, then cells were cultured with leu-ENK, met-ENK, and END for 24 h. Spontaneous IL-1 $\beta$  and TNF- $\alpha$  protein production were analyzed by immunoblotting. Results shown are mean + SEM of 5 independent experiments.

produced by synovium infiltrating lymphocytes and RA synovial cells, but are absent in noninflamed tissue, as reported<sup>11-13,15,17</sup>. Thus, opioid peptides and their receptors

expressed on various cell types within synovial tissue may constitute a regulatory circuit to control inflammatory reactions in RA.



**Figure 5.** Effects of leu-ENK, met-ENK, and END on nuclear translocation of CREB in RA synovial cells by immunocytochemical analysis. RA synovial cells ( $2 \times 10^4$  cells) were cultured in chamber slides. Cells were preincubated with met-ENK, leu-ENK, and END for 2 h, followed by stimulation with TNF- $\alpha$  5 ng/ml for 0 to 120 min. We studied effects of met-ENK, leu-ENK, and END on nuclear translocation of CREB of synovial cells, using anti-ser-133 phosphorylated CREB-specific antibody. Control staining using nonimmune rabbit IgG showed no positive staining cells (data not shown). Results shown are representative of 5 independent experiments with similar results. Note that CREB is detected within nuclei but not cytoplasm of TNF- $\alpha$  stimulated synovial cells, whereas CREB is mainly present within cytoplasm of the cells in the presence of leu-ENK, met-ENK, and END.

Several studies have reported that circulating opioid peptide levels are reduced in patients with RA<sup>36-38</sup>. Low circulating levels of END occur in various disease states, perhaps as part of a stress response<sup>39</sup>. It is possible that reduced opioid concentrations in systemic circulation contribute to the development of local inflammation of affected joints, whereas opioid peptides produced locally within the joints downregulate the inflammation. The sum of the influences of the systemic and local opioid peptides may affect the outcome of the arthritis.

Accumulation of intracellular cAMP by adenylate cyclase induces activation of catalytic subunits of protein kinase A, leading to phosphorylation of ser-133 of CREB, accompanying acquisition of transcription factor activity<sup>40</sup>. ENK and END inhibit adenylate cyclase activity and then cAMP accumulation, followed by deficient protein kinase A activity, and this process leads to the deficient CREB activity in other cell types<sup>28,29,40,41</sup>. We found that nuclear translocation and phosphorylation of CREB were induced by TNF- $\alpha$  stimulation in RA synovial cells; the translocation and phosphorylation were efficiently inhibited by the opioid treatment. We also studied effects of the opioids on basal CREB nuclear localization of RA synovial cells. The CREB nuclear translocation of non-stimulated synovial cells was too low a frequency and too faint to make a reliable comparison of the effects between nontreatment and opioid treatment on RA synovial cells.

The involvement of cAMP and cAMP responsive element in the upregulation of IL-1 $\beta$  synthesis have been described<sup>41</sup>. The antiinflammatory effects of the opioid peptides, such as reduction of IL-1 $\beta$  production, may be mediated by the reduced CREB activity.

Opioids inhibit the calcium dependent release of excitatory, proinflammatory compounds such as substance P from peripheral sensory nerve endings<sup>42</sup>, which may partly contribute to the antiinflammatory actions of opioids. However,

this is not the major cause of the action of opioid peptides in this study, because the opioid peptides directly inhibited TNF- $\alpha$  induced phosphorylation and nuclear translocation of CREB without participation of substance P.

It has been reported that opioids modulate lymphocyte and macrophage apoptosis<sup>43,44</sup>. Thus it is intriguing to study the effects of opioids on synovial cell apoptosis in RA.

Our results suggest potential regulatory roles of opioids in the inflammatory and destructive processes of the joints in RA. The opioid peptides may participate not only in pain control but also in pathophysiological control of inflammation within RA joints. Further, the antiinflammatory effects may be applicable for treating patients with RA.

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