Mod Rheumatol (2003) 13:326–332
DOI 10.1007/s10165-003-0249-2

ORIGINAL ARTICLE

Hidekazu Tanaka · Goro Asano · Shinichi Yoshino

The expression and localization of β-endorphin and μ,δ-opioid receptors in synovial tissues in patient with rheumatoid arthritis and osteoarthritis

Received: October 18, 2002 / Accepted: February 20, 2003

Abstract An examination of the localization and distribution of β-endorphin and opioid receptors in patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Immunohistochemical staining, reverse transcription polymerase chain reaction (RT–PCR) analysis, and in situ hybridization were performed using synovial tissues obtained from RA and OA patients. Immunohistochemical staining showed that β-endorphin was strongly expressed in synovial lining cells and in a few lymphocytes and macrophages surrounding the vessels, whereas μ- and δ-opioid receptors were expressed in lymphocytes and macrophages. However, we detected the weak expression of these opioid peptides in synovial tissues of OA patients. RT–PCR analysis showed that preproopiomelanocortin (POMC) mRNA, a precursor of β-endorphin, was strongly expressed in synovial tissues of RA patients, but these PCR products of synovial tissues obtained from OA patients were weakly detected compared with those from RA patients. POMC mRNA was also expressed in synovial tissues in RA patients. In in situ hybridization, the expression of POMC mRNA was detected in macrophages, synovial lining cells, and fibroblasts in synovial tissues of RA patients as well as β-endorphin. In RA patients, β-endorphin and μ- and δ-opioid receptors are synthesized and located in synovial lining cells, lymphocytes, and macrophages surrounding the vessels in synovial tissues, and may play a role in the regulation and modulation of inflammation.

Key words β-endorphin · δ-opioid receptor · μ-opioid receptor · Rheumatoid arthritis (RA) · Synovial tissues

H. Tanaka · S. Yoshino (✉)
Department of Joint Disease and Rheumatism, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan
Tel. +81-3-3822-2131; Fax +81-3-3822-2170
e-mail: hide-ra@kanagawa.hosp.go.jp

G. Asano
Department of Pathology, Nippon Medical School, Tokyo, Japan

Introduction

Recent studies have shown that the neuroimmune system is connected with the pathogenesis of rheumatoid arthritis (RA).[1] When RA patients are under some mental or social stress, their immune system may become highly active.[2–4] Since the mechanism by which stress affects the action of the immune system includes T cells, B cells, or monocytes, it is believed that neuropeptides are secreted from a nerve ending through the hypothalamus, i.e., a peripheral nervous system,[5,6] or that the immune cells responsible are influenced by the hypothalamus, i.e., corticotropin releasing factor (CRF), pituitaries, adrenocorticotropic hormone (ACTH), adrenal cortex, or the cortisol system.[7]

Endogenous opioid peptides as well as neuropeptides are also morphine-like in that they combine with morphine receptors.[8] These peptides, β-endorphin, enkephalin, and dynorphin, have been identified in the adrenal medulla, interstitial mucosa, and sympathetic nerves, as well as in brain structures.[9] Endogenous opioid peptides and their receptors have been reported to be produced in brain structures as well as several other organs. Three major classes, μ, δ, and κ, of opioid receptors have been defined according to their ligand-binding affinities: μ- and δ-opioid receptors are specific to β-endorphin.[10,11] Several investigators have reported that opioid peptides, as studied using immunohistochemistry, were localized and secreted from synovial tissues of RA patients.[12,13] We studied the expression and distribution pattern of β-endorphin and its receptors, μ- and δ-receptors, in synovial tissue of RA and osteoarthritis (OA) patients using immunohistochemistry, reverse transcription polymerase chain reaction (RT–PCR) analysis, and in situ hybridization.

Material and methods

Specimens were obtained from 12 patients diagnosed as having RA (stages III and IV) according to the criteria of

the American College of Rheumatology, and with a disease duration of 10–20 years, and also from patients with OA, after obtaining their informed consent. Synovial tissues were obtained by synovectomy (knee-joint) or total knee joint arthroplasty using the method prescribed by the Ethical Committee of Nippon Medical School. These samples were used for immunohistochemical observation, RT–PCR analysis, and in situ hybridization.

Immunohistochemical observation

The tissues were fixed in 4% paraformaldehyde in phosphate-buffered solution (PBS) for 16–18 h at 4°C, and then immersed in 30% (w/v) sucrose in PBS containing 0.02% (v/v) diethylpyrocarbonate (Sigma, St. Louis, MO, USA) at 4°C for 16–18 h and frozen at −80°C until use. Frozen sections were then cut using a cryostat microtome at a thickness of 6 μm and mounted onto 3-aminopropylmethoxysilane-coated slides. An anti-β-endorphin polyclonal antibody (Organon Teknika NV, USA) was used at a 1:100 dilution in 0.1% BSA. An anti-μ-opioid receptor polyclonal antibody and anti-δ-opioid receptor polyclonal antibody (Santa Cruz Biochemicals, Santa Cruz, USA) were used at a 1:2000 dilution in 0.1% bovine serum albumin (BSA). The streptavidin–biotin–peroxidase complex method was carried out using an ABC kit (Nichirei, Tokyo, Japan). Sections without primary antibodies were prepared as negative controls.

RT–PCR analysis

Total RNA was extracted from the tissues, and cultured synoviocytes using Isogen (Nippon Gene, Tokyo, Japan), and poly (A) mRNA was isolated using a poly AT tract mRNA isolation kit (Promega, Madison, WI, USA). The first strands of cDNA were synthesized from 100 ng polyadenylated RNA with a 1st Strand cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). The cDNA sequence of β-endorphin is so short that β-endorphin mRNA expression cannot be detected precisely. We therefore used preproopiomelanocortin (POMC) β-endorphin precursor gene in the RT–PCR analysis. Oligonucleotide primers were produced using a DNA synthesizer according to the human POMC cDNA sequence.[14,15] The sequences of the primers were 5′-CCTCAGCCTGCCTGGAAGA TGC-3′ and 5′-CGAGGATGAGGTACCTCGAGAA

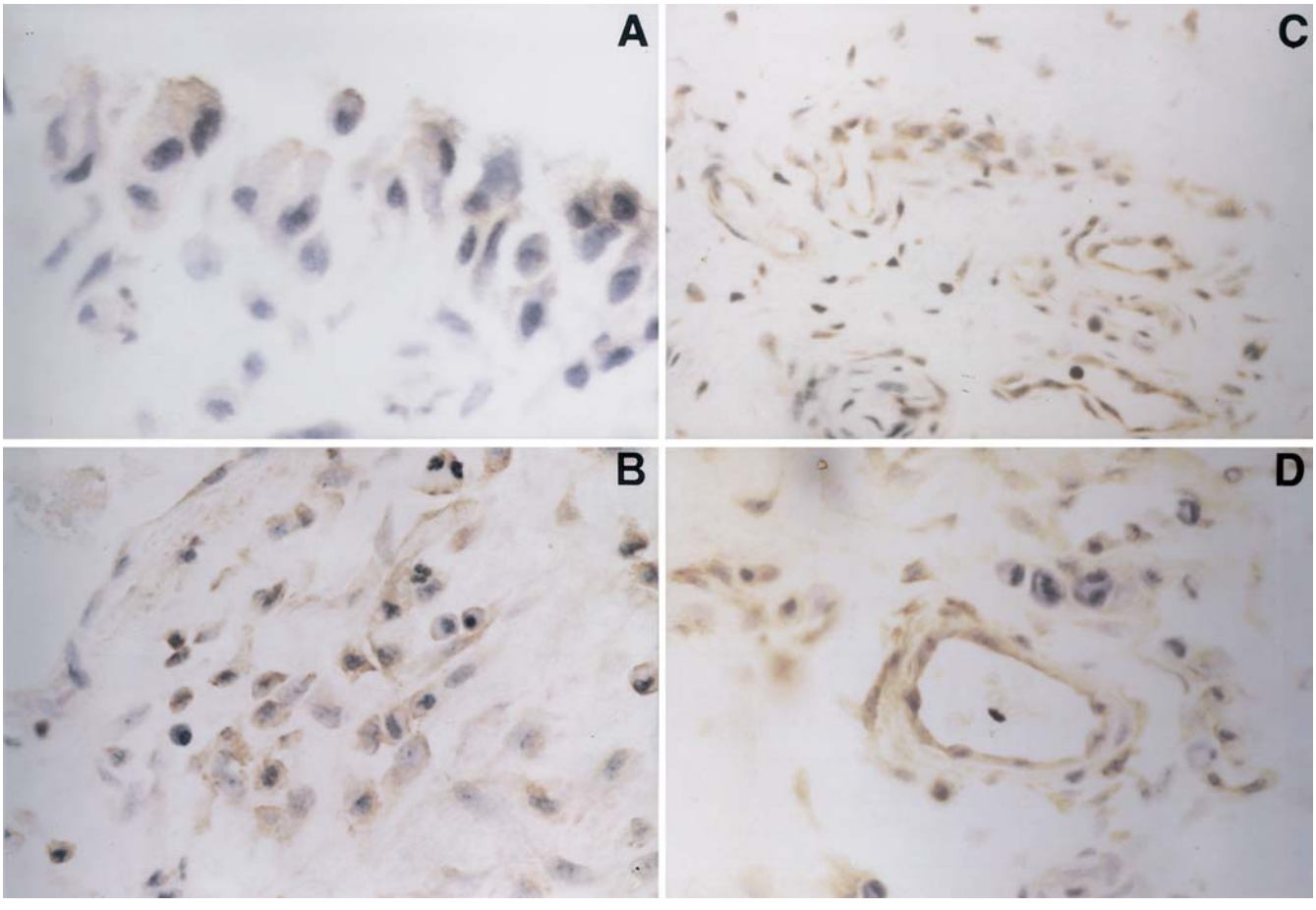

Fig. 1. Immunohistochemical staining for β-endorphin in synovial tissues in rheumatoid arthritis (RA). Synovial lining cells are proliferating, and β-endorphin are strongly detected mainly in synovial lining cells (**A**, ×200), a few lymphocytes and macrophages (**B**, ×200), and vascular endothelial cells (**C**, ×100; **D**, ×200)

GG-3′ for POMC. A 50-μl aliquot of the reaction mixture contained 5 μl 10 × PCR buffer (Takara, Tokyo, Japan), 1 μl 200 μM deoxynucleoside triphosphate (dNTP), 2.5 units of Ampli Taq DNA polymerase (Takara), 1 μg template cDNA, and 100 pmol each of 5′ and 3′ primers. PCR was carried out in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 30 cycles, each cycle consisting of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, and the PCR products were analyzed on a 2% agarose gel.

In situ hybridization

Probe preparation

According to human POMC cDNA, a 48-mer oligonucleotide encoding position 7106–7153 was synthesized for use as an antisense probe,[14,16,17] and the sequence of the oligonucleotide was 5′-GCCCACCGGCTTGCCCCAGCGGA AGTGCTCCATG GAGTAGGAGC GCTT-3′. In addition, the oligonucleotide with the reverse sequence was synthesized for use as a sense probe. Then the oligonucleotides were labeled with digoxigenin (DIG)-deoxyuridine triphosphate (dUTP) by the 3′-tailing method using a DIG oligonucleotide tailing kit (Boehringer Mannheim). The labeling reaction mixture consisted of the following components: 100 pmol oligonucleotide, 4 μl tailing buffer, 4 μl 25 mM $CoCl_2$, 1 μl digoxigenin-dUTP (1 mM stock solution, Boehringer Mannheim), 1 μl 10 nM d-adenosine triphosphate and 50 units of terminal transferase. Water was added to a final volume of 20 μl. The mixture was incubated at 37°C for 15 min and then placed on ice. The reaction was stopped by adding 2 μl of the reaction mixture (1 μl glycogen solution with 200 μl 200 mM ethylene diaminetetraacetate (EDTA) solution, pH 8). Ethanol precipitation was then performed.

Hybridization and detection

The tissue sections were rehydrated with PBS, and incubated in PBS with 0.2 N HCl for 20 min and with proteinase K (5 μg/ml, Sigma) for 30 min at 37°C. The sections were then postfixed in 4% paraformaldehyde/PBS for 5 min, and quenched twice in PBS with glycine (2 mg/ml), before storage in 50% (v/v) deionized formamide/2 × SSC. The sections were incubated for 16 h at 37°C in a buffer consisting of 600 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 120 μg/ml herring sperm DNA (Boehringer Mannheim), 200 μg/ml yeast RNA (Boehringer Mannheim), 1 ×

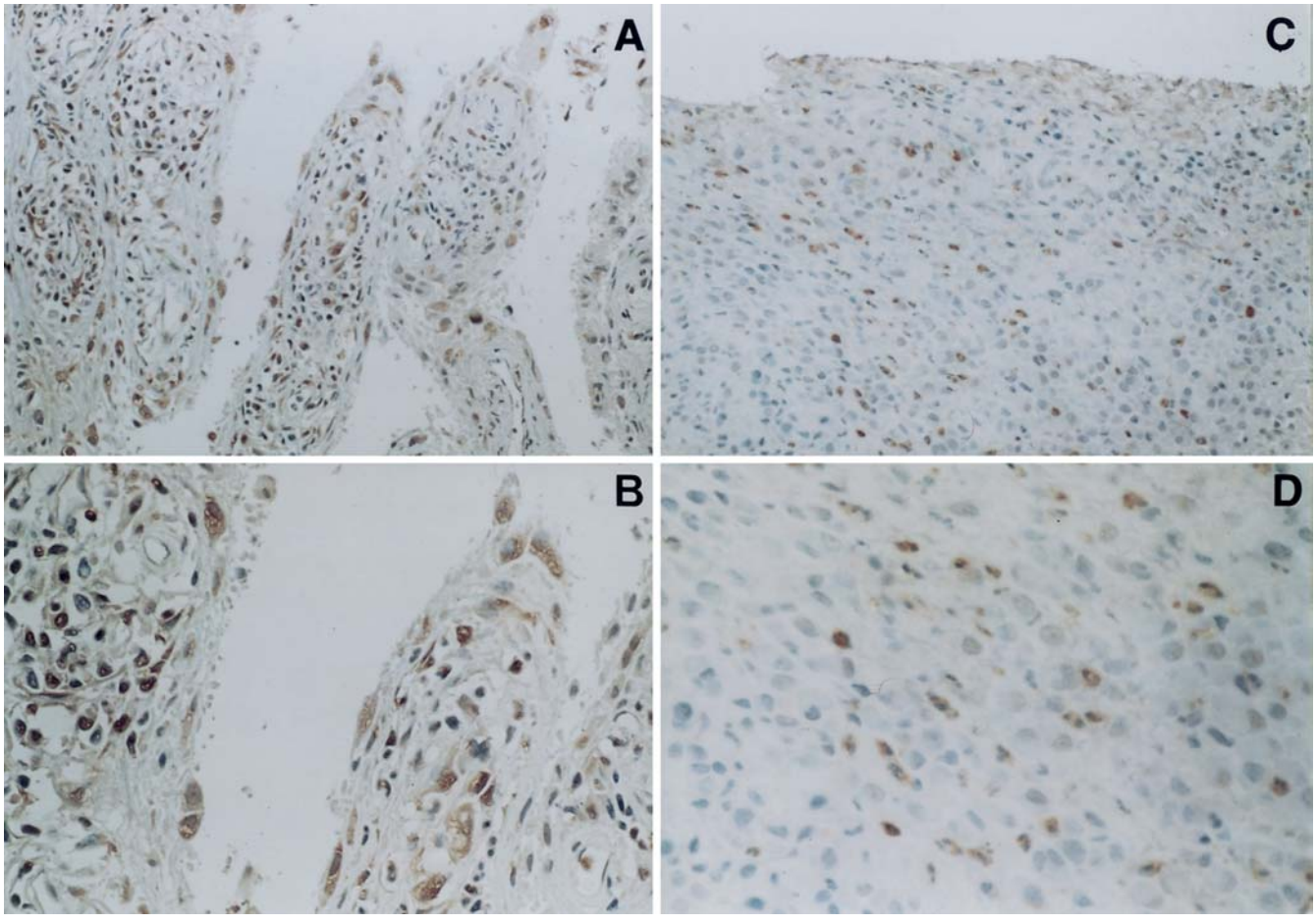

Fig. 2. Immunohistochemical staining for μ,δ-receptor in synovial tissues in RA. μ- and δ-receptor are clearly detected in lymphocytes and macrophages (μ-receptor: **A**, ×80; **B**, ×150. δ-receptor: **C**, ×80; **D**, ×150)

Denhardt's solution, 10% (w/v) dextran sulfate (Sigma Chemical), and 100–300 ng/ml labeled oligonucleotide probe. The sections were then washed twice with 50% formamide/2 × SSC for 30 min at room temperature (RT), and once with 2 × SSC for 30 min at RT. For immunological detection, a nucleic acid detection kit (Boehringer Mannheim) was used. The sections were washed briefly with buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.6) and incubated with 1% (w/v) blocking reagent in buffer 1 solution for 30 min at RT. After washing again briefly with buffer 1 solution, the sections were incubated with a 1/4000 dilution of a polyclonal sheep antidigoxigenin Fab fragment conjugated to alkaline phosphatase in buffer 1 solution for 30 min at RT. The sections were washed three times with buffer 1 solution for 15 min at RT, and equilibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. The sections were then incubated in a staining solution containing nitroblue tetrazolium and BCIP in a dark box for 5–6 h. The reaction was stopped by adding Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and the sections were mounted onto an aqueous mounting medium (Mutoh Chemical, Tokyo, Japan).

Results

Immunohistochemical observation

Immunohistochemically, β-endorphin was mainly localized and strongly expressed in synovial lining cells, and also in a few lymphocytes and macrophages surrounding the blood vessels (Fig. 1), while μ- and δ-receptors were located in lymphocytes and macrophages in synovial tissues of the RA patients (Fig. 2). However, we only detected slight traces of β-endorphin in the synovial tissues of OA patients (Fig. 3).

RT–PCR analysis

The products of PCR amplification for POMC mRNA were strongly expressed in synovial tissues of RA patients at 284 bp. In these tissues of the OA patients, all the POMC mRNA were weakly expressed compared with those of the RA patients. POMC mRNA in the human thalamus and pituitary (positive control) were also detected in RA patients (Fig. 4). We thus confirmed that β-endorphin is produced in synovial cells.

In situ hybridization

In situ hybridization for POMC mRNA was performed under stringent conditions using frozen synovial sections from RA and OA patients. In the RA patients, the expression of POMC mRNA with an antisense probe appeared in macrophages and synovial lining cells as a homogeneous staining in the cytoplasm (Fig. 5).

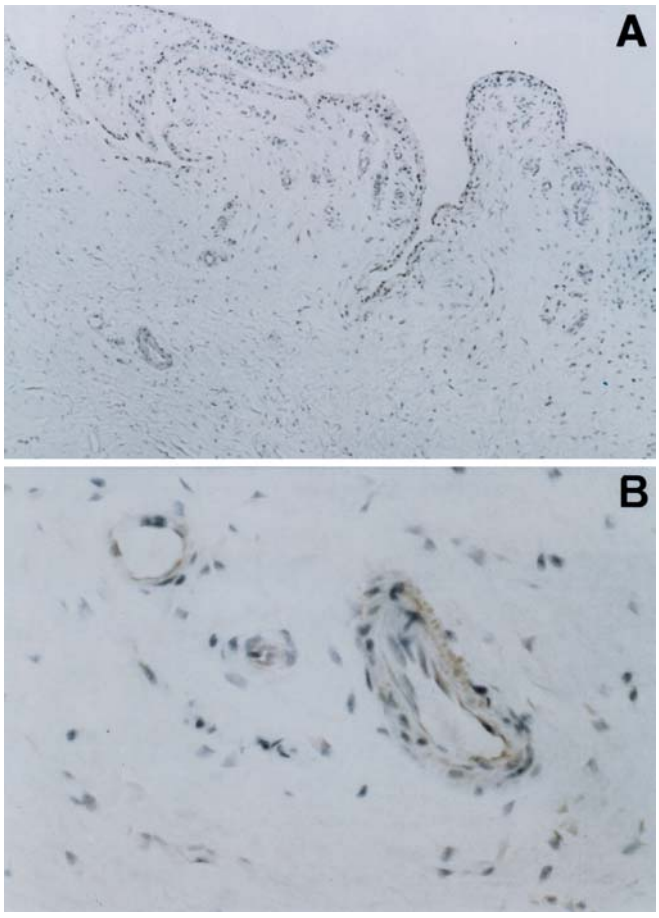

Fig. 3. Immunohistochemical staining for β-endorphin in synovial tissues in osteoarthritis (OA). β-endorphins are slightly detected mainly in synovial lining cells (**A**, ×40), a few lymphocytes and macrophages and vascular endothelial cells (**B**, ×100)

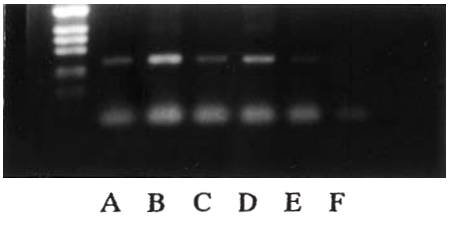

Fig. 4. Reverse transcription – polymerase chain reaction (RT–PCR) for preproopiomelanocortin (POMC) mRNA in synovial tissues in RA. A band of each lane near 284 bp shows that POMC mRNA exists in synovial tissues of RA, OA, and human thalamus and pituitary (positive control). The first lanes each show a molecular weight marker. Lane A shows human thalamus, B shows human pituitary, C and D show RA, and E and F show OA. First lane, 1 kb DNA ladder (Gibco BRL, USA)

Discussion

The presence of cytokines such as IL-1-β, IL-6, and angiogenic factors such as b-FGF and VEGF has been shown in RA synovial tissues.[18] These cytokines are thought to play an important role in the infiltration of immune cells into

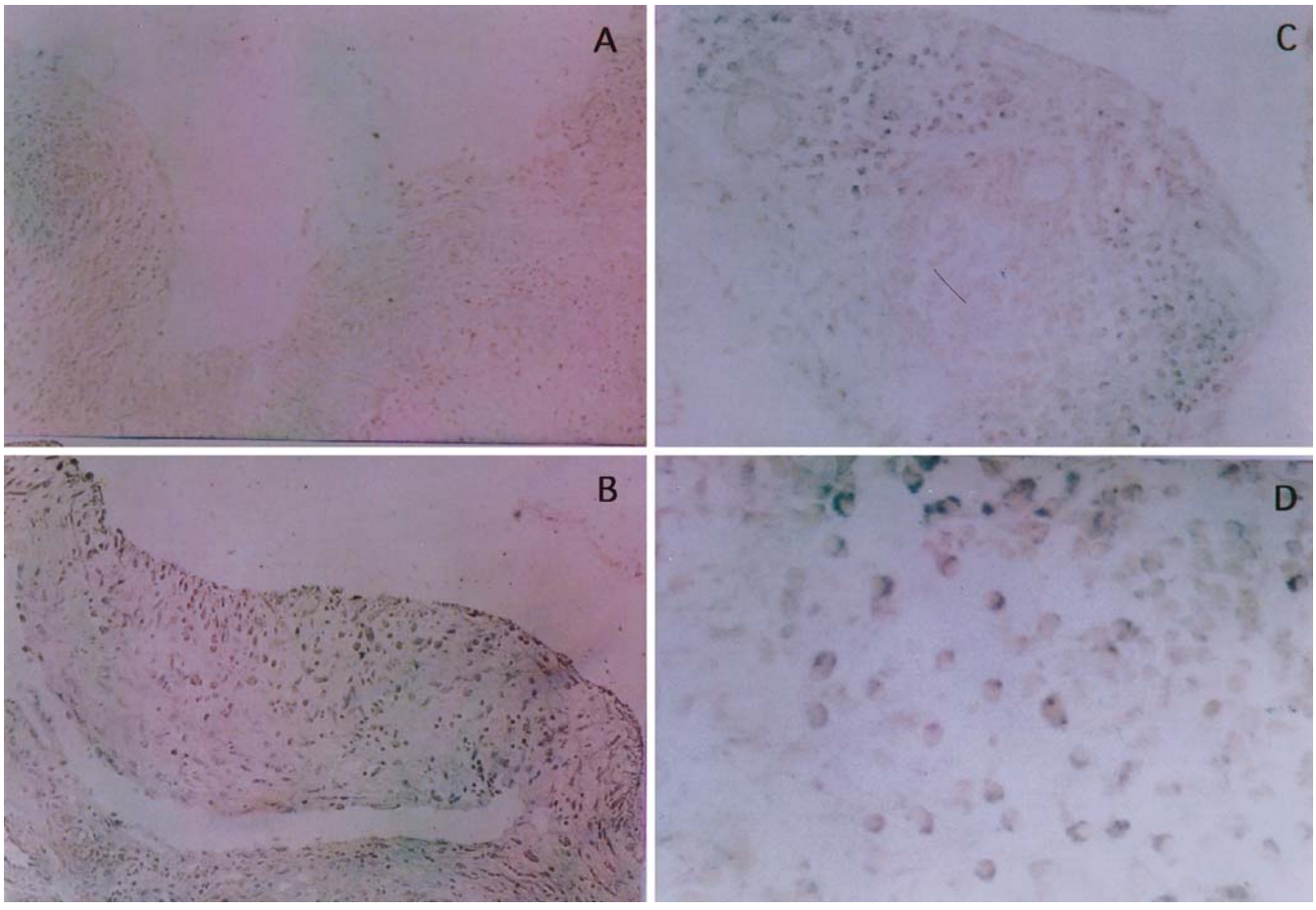

Fig. 5. In situ hybridization for POMC mRNA in synovial tissues in RA. Positive signals are shown in macrophage, fibroblasts, vascular smooth muscle cells, and synovial lining cells. **A**, sense; **B**, **C**, **D** antisense. **A**, ×100; **B**, ×100; **C**, ×100; **D**, ×200

synovial tissues, and the destruction of bones and cartilage in RA patients.

Opioid peptides have been shown to exert various physiological effects such as analgesic effects on mental function, endocrine effects, and effects on lymphocytes, but much remains unknown. Sharp[19] reported that T cells produce opioid immunopeptides that control pain at sites of inflammation, and Jones et al.[20] reported that in active RA there were substantial changes in opioid receptor binding in the brain. Using the immunohistochemistry of synovial tissues from the RA patients, Koiwa et al.[12] showed the localization of β-endorphin mainly in synovial lining cells and also in a few synovial interstitial cells, and detected it at high concentrations in the supernatant of fluids from cultured synovial tissues from RA patients. Immunohistochemical findings have shown that neurofilaments are also present around the blood vessels in synovial tissues of RA and OA patients,[5] whereas neurofilaments have previously been found only in interstitial cells in synovial tissues of RA and OA patients. Gronblad et al.[21] speculated that RA patients show a marked release of neuropeptides (methionine–enkephalin and leucine–enkephalin) from neuronal terminals into synovial cells.

In this study, we showed the localization and production of β-endorphin, μ- and δ-opioid receptors in the synovial tissues of RA and OA patients using immunohistochemical staining, RT–PCR analysis, and in situ hybridization. In RA patients, immunohistochemical observations showed the localization of β-endorphin mRNA expression in synovial lining cells, perivascular lymphocytes, and macrophages of synovial tissues. Using in situ hybridization, POMC mRNA was expressed in synovial lining cells and perivascular macrophages, fibroblasts, and lymphocytes, as well as the sites shown by immunohistochemistry. μ- and δ-opioid receptor mRNA were detected in lymphocytes and macrophages in synovial tissues. On the other hand, in OA patients, the localization of β-endorphin was detected slightly by immunohistochemistry, and POMC mRNA was also detected slightly by RT–PCR and in situ hybridization.

The amounts of opioid peptides produced by macrophages and lymphocytes are reported to increase in inflammatory tissues, suggesting that they are produced by inflammatory cells.[7,19,22] Opioid peptides thus produced are believed to relieve inflammation and inhibit the production and release of bradykinin, a mediator of inflammation and pain, by reducing the amount of released substance P, a pain-producing substance.[23]

μ- and δ-receptors are mainly present in lymphocytes, macrophages, and polymorphonuclear leukocytes of immune or inflammatory cells. β-endorphin, Met-enk, and

Leu-enk exert various effects by binding to the μ- or δ-opioid receptor, such as the promotion of monocyte chemotaxis, the stimulation of the natural killer activity, and an increase in antibody production by lymphocytes. Stein and co-workers[24–27] have reported that lymphocytes, T and B cells, and macrophages found in inflammatory infiltrates of the rat hindpaw injected with Freund's complete adjuvant are immunoreactive for β-endorphin, enkephalin, and dynorphin.

Gunji et al.[28] reported that κ-opioid receptor mRNA in the peripheral blood are mainly expressed in lymphocytes, monocytes, and natural killer cells in patients with RA. Moreover the expression level of κ-opioid receptor mRNA is lower in RA patients than in healthy volunteers. The expression of κ-opioid receptor mRNA is correlated with NK cell activity in RA patients, and the expression of κ-opioid receptor mRNA is related to anti-inflammatory effects in RA patients. IL-1 stimulates the hypothalamus and induces secretion of CRF, which affects the pituitary glands, which not only produce and secrete ACTH, but also release opioid peptides, and are closely related to the endocrine system.[29–31] In this study, synovial tissues were obtained from RA patients treated with steroids. This may have served as a negative feedback, resulting in the inhibition of CRF and opioid peptide production. Mediators of inflammation, such as corticotropin-releasing hormone and IL-1, stimulate the release of β-endorphin from immune cells in inflamed local lymph nodes.[32,33] These findings suggest that β-endorphin, an opioid peptide, is locally produced in synovial lining cells, macrophages, fibroblasts, and lymphocytes in synovial tissues of patients with RA, and exerts its effects by binding to μ- and δ-opioid receptors and other specific receptors present on the surface of lymphocytes and monocytes which have infiltrated into synovial tissues. Thus, β-endorphin may play an important role in the regulation and modulation of inflammation.

References

1. Ansar Ahmed S, Penhale WJ, Talal N. Sex hormones, immune responses, and autoimmune diseases. Am J Pathol 1985;121:531–51.
2. Denko CW, Aponte J, Gabriel P, Petricevic M. Serum β-endorphin in rheumatic disorders. J Rheumatol 1982;9:827–33.
3. Taylor R. Reguation of the immune response. 2nd ed. London: Gower; 1989. p. 10.1–10.10.
4. Nakamura H, Yoshino S. Opioid peptides in RA: modulation of immunologic mechanisums and mental states. Arerugi 1990;39: 110–7.
5. Mapp PI, Walsh DA, Garrett NE, Kidd BL, Cruwys SC, Polak JM, et al. Effect of three animal models of inflammation on nerve fibers in the synovium. Ann Rheum Dis 1994;53:240–6.
6. Stein C, Hassan AH, Przewlocki R, Gramsch C, Peter K, Herz A. Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. Proc Natl Acad Sci USA 1990;87:5935–9.
7. Fujimoto K, Yokoyama M. Interaction between emotive–behavioral science and neuroimmunology. Med Immunol 1989;7:153–60.
8. Stein C, Pfluger M, Yassouridis A, Hoelzl J, Lehrberger K, Welte C, et al. No tolerance to peripheral morphine analgesia in the presence of opioid expression in inflamed synovia. J Clin Invest 1996;98:793–9.
9. Nakai Y. Opioid peptides and its clinical application. Metabolism 1985;22:49–61.
10. Atcheson R, Lambert DG. Update on opioid receptors. Br J Anaesth 1994;73:132–4.
11. Meites J, Bruni JF, Van Vugt DA, Smith AF. Relation of endogenous opioid peptides and morphine to neuroendocrine functions. Life Sci 1979;24:1325–36.
12. Koiwa M, Shiga H, Nakamura H, Yoshino S. Role of opioid peptide in rheumatoid arthritis–detection of beta-endorphin in synovial tissue. Arerugi 1992;41:1423–9.
13. Shiga H, Yoshino S, Nakamura H, Koiwa M. Role of opioid peptide in rheumatoid arthritis–detection of methionine–enkephalin in synovial tissue. Arerugi 1993;42:243–9.
14. Takahashi H, Hakamata Y, Watanabe Y, Kikuno R, Miyata T, Numa S. Complete nucleotide sequence of the human corticotropin–beta-lipotropin precursor gene. Nucleic Acids Res 1983;11:6847–58.
15. Noda M, Teranishi Y, Takahashi H, Toyosato M, Notake M, Nakanishi S, et al. Isolation and structural organization of the human preproenkephalin gene. Nature 1982;297(5865):431–4.
16. Sukhov RR, Walker LC, Rance NE, Price DL, Young WS 3rd. Opioid precursor gene expression in the human hypothalamus. J Comp Neurol 1995;353:604–22.
17. Comb M, Seeburg PH, Adelman J, Eiden L, Herbert E. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. Nature 1982;295(5851):663–6.
18. Nagashima M, Yoshino S, Ishiwata T, Asano G. Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. J Rheumatol 1995;22:1624–30.
19. Sharp B, Yaksh T. Pain killers of the immune system. Nat Med 1997;3:831–2.
20. Jones AK, Liyi Q, Cunningham VV, Ha-kawa S, Fujiwara T, Friston KF, et al. Endogenous opiate response to pain in rheumatoid arthritis and cortical and subcortical response to pain in normal volunteers using positron emission tomography. Int J Clin Pharmacol Res 1991;11:261–6.
21. Gronblad M, Konttinen YT, Korkala O, Liesi P, Hukkanen M, Polak JM. Neuropeptides in synovium of patients with rheumatoid arthritis and osteoarthritis. J Rheumatol 1988;15:1807–10.
22. Wick MJ, Minnerath SR, Roy S, Ramakrishnan S, Loh HH. Differential expression of opioid receptor genes in human lymphoid cell lines and peripheral blood lymphocytes. J Neuroimmunol 1996;64:29–36.
23. Kar S, Rees RG, Quirion R. Altered calcitonin gene-related peptides, substance P and enkephalin immunoreactivities and receptor binding sites in the dorsal spinal cord of the polyarthritic rat. Eur J Neurosci 1994;6:345–54.
24. Przewlocki R, Hassan AH, Lason W, Epplen C, Herz A, Stein C. Gene expression and localization of opioid peptides in immune cells of inflamed tissue: functional role in antinociception. Neuroscience 1992;48:491–500.
25. Schafer M, Carter L, Stein C. Interleukin-1-beta and corticotropin-releasing factor inhibit pain by releasing opioids from immune cells in inflamed tissue. Proc Natl Acad Sci USA 1994;91:4219–23.
26. Faith RE, Liang HJ, Murgo AJ, Plotnikoff NP. Neuroimmunomodulation with enkephalins: enhancement of human natural killer (NK) cell activity in vitro. Clin Immunol Immunopathol 1984;31:412–8.
27. Hsueh CM, Chen SF, Huang HJ, Ghanta VK, Hiramoto RN. Activation of mu-opioid receptors are required for the conditioned enhancement of NK cell activity. Brain Res 1996;737:263–8.
28. Gunji N, Nagashima M, Asano G, Yoshino S. Expression of kappa-opioid receptor mRNA in human peripheral blood lymphocytes and the relationship between its expression and the inflammatory changes in rheumatoid arthritis. Rheumatol Int 2000;19:95–100.
29. Sibinga NE, Goldstein A. Opioid peptides and opioid receptors in cells of the immune system. Ann Rev Immunol 1988;6:219–49.
30. Jones AK, Cunningham VJ, Ha-kawa S, Fujiwara T, Luthra SK, Silva S, et al. Changes in central opioid receptor binding in relation to inflammation and pain in patients with rheumatoid arthritis. Br J Rheumatol 1994;33:909–16.

31. Mascarenhas G, Quirico-Santos T. Inhibitory effect of tumor growth by methionine–enkephalin. Arq Neuropsiquiatr 1992;50: 84–90.
32. Mousa SA, Schafer M, Mitchell WM, Hassen AH, Stein C. Local upregulation of corticotropin-releasing hormone and interleukin-1 receptors in rats with painful hindlimb inflammation. Eur J Pharmacol 1996;311:221–31.
33. Cabot PJ, Carter L, Gaiddon C, Zhang Q, Schafer M, Loeffler JP, et al. Immune cell-derived beta-endorphin. Production, release, and control of inflammatory pain in rats. J Clin Invest 1997; 100:142–8.