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Antiinflammatory Role of Endomorphins in Osteoarthritis, Rheumatoid Arthritis, and Adjuvant-Induced Polyarthritis

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Objective. **Pain sensitization and the related secretion of neuropeptides from sensory nerve terminals are proinflammatory in osteoarthritis (OA), rheumatoid arthritis (RA), and adjuvant-induced polyarthritis. In contrast, endogenous opioids such as the recently discovered endomorphins (EMs) are antiinflammatory. However, the role of endogenous EMs such as EM-1 and EM-2 has never been investigated in OA and RA.**

Methods. **We established a highly sensitive radioimmunoassay to detect EM-1 and EM-2. In patients with RA and patients with OA, immunohistochemistry for EM-1 and EM-2 was performed, and double-staining was used to identify EM-positive cells. The effects of EM-1 and EM-2 on the secretion of interleukin-6 (IL-6) and IL-8 from human synovial tissue were studied by tissue superfusion, and the therapeutic effects of EM-1 were tested in a rat model of adjuvant-induced polyarthritis.**

Results. **EM-positive cells were located in the sublining area and vessel walls but were particularly evident in the highly inflamed lining area. Human macrophages, T cells, and fibroblasts stained positive for EMs. The synovial density of EM-positive cells was higher in patients with OA than in those with RA. EM-1 inhibited synovial secretion of IL-6 in patients with RA and secretion of IL-8 in patients with RA and those with OA (maximum $10^{-10}M$). EM-2 inhibited IL-8 secretion only from RA tissue (maximum $10^{-10}M$). In rats with adjuvant-induced polyarthritis, thymus, spleen, and synovial tissue contained significantly more EM-1 than was observed in controls. Rats with adjuvant-induced polyarthritis benefited from EM-1 treatment.**

Conclusion. **EM-1 had antiinflammatory effects in patients with OA or RA and in a model of adjuvant-induced polyarthritis. Local enhancement of EM-1 might be an interesting therapeutic option in different forms of arthritis.**

Pain is a hallmark of the arthritic process, and sensitization of pain pathways has been documented in arthritis (for review, see ref. 1). However, pain is not simply an unwanted symptom without connections to inflammation, because the most important neuropeptide of sensory nerve fibers, substance P, is a strong proinflammatory signal for acute and chronic inflammation (2,3). In addition, cytokines such as interleukin-6 (IL-6) in the local inflammatory environment contribute to pain sensitization (4).

Endogenous opioids such as β-endorphin, methionine–enkephalin, and leucine–enkephalin counteract pain pathways and related inflammation (for review, see refs. 5 and 6). This is achieved by binding of

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endogenous opioids to opioid receptors on peripheral sensory nerve terminals inhibiting substance P release (7–10), and by directly influencing immune and other cells involved in the proinflammatory process (11). Thus, endogenous and exogenous opioids are not only analgesic but can be antiinflammatory, which is in part mediated via μ-opioid receptor agonism, as shown in animal models and in humans (12,13). In order to study these phenomena at μ-opioid receptors, the endogenous opioid β-endorphin has often been used. However, β-endorphin is not highly specific for this particular receptor, and morphine, the exogenous μ-opioid receptor agonist, has never found a place in the modern arsenal of antiinflammatory agents, due to systemic adverse events.

With the discovery of the two endogenous tetrapeptides endomorphin 1 (EM-1) and EM-2, two endogenous, highly specific μ-opioid receptor agonists have been identified (14). The mechanism of action for EMs is through G protein coupling and consequent inhibition of adenylate cyclase and Ca^{2+}-channel currents (15). Selective activation of μ-opioid receptors with EMs reduces joint mechanosensitivity to noxious stimuli, which is lost during inflammation due to down-regulation of the receptor (16). Similarly, EM-1 reduces blood flow to the joint, which is lost during inflammation due to receptor down-regulation (17). EM-1 and EM-2 were also shown to have analgesic effects, which might lead to antiinflammatory conditions (18,19). In a blister skin model, it was also shown that substance P–induced inflammation can be directly inhibited by EM-1 (20). These studies indicate that EMs might be good candidates to study in osteoarthritis (OA) and rheumatoid arthritis (RA).

The questions remain whether EMs are expressed in the inflamed synovial tissue of patients with RA and patients with OA, whether a difference in expression exists between RA and OA, whether cells other than neurons express and secrete EMs, whether EMs have antiinflammatory activities in the synovial tissue of patients with RA and patients with OA, whether EMs are up-regulated in an experimental form of arthritis, and whether EMs inhibit the inflammatory process in arthritis. The aim of this study was to provide some answers to the above-mentioned questions. In a comparative manner, we studied tissue obtained from patients with RA and patients with OA, and we combined these investigations using human material with experimental studies in an animal model of adjuvant-induced polyarthritis.

PATIENTS AND METHODS

Patients. Ten patients with refractory chronic RA and 10 patients with chronic OA, who underwent elective knee joint replacement surgery, were included without further selection. The diagnosis of RA was based on the established criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (21). All patients were informed about the purpose of the study and gave written consent. The study was approved by the ethics committee of the University of Regensburg. Clinical and laboratory data are shown in Table 1 (the determination of markers of synovial inflammation is described below), and the erythrocyte sedimentation rate was measured by standard techniques.

Radioimmunoassay for detection of EM-1 and EM-2. Tissue samples were collected, acid-extracted, vacuum-dried, and reconstituted in phosphate buffer for radioimmunoassay (RIA) of EM-1 and EM-2. Details of EM-1 and EM-2 RIAs have been previously published (22). Briefly, RIAs for EM-1 and EM-2 were fully developed and validated in our laboratories. Antisera for C-terminally amidated EM-1 and EM-2 conjugated to keyhole limpet hemocyanin were raised in rabbits and supplied by Advanced Chemtech (Louisville, KY). Final antiserum titers used in the RIAs were 1:12,000 and 1:300,000 for EM-1 and EM-2, respectively. Cross-reactivity of EM-1 antiserum with synthetic EM-2 was 0.5%, and cross-reactivity of EM-2 antiserum with synthetic EM-1 was 0.01%. The antiserum did not cross-react with other opioid peptides such as β-endorphin, dynorphin A, Met-enkephalin, or orphanin FQ. The assay limit of detection for both EM-1 and EM-2 was 5 pg/tube. Intraassay coefficients of variation at a dose of 10 pg of EM-1 or EM-2 were 5.5% and 4.7%, respectively.

Synthetic EM-1 and EM-2 (NeoMPS, Strasbourg, France) were iodinated with ^{125}I (Amersham, Herts, UK) using the chloramine T method, and tracers were purified on Sep-Pak columns (Waters), using a gradient of acidified 1-propanol. RIA reagents were incubated in plastic assay tubes at 4°C for 24 hours. Bound tracer was separated from unbound reagents using sheep anti-rabbit antiserum (Therapeutic Antibodies, Llandysul, Wales, UK) in a solution of 4% polyethylene glycol. Pellets were counted for gamma radioactivity.

Synovial tissue preparation and histologic evaluation in RA and OA. Synovial tissue samples were obtained immediately after opening the knee joint capsule. Preparation of the tissue for histologic analysis was performed as previously described (23). Briefly, a piece of synovial tissue of up to 9 cm^2 was dissected. Fat tissue and tissue with a large number of blood vessels were removed. Twelve pieces of ~16 mm^2 from every patient were loaded into 12 superfusion chambers (see below for a description of the superfusion technique), and ~8 pieces of roughly 0.8 cm^2 from the same synovial area were used for histologic evaluation. Samples were immediately placed in protective freezing medium (TissueTek; Sakura Finetek Europe, Zoeterwoude, The Netherlands) and then quick-frozen floating on liquid nitrogen. All tissue samples were stored at −80°C.

Histologic evaluation was carried out as described previously (23). Briefly, frozen tissue samples were cut into 6–8-μm–thick sections, and cell density and lining layer thickness were determined using standard hematoxylin and eosin staining of ~45 sections from at least 2 different tissue samples

per patient. The overall cell density was determined by counting all stained cell nuclei in 17 randomly selected high-power fields of view (400× magnification) and expressed per square millimeter. The lining layer thickness was analyzed by averaging the number of cells in a lining layer cross-section at 9 different locations (400× magnification). To determine the number of T cells (CD3; Dako, Hamburg, Germany), macrophages (CD163; Dako), and vessels (type IV collagen; Dako) in synovial tissue from each patient, 8 cryosections were investigated using alkaline phosphatase–anti–alkaline phosphatase staining, and the number of identified structures was averaged from 17 randomly selected high-power fields of view (400× magnification) and expressed per square millimeter.

Detection of EM-1– and EM-2–positive cells, and double staining in RA and OA. Cryosections (5 μm) from at least 2 different formaldehyde-fixed synovial tissue samples from each patient were air-dried for 1 hour and then rehydrated in 0.05*M* Tris buffered saline (TBS). Nonspecific binding sites were blocked with 0.05*M* TBS containing 10% fetal calf serum (FCS), 10% bovine serum albumin, and 10% normal goat serum for 1 hour at room temperature. After washing with TBS 3 times for 5 minutes, the sections were incubated with the polyclonal antibodies against EM-1 and EM-2 for 12–18 hours at 4°C (polyclonal antisera specific for C-terminally amidated EM-1 or EM-2 conjugated to keyhole limpet hemocyanin were raised in rabbits; a generous gift from Advanced Chemtech, Louisville, KY). The sections were washed 3 times for 5 minutes and then incubated with an alkaline phosphatase–conjugated secondary antibody directed against rabbit IgG. After another wash, the positively marked cells were visualized using BCIP–nitroblue tetrazolium substrate (Dako). Control staining with nonspecific rabbit IgG as the primary antibody was carried out in parallel, which did not reveal any positive staining. In addition, in control stainings without the primary antibody or after neutralizing the primary antibody with authentic EMs, no staining was observed. The density of EM-1– and EM-2–positive cells was averaged from 17 randomly selected high-power fields of view (400× magnification) and expressed per square millimeter.

Immunohistochemical double staining was carried out with the above-mentioned polyclonal antibodies against the 2 EMs each, together with a monoclonal antibody against macrophages (CD163; DakoCytomation, Carpinteria, CA), T lymphocytes (CD3; DakoCytomation), or fibroblasts (prolyl 4-hydroxylase; DakoCytomation). In the first step, staining of EM-1 and EM-2 was carried out as described above. In the second step, double staining of cells was achieved by incubating the sections with the respective monoclonal antibodies against CD163, CD3, and prolyl 4-hydroxylase for 12–18 hours at 4°C. After a further 3 washes with TBS, samples were incubated with a secondary anti-mouse Alexa Fluor 546 IgG (catalog no. A-11030; Molecular Probes, Eugene, OR). Control staining was performed with nonspecific mouse IgG instead of the above-mentioned monoclonal antibodies.

Superfusion of synovial tissue in RA and OA and determination of cytokine levels. As described in detail previously (23), we used a microsuperfusion chamber apparatus to superfuse slices of synovial tissue with culture medium (RPMI 1640, 25 m*M* HEPES [without FCS], 1% penicillin–streptomycin, 30 μ*M* mercaptoethanol, 0.57 m*M* ascorbic acid, 1.3 m*M* calcium; all from Sigma, Munich, Germany). These superfusion chambers had a volume of ~80 μl. Superfusion was performed for 330 minutes at a temperature of 37°C and a flow rate of 66 μl/minute (1 piece per chamber; 12 chambers in parallel). Synovial tissue pieces had a standard size of 5 μm in diameter and were obtained using a precision biopsy punch (Stiefel, Offenbach, Germany). Using 12 chambers, we were

Table 1. Characteristics of the patients with osteoarthritis and rheumatoid arthritis*

Characteristic	Osteoarthritis (n = 10)	Rheumatoid arthritis (n = 10)
Age, years	64.3 ± 3.8	67.0 ± 3.1
No. of women/no. of men (%)	7/3 (70/30)	7/3 (70/30)
ESR, mm/hour	7.5 ± 1.9	20.2 ± 9.4
Synovial lining layer thickness, no. of cells	0.75 ± 0.55	4.33 ± 0.12†
Synovial cellularity, cells/mm^2	520 ± 312	1,807 ± 304‡
Synovial T cell density, cells/mm^2	11.7 ± 3.9	31.2 ± 17.1
Synovial macrophage density, cells/mm^2	27.9 ± 7.8	43.2 ± 13.8
Synovial vascularity, vessels/mm^2	25.7 ± 7.0	32.7 ± 8.4
Medication		
Prednisolone, no. (%)	0 (0)	7 (70)
Prednisolone dosage, mg/day	0 ± 0	6.8 ± 3.2§
Methotrexate, no. (%)	0 (0)	3 (30)
NSAIDs, no. (%)	4 (40)	9 (90)
Sulfasalazine, no. (%)	0 (0)	2 (20)
Hydroxychloroquine, no. (%)	0 (0)	1 (10)
Oral opioid, no. (%)	0 (0)	1 (10)

* Except where indicated otherwise, values are the mean ± SEM. ESR = erythrocyte sedimentation rate; NSAIDs = nonsteroidal antiinflammatory drugs.
† $P < 0.05$ versus osteoarthritis patients.
‡ $P < 0.10$ versus osteoarthritis patients.
§ $P < 0.01$ versus osteoarthritis patients.

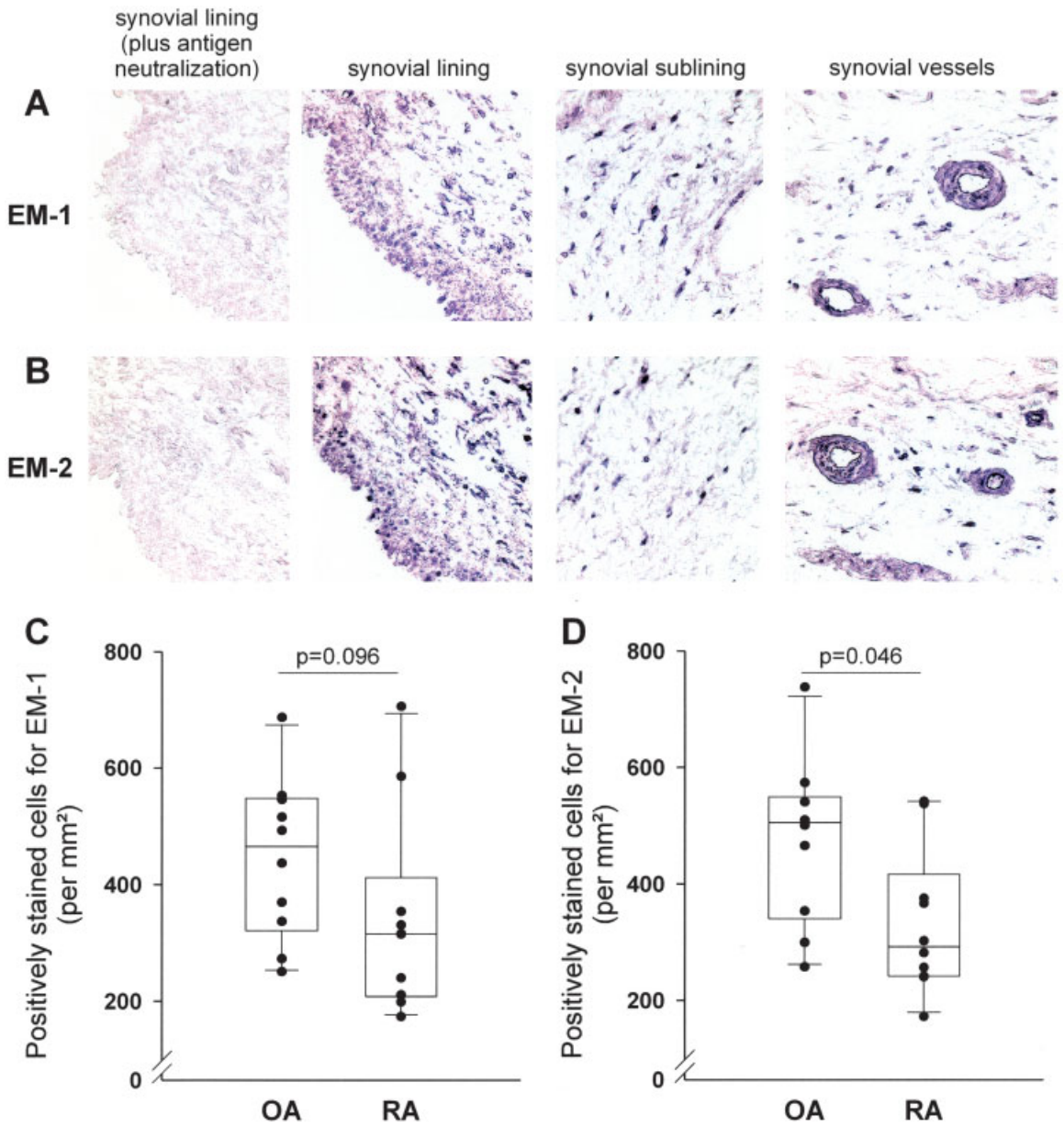

Figure 1. Detection of endomorphin 1 (EM-1) and EM-2. **A** and **B,** Staining of EM-1 and EM-2 in synovial tissue of a patient with rheumatoid arthritis (RA). In addition to vessels, the lining and sublining area are demonstrated. Control stainings with neutralization of the antibodies by the authentic peptides are demonstrated in the left column (original magnification × 200). **C** and **D,** Comparison of density of EM-1–positive and EM-2–positive cells in synovial tissue of 10 patients with RA and 10 patients with osteoarthritis (OA). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Each symbol represents the mean number of cells from 1 patient from 17 high-power fields.

able to investigate 12 slices in 1 experiment involving 1 synovial tissue sample. At 120 minutes, superfusate was collected the first time in order to measure baseline superfusate cytokine concentrations in a fraction of ~1 ml (collected over 15 minutes).

In the second period of superfusion for another 210 minutes, 4 different conditions were tested, as follows: 3 control slices (medium only), 3 slices with EM-1 or EM-2 at $10^{-10}M$, 3 slices with EM-1 or EM-2 at $10^{-9}M$, and 3 slices with EM-1 or EM-2 at $10^{-8}M$. EM-1 and EM-2 were pur-

chased from NeoMPS. Beginning at 315 minutes, superfusate was collected a second time for 15 minutes, and IL-6 and IL-8 were determined in the second sample. Due to technical reasons such as insufficient superfusion flow in a single pumping tube, the number of observations was not always a multiple of 3.

Levels of human IL-6 and IL-8 were determined by enzyme immunometric assay (detection limit in the 2 assays <2 pg/ml) (Endogen via Perbio, Cologne, Germany). The interassay and intraassay coefficients of variation were $<10\%$ for both cytokines.

The cytokine concentration at 120 minutes (Cyt_{120min}) was used to standardize the cytokine-secreting capacity of the different slices. The dimensionless ratio $\Psi = 100 \times (Cyt_{330min}/Cyt_{120min})$ was used to standardize cytokine secretion of each slice at 330 minutes, the same technique as used for spleen slices (24,25). This standardization technique was found to be superior compared with standardization using wet weight, dry weight, protein content, and volume of the slice.

Animals, polyarthritis model, and tissue removal. Polyarthritis was induced in adult male Wistar rats by a single intradermal injection (0.1 ml) of a suspension of ground, heat-killed *Mycobacterium butyricum* into the base of the tail. Rats (an arthritic group with inflamed hind paws and a control nonarthritic group; n = 6 per group) were killed 14 days after injection, and spleens, thymuses, and hind paws were collected on dry ice and stored at −80°C. Spleens and thymuses and synovial tissue harvested from the ankle joints of hind paws (n = 8 of the most inflamed joints) were acid-extracted and measured for EM-1 and EM-2 by RIA (see above).

In a separate experiment, rats were injected with *M butyricum* on day 1, and basal hind paw volumes were measured by plethysmometry (Ugo Basile, Milan, Italy). After being returned to their home cages for 9 days, on the morning of days 9, 10, 11, 12, and 13, rats were given a single intraperitoneal injection of EM-1, either 0.1 μmole or 1 μmole, or saline control. Rats were killed on day 14, and hind paw volumes were measured by plethysmometry. We considered performing an experiment with a μ-opioid receptor antagonist (e.g., CTOP; D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2), but the extremely high costs of the drug at the in vivo dose required were prohibitive.

Statistical analysis. All data are presented as the mean ± SEM. Medians were compared by the Mann-Whitney U test in case of non-normally distributed data and by *t*-test in case of normally distributed data (SPSS/PC Advanced Statistics, version 12.0; SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Localization and density of EM-1– and EM-2–positive cells in synovial tissue from patients with RA and patients with OA. In patients with RA, EM-1– and EM-2–positive cells were particularly located in the proliferating lining zone (Figures 1A and B). However, a proportion of cells in the sublining area also stained positive for EM-1 and EM-2. In addition, vessels were intensely positive for EM-1 and EM-2. It appears that particularly smooth muscle cell stained positive for the 2 endomorphins. This demonstrates that these 2 endogenous opioids are expressed mainly in highly inflamed regions of synovial tissue. Staining in patients with OA appropriately matched the results of immunohistochemistry observed in patients with RA (data not shown).

The density of EM-1–positive cells tended to be increased in patients with OA compared with patients with RA (Figure 1C). However, the density of EM-2–positive cells differed significantly between the 2 patient groups, with the density being lower in patients with RA than in those with OA (Figure 1D). Considering the fact that the cellularity of synovial tissue is generally higher in patients with RA than in those with OA, the lower numbers of EM-2–positive cells in patients with RA indicate a relative loss of these endogenous opioids compared with that in patients with OA.

Double immunohistochemistry revealed that macrophages (CD163), T cells (CD3), and fibroblasts (prolyl 4-hydroxylase) stained positive for EM-1 and EM-2 (Figure 2). Using these immunohistochemistry techniques, double staining was not positive for B cells and neutrophils (data not shown).

Production of EM-1 and EM-2 in various tissues from rats with adjuvant-induced polyarthritis. The results presented above indicated that the levels of EM-1 and EM-2 are increased in inflamed tissue such as human synovium. In order to test whether this is also true in rats with adjuvant-induced polyarthritis, different organs were investigated for their content of EM-1 and EM-2. Animals with adjuvant-induced polyarthritis demonstrated a significantly higher content of EM-1 in the spleen and synovial tissue compared with control rats (Figures 3A and B). Similarly, the level of EM-1 tended to be elevated in the thymus (Figure 3A). Levels of EM-2 in the thymus and the spleen were not significantly different in control rats and rats with adjuvant-induced polyarthritis (Figure 3C). Similarly, there was only a trend toward an increased level of EM-2 in synovial tissue in rats with adjuvant-induced polyarthritis compared with control rats (Figure 3D).

Influence of EM-1 and EM-2 on synovial secretion of IL-6 and IL-8 in RA and OA. In order to identify the influence of EM-1 and EM-2 on secretion of proinflammatory cytokines, superfusion of synovial tissue samples was performed. In patients with OA, EM-1 at

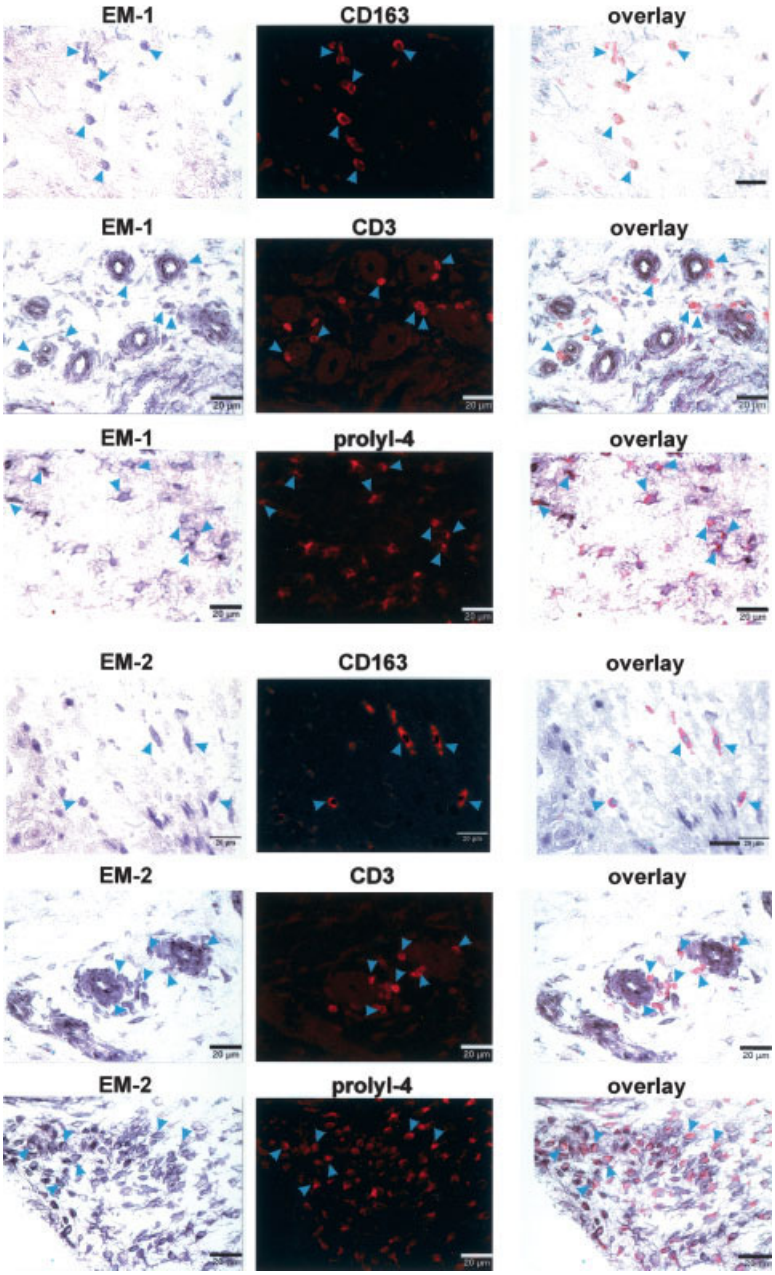

Figure 2. Immunohistochemical double staining of endomorphin 1 (EM-1) or EM-2 and macrophages (CD163), T cells (CD3), and fibroblasts (prolyl 4-hydroxylase). Results are representative of a patient with rheumatoid arthritis. Left lane, Staining of EM-1 and EM-2; middle lane, identification of the respective cell types with immunofluorescence; right lane, overlay of the 2 other panels. Positive results of double immunohistochemistry are shown as red staining in the overlay (**arrowheads**). Bar = 20 μM.

concentrations of $10^{-10}M$ and $10^{-8}M$ significantly reduced IL-8 secretion (Figure 4C). In patients with RA, EM-1 at concentrations of $10^{-9}M$ and $10^{-8}M$ decreased the level of IL-6 and reduced secretion of IL-8 at a concentration of $10^{-10}M$ (Figures 4A and C). Importantly, secretion of IL-8 was more strongly inhibited in

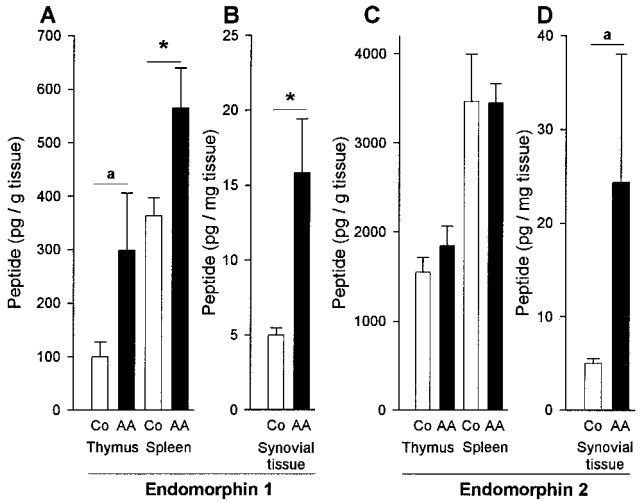

Figure 3. Role of endomorphin 1 (EM-1) and EM-2 in adjuvant-induced polyarthritis (AA). Tissue levels of EM-1 (**A** and **B**) and EM-2 (**C** and **D**) in thymus, spleen, and synovium in control (Co) rats and rats with AA are shown. At least 6 animals were used for each condition. Values are the mean and SEM. $* = P < 0.05$; a = $P < 0.10$, versus the respective control.

OA compared with RA when using $10^{-10}M$ of EM-1 (Figure 4C).

Superfusion with EM-2 did not lead to similarly strong effects on secretion of IL-6 and IL-8 (Figures 4B and D). In patients with RA, EM-2 at a concentration of $10^{-10}M$ decreased secretion of IL-8, but this decrease was not significant in patients with OA (Figure 4D). EM-2 had no effect on the secretion of synovial IL-6 (Figure 4B).

Beneficial effects of EM-1 in adjuvant-induced polyarthritis. On the basis of the data presented in Figure 3A showing that tissue contents of EM-1 are generally more modifiable than EM-2 to inflammation in rats with adjuvant-induced polyarthritis, we investigated the effects of EM-1 in adjuvant-induced polyarthritis in vivo. Intraperitoneal injection of 2 separate concentrations of EM-1 on days 9, 10, 11, 12, and 13 after injection of adjuvant attenuated the inflammatory response on day 14 (Figure 5). This indicates that EM-1 similar as in the human situation exerts beneficial effects on parameters relevant in inflammation.

DISCUSSION

As early as 1986, endogenous opioids have been described in the synovial fluid of patients with RA (26). Similarly, nerve fibers containing endogenous opioids have been observed in the synovial tissue of patients with RA and patients with OA (27). Detection of endogenous opioids in the local inflammatory process has been confirmed in arthritic animals and in human arthritic tissue (28–30). In parallel, these endogenous opioids

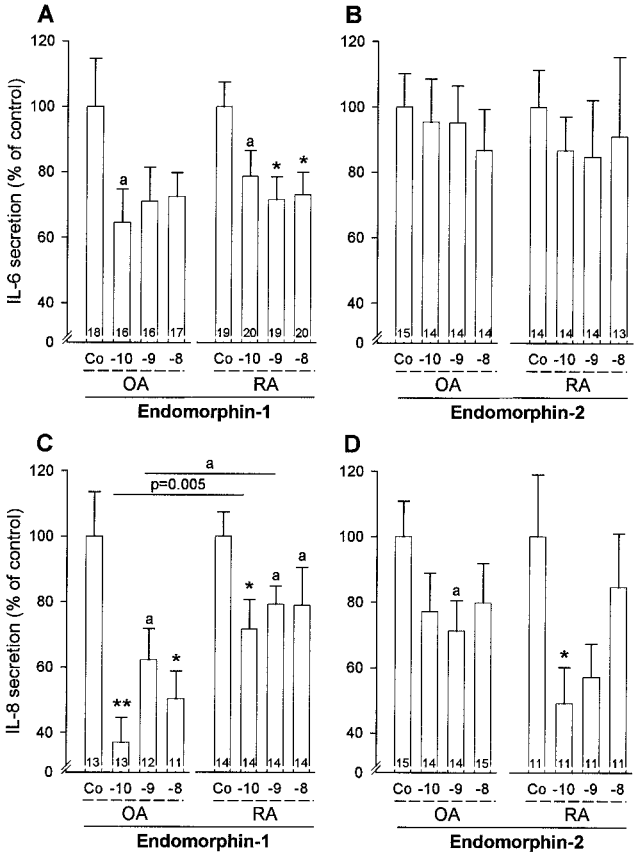

Figure 4. Influence of endomorphin 1 (EM-1) and EM-2 on spontaneous secretion of interleukin-6 (IL-6) and IL-8 from synovium of patients with osteoarthritis (OA) and patients with rheumatoid arthritis (RA). Synovial tissue slices were transferred to microsuperfusion chambers and superfused at 37°C and 66 μl/minute for 330 minutes. After superfusion for 120 minutes, a first 15-minute fraction was collected to determine the cytokine superfusate concentration. Between 120 and 310 minutes of superfusion, EM-1 or EM-2 was applied at the indicated concentrations, shown as log(M) values. At 310 minutes, a second 15-minute fraction was collected, and superfusate concentrations of cytokines were determined. Results are presented as percentages of control (Co) values (set at 100%). In **A,** the average Ψ value of the OA control (pg/ml) was $100 \times (992/259)$ and that of the RA control was $100 \times (403/147)$. In **B,** the average Ψ value of the OA control was $100 \times (1{,}214/645)$ and that of the RA control was $100 \times (511/103)$. In **C,** the average Ψ value of the OA control was $100 \times (283/21)$ and that of the RA control was $100 \times (335/134)$. In **D,** the average Ψ value of the OA control was $100 \times (1{,}625/233)$ and that of the RA control was $100 \times (335/74)$. Values are the mean and SEM. Numbers in the bars are the numbers of slices for each condition. For each panel, synovial tissue from at least 5 patients with OA and 5 patients with RA was included. a = $P < 0.10$; $* = P < 0.05$; $** = P < 0.01$, versus control.

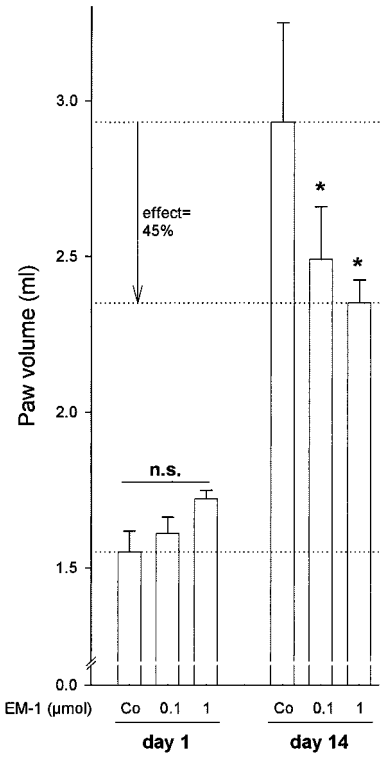

Figure 5. Beneficial effects of 2 intraperitoneal doses of endomorphin 1 (EM-1) in the rat model of adjuvant-induced polyarthritis. Values are the mean and SEM. NS = not significant. $* = P < 0.05$ versus control (Co).

have been described in the spinal cord of arthritic animals, and this was linked to chronic pain (31–38). Along with recognition of κ- and δ-opioid receptors (39), the μ-opioid receptor has been detected in the inflamed tissue of patients with RA (40,41). In articular cells, the μ-opioid receptor is coupled to an inhibitory G protein, leading to decreased phosphorylation of the transcription factor CREB (41,42). Although some beneficial effects of β-endorphin, a partial endogenous ligand at the μ-opioid receptor, have been described in experimental arthritis (43), the proinflammatory or antiinflammatory role of μ-opioid receptor ligation has long been obscured, because no specific endogenous agonists were known. With the discovery of EM-1 and EM-2 (14), endogenous ligands that were highly μ-opioid receptor–specific were available to further investigate the role of endogenous μ-specific opioids. Because μ-opioid receptors were thought to play an important antiinflammatory role (11), these particular receptors were thought to be important in arthritis.

The present study is the first to demonstrate the expression of EMs in inflamed tissue from patients with RA and patients with OA. This confirms studies in the knee joints of rats with adjuvant-induced monarthritis, in which the authors demonstrated a 9-fold increase in the expression of EM-1, which notably demonstrated the up-regulation of this peptide (17). Expression is particularly evident in highly inflamed areas such as the lining layer but also in vessel walls (smooth muscle cells) and sublining zones. In RA and OA, important cells involved in the proinflammatory process such as macrophages, T cells, and fibroblasts abundantly express EM-1 and EM-2. This generally demonstrates the ability of immune cells to express these opioids, most probably upon activation (5).

In our study, it is noteworthy that general cellularity was higher in patients with RA than in patients with OA (Table 1), but EM-positive cells were more abundant in OA than in RA. This is an important differential aspect in these 2 diseases, which might contribute to increased pain processing in RA as compared with OA. The reason for this differential effect is currently not known, but the underlying mechanism might be a target for future therapeutic approaches. The data strongly suggest that μ-opioidergic EMs play a critical role in inflamed tissue of patients with RA and those with OA. However, the function of EMs in cells or tissue from patients with RA and patients with OA has never been studied.

In a functional analysis, we demonstrated that particularly EM-1 is able to inhibit secretion of IL-6 and IL-8 in the superfusion model of inflamed tissue ex vivo. Importantly, secretion of IL-8 was inhibited by >50% in patients with OA and by 30% in patients with RA. Because IL-6 and IL-8 are proinflammatory in nature, locally expressed EMs might play an important antiinflammatory role in human joint inflammation. We did not observe clear dose-response effects, and it seems that lower doses of the peptides exerted stronger antiinflammatory effects. The lack of a dose response in the given range can be attributable to the fact that we did not reach the optimum concentration range. In addition, because we treated whole tissue samples with many different cell types, quite opposite effects can appear due to the stimulating and inhibiting activities on different cell types. In our earlier superfusion experiments with spleen slices, we always observed optimum effects in a U-shaped or bell-shaped manner (25,44). Thus, the inhibiting effects of EMs on cytokine secretion might be present at somewhat lower doses. Furthermore, the well-recognized biphasic effects of another endogenous opioid, β-endorphin (45–47), may also lead to empirical

confusion because, depending on the concentration, β-endorphin may inhibit, stimulate, or leave unaffected cell proliferation and activity. The pharmacology of action of peptides in immune tissues may be quite different from their actions in the central nervous system.

However, the question arises why inhibition of IL-8 was weaker in patients with RA than in patients with OA. This open question cannot be answered in detail, but we suggest that opioidergic pathways are down-regulated due to receptor desensitization or cross-desensitization by proinflammatory cytokines or chemokines (48). Such a desensitization has recently been described in functional experiments for the μ-opioid receptor and EMs in inflammation (16,17). These findings point to an important aspect of inflammation in patients with RA and patients with OA, because local production of normally antiinflammatory factors such as endogenous opioids, catecholamines (49,50), somatostatin (51), or vasoactive intestinal peptide (52) may not exert the expected full antiinflammatory activities due to receptor desensitization. This is an important aspect that has not been studied in the inflamed tissue of patients with RA or OA. Nevertheless, we know that endogenous opioids such as EMs have beneficial effects in models of inflammation (20,53).

The present study further demonstrated that rats with polyarthritis also had up-regulated expression of EM-1, and to a lesser extent of EM-2, in primary and secondary lymphoid organs as well as in inflamed synovial tissue. Thus, the results obtained in the human tissue were corroborated by findings in the rat. In addition, EM-1 exerted a beneficial effect in polyarthritic rats. It appeared that administration of additional EMs, at least during the very early phase of the disease, ameliorated signs of inflammation during the symptomatic phase of the disease. Whether or not EMs have a similarly favorable effect in already symptomatic animals needs to be investigated. It is possible that EMs administered during the more chronic symptomatic phase exert less beneficial effects due to ligand-dependent down-regulation of signaling pathways. Thus, EM administration can be favorable, particularly, during flares of the disease, with a strong contribution of the neurogenic part of inflammation.

Another important finding in the present study is the smaller effect of EM-2 compared with EM-1 on synovial cytokine secretion. EM-1 inhibited secretion of IL-6 and IL-8, and this effect was not similarly observed with EM-2. Both EMs increase GTPγS signaling via μ-opioid receptor binding (54), but it appears that EM-1 and EM-2 exert their effects via different subtypes of μ-opioid receptors (55,56). It is possible that these different μ-opioid receptor subtypes are differentially coupled to IL-6 and IL-8 secretion. In addition, up-regulation of different μ-opioid receptors might change the outcome with respect to cytokine secretion in the 2 patient groups.

In conclusion, EMs are abundant in the tissue of patients with RA and patients with OA, and EMs exert antiinflammatory effects in human tissue and in the rat model of adjuvant-induced polyarthritis. The present study, together with some other important investigations (53,57), emphasizes a probably beneficial role of EMs in arthritis. On the basis of the already known therapeutic effects of morphine in human arthritis (13), these investigations clearly point toward a possible role of EMs as therapeutic agents in RA and OA, especially during acute flares of arthritis due to the large contribution of the nervous system (the neurogenic component of inflammation). The fact that these endogenous opioids have a very high affinity for the μ-opioid receptor most probably suggests application of EMs at very low levels in order to avoid systemic adverse effect.

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AUTHOR CONTRIBUTIONS

Drs. Straub and Jessop had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Straub, Jessop.

Acquisition of data. Straub, Wolff, Fassold, Hofbauer, Chover-Gonzalez, Richards, Jessop.

Analysis and interpretation of data. Straub, Jessop.

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Statistical analysis. Straub, Jessop.

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