EXTENDED REPORT

β -Endorphin, Met-enkephalin and corresponding opioid receptors within synovium of patients with joint trauma, osteoarthritis and rheumatoid arthritis

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Opioids elicit analgesic effects by activating both central and peripheral opioid receptors (ORs).^{1–2} Peripheral ORs have been demonstrated on sensory nerve terminals within subcutaneous tissue in animals.^{3–5} The activation of such peripheral ORs by exogenous and endogenous agonists can produce significant antinociceptive effects.^{5–8} Recently, ORs were identified by immunohistochemical staining and radioligand-binding assay in synovial cells of patients with RA^{9–10} and in equine joints.¹¹ Keates *et al*¹² reported that the density of opioid-binding sites was markedly enhanced in inflamed compared with control canine joint tissue. A growing body of clinical data documents the successful use of small doses of intra-articular (IA) morphine for pain control in patients with osteoarthritis (OA), rheumatoid arthritis (RA) and joint trauma (JT; ie, after arthroscopic knee surgery) without central side effects.^{13–18} Opioid peptides are synthesised and processed within inflammatory cells¹⁹ and, on release, inhibit inflammatory pain by activating peripheral ORs.^{19–22} In humans, β -endorphin (END) and Met-enkephalin (ENK) have been found in synovial tissue.^{14–23–24} Such endogenous opioid peptides contribute to clinical pain inhibition as studies have shown that the IA administration of the opioid receptor antagonist naloxone can exacerbate postoperative pain.²⁵ However, the exact cell types and disease-specific differences in the occurrence of opioid peptides and receptors have not been examined so far. Therefore, we examined in detail the localisation of END and ENK and their corresponding ORs in synovium of patients with JT, OA and RA. We investigated the differences in the number and types of cells expressing END and ENK by use of different markers for macrophages/monocytes (CD68), granulocytes (CD15), T lymphocytes (CD3) and B lymphocytes

Objective: Intra-articularly applied opioid agonists or antagonists modulate pain after knee surgery and in chronic arthritis. Therefore, the expression of β -endorphin (END), Met-enkephalin (ENK), and μ and δ opioid receptors (ORs) within synovium of patients with joint trauma (JT), osteoarthritis (OA) and rheumatoid arthritis (RA) were examined.

Methods: Synovial samples were subjected to double immunohistochemical analysis of opioid peptides with immune cell markers, and of ORs with the neuronal markers calcitonin gene-related peptide (CGRP) and tyrosine hydroxylase (TH).

Results: END and ENK were expressed by macrophage-like (CD68⁺) and fibroblast-like (CD68[−]) cells within synovial lining layers of all disorders. In the sublining layers, END and ENK were mostly expressed by granulocytes in patients with JT, and by macrophages/monocytes, lymphocytes and plasma cells in those with OA and RA. Overall, END- and ENK-immunoreactive (IR) cells were more abundant in patients with RA than in those with OA and JT. ORs were found on nerve fibres and immune cells in all patients. OR-IR nerve fibres were significantly more abundant in patients with RA than in those with OA and JT. μ ORs and δ ORs were coexpressed with CGRP but not with TH.

Conclusions: Parallel to the severity of inflammation, END and ENK in immune cells and their receptors on sensory nerve terminals are more abundant in patients with RA than in those with JT and OA. These findings are consistent with the notion that, with prolonged and enhanced inflammation, the immune and peripheral nervous systems upregulate sensory nerves expressing ORs and their ligands to counterbalance pain and inflammation.

(CD23). We also examined the expression and colocalisation of μ ORs and δ ORs with markers for primary afferent neurones (calcitonin gene-related peptide (CGRP)) and for postganglionic sympathetic neurones (tyrosine hydroxylase (TH)).

MATERIALS AND METHODS

Patients

Synovial tissue was obtained from 28 patients (aged 23–81 years) during routine arthroscopy or open joint surgery for diagnostic and therapeutic procedures in patients with JT, or during elective knee joint replacement in those with OA and RA. Nine patients fulfilled the American College of Rheumatology criteria for RA,²⁶ and nine patients met the criteria for OA.²⁷ Ten patients with JT were operated following a 10–30-day interval after knee injury (four patients with meniscal injury, three with patellar injury and three with cartilage damage). Histologically normal synovium (n = 2) was obtained during diagnostic arthroscopy from patients with minor knee JT. Before surgery, patients were informed about the purpose of the study and gave their written consent. The ethical committees of the Charité—Universitätsmedizin Berlin, Berlin, Germany, and the University Hospital Regensburg, Regensburg, Germany, approved the study. Table 1 gives the clinical and laboratory data of the patients. Parameters such as

Abbreviations: ABC, avidin-biotin-peroxidase complex; ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; DAB, 3',3'-diaminobenzidine tetrahydrochloride; DPX, distrene, tricresyl phosphate and xylene; END, endorphin; ENK, enkephalin; IA, intra-articular; IR, immunoreactive; JT, joint trauma; OA, osteoarthritis; OR, opioid receptor; PBS, phosphate buffered saline; RA, rheumatoid arthritis; TH, tyrosine hydroxylase

Table 1 Clinical, laboratory and histological parameters of patients with joint trauma, osteoarthritis and rheumatoid arthritis

Patients	JT (n = 10)	OA (n = 9)	RA (n = 9)
Age (years)	41 (5)	75.0 (1.7)	78.0 (4.7)
Sex (F/M)	5/5	7/2	5/4
Disease duration	(10–30 days)	(2–10 years)	(2–10 years)
C reactive protein (mg/l)	1.9 (0.3)	3.6 (0.2)*	34 (17)†
Positive for rheumatoid factor O, n (%)	0	5 (55)	
Lining-layer thickness (number of cells)	1.4 (0.1)	3.1 (0.2)*	4.1 (0.2)†
Overall cellularity (cells/mm ²)	476 (102)	1016 (387)	1749 (484)†
CD3 T cell density (cells/mm ²)	10.6 (1.3)	28.9 (17.1)	87 (47)*
CD163 macrophages (cells/mm ²)	14.8 (1.6)	34.7 (14.7)	106 (47)*
Vascularity (vessels/mm)*	11.9 (0.8)	23.6 (5.8)*	27.6 (6.3)*
ESR (mm/first hour)	NR	9.0 (4.5)	21.8 (9.6)†
Drugs, n (%)			
Prednisolone	0	0	5 (55.5)
NSAIDs	4 (40)	3 (33.3)	5 (55.5)
Methotrexate	0		8 (88.8)
Leflunomide	0		2 (22.2)
Ciclosporin A	0	0	2 (22.2)
Opioids	0	1 (11.1)	1 (11.1)

ESR, erythrocyte sedimentation rate; F, female; JT, joint trauma; M, male; NR, not recorded; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; RA, rheumatoid arthritis.

Except where indicated, values are means (SEM).

*Values were significantly higher than that of JT ($p < 0.05$, analysis of variance (ANOVA) on ranks followed by Dunn's test).

†Values were significantly higher than that of JT and OA ($p < 0.05$, ANOVA on ranks followed by Dunn's test).

plasma C-reactive protein and rheumatoid factor were measured by standard techniques, as described previously.²⁸

Reagents

The following antibodies were used: rabbit polyclonal antibodies against human END and ENK (Peninsula Laboratories, Merseyside, UK), μ OR (Drs S Schulz and V Höllt, Magdeburg, Germany) and δ OR (Dr R Elde, Minneapolis, Minnesota, USA); polyclonal guinea pig antibody against human CGRP (Peninsula Laboratories); monoclonal antibodies against human CD3 (clone PC3/188A; marker for T lymphocytes), CD15 (clone C3D-1; granulocytes), CD23 (clone MHM6; B-lymphocytes), CD68 (clone KP1; macrophages), CD163 (clone Ber-MAC3; macrophages), type IV collagen (clone CIV 22, capillary vessels; all from Dako, Hamburg, Germany) and human TH (Incstar, Minneapolis, Minnesota, USA). Further, materials included the VectaStain Elite Kit, Vectashield, Texas red-conjugated goat anti-rabbit and fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibodies (Vector Laboratories, Burlingame, California, USA) and HistoGreen Peroxidase-Substrate Kit (Linaris, Wertheim-Bettingen, Germany).

Tissue preparation and histological evaluation

Immediately after surgery, synovial tissue was taken from all three groups, then fat tissue and tissue with a large number of vessels were removed. To obtain optimal tissue morphology, only intact synovial tissue was fixed in 4% (weight/volume) paraformaldehyde in 0.16 M phosphate buffered saline (PBS; pH 7.4) for 4 h and then cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was then embedded in tissue-Tek compound (OCT, Miles, Elkhart, Indiana, USA) and frozen. These procedures are analogous to our previous studies that included positive controls—for example, in spinal cord²⁹—thus precluding false-negative results. All tissue samples from patients with JT, OA and RA were subjected to the same treatment protocol. For OR detection 40 μ m thick sections were prepared on a cryostat and collected in PBS (floating sections).

For histological examination, opioid peptide immunohistochemistry and cell identification, 7 μ m thick sections from the same patient were mounted on gelatin-coated slides. Histological evaluation was performed as described previously.²⁸ For best results of OR demonstration, we used the most common method (floating thick tissue sections of 40 μ m) to increase exposure and penetration of tissue by OR antibodies. In general, many antigens on nerve fibres can be more successfully demonstrated in floating thick tissue sections than in slide-mounted tissue sections, which may, partly, be due to the three-dimensional course of nerve fibres within tissue.^{3, 30, 31, 29, 4} However, the use of 7 μ m thick sections mounted on slides is sufficient for demonstration of opioid peptide and identification of cell type. Cell density and lining layer thickness were determined in sections from at least three different tissue samples (H&E stained) per patient. The thickness of the lining layer was analysed by averaging the number of cells in a cross section at nine different locations ($\times 400$ magnification). The overall cell density was determined by counting all stained cell nuclei in 17 randomly selected high-power fields of view ($\times 400$ magnification). The numbers of T cells, macrophages and vessels were evaluated in eight cryosections stained with antibodies against human CD3, CD163 and type IV collagen, respectively. The number of stained structures was averaged from 17 randomly selected high-power fields ($\times 400$ magnification). The number of high-power fields was derived from a histological study by Bresnihan *et al.*³²

Single staining procedures

All sections were processed for immunohistochemistry with a vectastain avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories) as described previously.²⁹ Unless otherwise stated, all incubations were performed at room temperature and PBS was used for washing (three times for 10 min) after each step. The floating (for ORs) and mounted (for opioid peptides) sections were incubated for 45 min in PBS with 0.6% H₂O₂ and 50% methanol to block endogenous peroxidase, and for 60 min

in PBS containing 0.3% Triton X-100, 1% bovine serum albumin, 5% goat serum and 5% donkey serum (blocking solution) to prevent non-specific binding. The sections were then incubated overnight with antibodies against END, ENK, μ OR or δ OR (1:1000), and thereafter for 90 min with a goat anti-rabbit biotinylated secondary antibody and for another 90 min with ABC. Finally, the sections were washed and stained with 3',3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, Saint Louis, USA) containing 0.01% H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6) for 3–5 min. After the enzyme reaction, the floating sections (for OR detection) were mounted on gelatin-coated slides. The slide-mounted sections were washed in tap water, counterstained with thionin, dehydrated in alcohol, cleared in xylene and mounted in distrene, tricresyl phosphate and xylene (DPX; Merck, Darmstadt, Germany).

In addition to double staining, immunoreactive (IR) cells were identified by the following morphological criteria: (1) macrophages/monocytes by large cell bodies, vacuolated cytoplasm and irregular-shaped nuclei; (2) lymphocytes by small cell bodies, large nuclei and small amounts of cytoplasm; (3) polymorphonuclear leucocytes by large cell bodies and multi-segmented nuclei; (4) plasma cells by vacuolated cytoplasm and small dense eccentric nuclei; and (5) fibroblast-like synoviocytes by dendritic-shaped processes.

Double staining procedures

Double staining of opioid peptides with cell markers

Double staining was performed as described previously.¹⁹ Sections stained in the first sequence with an antibody against either END or ENK (as described above) were treated with 1% H_2O_2 for 30 min to inactivate peroxidase in ABC, washed in several changes of PBS and incubated with blocking solution. In a second step, sections mounted on gelatin-coated slides were incubated with monoclonal mouse antibodies against human CD15, CD68, CD3 or CD23. All incubations were maintained overnight at 4°C, then slides were washed in PBS and exposed to the appropriate biotinylated secondary antibody for 1 h and to ABC for 45 min. Finally, these sections were washed and stained using Histogreen. The chromogen DAB used for the primary antiserum (END and ENK) appeared brown, whereas the Histogreen used for the second antiserum (cell markers) appeared green. After the enzymatic reaction, sections were mounted on gelatin-coated slides. Without counterstaining, sections were washed in distilled water, dehydrated in alcohol, cleared in xylene and mounted in DPX.

Double staining of ORs and neuronal markers

Floating sections stained in the first sequence with an antibody against either μ OR or δ OR (as described above) were treated with 1% H_2O_2 for 30 min to inactivate peroxidase in ABC, washed in several changes of PBS and incubated with blocking solution. Then, sections were incubated with a guinea pig antibody against human CGRP overnight at 4°C. The slides were then washed in PBS, exposed to the appropriate biotinylated secondary antibody (Vector Laboratories) for 1 h and to ABC for 45 min. Finally, the sections were washed and stained using DAB. During staining with DAB, nickel solution was used to differentiate the double immunostaining. The chromogen DAB/nickel used for the primary antisera (μ OR and δ OR) appeared grey/black, whereas the one (DAB) used for the second antiserum (CGRP) appeared brown. After the enzymatic reaction, the floating sections were mounted on gelatin-coated slides, washed in distilled water, dehydrated in alcohol, cleared in xylene and mounted in DPX. For double immunofluorescence staining for TH with μ OR or δ OR, floating sections were incubated with rabbit polyclonal antibodies against μ OR or δ OR in combination with a mouse monoclonal antibody against TH

(1:1000), washed with PBS and then incubated with secondary Texas red-conjugated goat anti-rabbit antibody in combination with fluorescein isothiocyanate-conjugated donkey anti-mouse antibody. Thereafter, the floating sections were washed with PBS, mounted on gelatin-coated slides and Vectashield, and viewed under a Zeiss 510 laser-scanning microscope.

Specificity controls

To demonstrate specificity of staining, the following controls were included (see also Mousa *et al*¹⁹): (1) preabsorption of diluted antibody against END, ENK, μ OR or δ OR with 5 μ g/ml of purified END, ENK (Peninsula Laboratories) or synthetic peptide antigen for μ OR and δ OR (Gramsch Laboratories-Biotechnology, Schwabhausen, Germany), respectively, for 24 h at 4°C; (2) preincubation of END antiserum with 7 μ g/ml purified ENK, and preincubation of ENK antiserum with 7 μ g/ml purified END to exclude cross reactivity; and (3) omission of either the primary antisera, the secondary antibodies or ABC.

Quantification of immunostaining

END- or ENK-IR cells as well as OR-IR nerve fibres or cells were counted using only intact tissue exhibiting optimal morphology to avoid misleading results. END- or ENK-IR cells were counted by a blinded experimenter in four sections per patient. Fifteen squares (384 μm^2 each) per section were analysed using a Zeiss microscope (objective $\times 20 \times 10$) as described previously.^{29, 19} The percentage of stained (for END or ENK) polymorphonuclear or mononuclear cells was determined by the formula: stained polymorphonuclear or mononuclear cells/total number of stained cells $\times 100$. OR-IR nerve fibres were counted by a blinded experimenter in five sections per patient and 10 squares (38.4 mm^2 each) per section using a Zeiss microscope (objective $\times 20 \times 10$). Seven patients per group were used for quantitative analysis.

Statistical analysis

Data are represented as means (SEM). Sample comparisons were made using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls or Dunnett's post hoc test in the case of normally distributed data, and Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's test in the case of data not distributed normally. Differences were considered significant if $p < 0.05$. All tests were performed using Sigma Stat V.2.03 statistical software.

RESULTS

Parameters of inflammation in serum and synovial tissue

To compare the severity of inflammation among patients with JT, OA and RA, we investigated laboratory serum data, the thickness of the lining layer, overall cellularity, density of CD3 T cells and CD163 macrophages, and vascularity. Parameters of inflammation were more overt in patients with RA than in those with JT and OA (table 1). C-reactive protein, cellularity and thickness of the synovial lining increased from patients with JT to OA to RA and were significantly higher in patients with RA than in those with JT and OA ($p < 0.05$; table 1). Synovial vascularity was similar in patients with OA and RA and significantly higher than in those with JT (table 1). The overall density of CD3 T cells and CD163 macrophages increased from patients with JT to OA to RA. This increase was significant for CD163 macrophages in patients with RA ($p < 0.05$, ANOVA on ranks followed by Dunn's test). Erythrocyte sedimentation rate (ESR) was significantly higher in patients with RA than in those with OA ($p < 0.05$; table 1).

Localisation of END and ENK

Lining layers

Staining of tissue of patients with JT demonstrated END- and ENK-IR cells scattered within and directly under the lining layer of inflamed, but rare in non-inflamed, synovial tissue of patients with minor trauma (figs 1A, 2C). The number of END- and ENK-IR cells was similar, and both cell types were significantly more abundant in patients with OA and RA than in those with JT (table 2). The morphological appearances (dendritic-shaped processes) of some END- and ENK-IR cells were consistent with fibroblast-like (type B) synoviocytes (fig 1A,C,E). In all diseases, double immunohistochemistry demonstrated that most of the cells containing END or ENK were also positive for CD68 (macrophages; data not shown).

Sublining layers

In JT, 69.9% (1.7%) of END-IR and 66.4% (2.7%) of ENK-IR cells were granulocytes (fig 1B). Consistently, the majority of END-IR and ENK-IR cells were positive for CD15 (granulocytes) and only a few cells were positive for CD3 (fig 3A,B).

In contrast, in OA, 94.6% (2.4%) of END-IR and 90.4% (1.5%) of ENK-IR cells were mononuclear cells. Similarly, in RA, 91.1% (0.6%) of END-IR cells and 93.0% (1.2%) of ENK-IR cells were mononuclear cells (figs 1D,F, 2A,C). Accordingly, a high number of END-IR and ENK-IR cells were positive for CD68 (macrophages) and CD3 (T lymphocytes) in OA and RA

(fig 3C–H). Some END-IR and ENK-IR cells were also positive for CD23 (B lymphocytes) in RA and OA (data not shown). In some samples of synovium from patients with OA or RA, fibroblasts and plasma cells, respectively, were the main population of cells expressing END and ENK (fig 2B,C). Overall, END- and ENK-IR immune cells were more abundant in RA than in JT and OA ($p < 0.05$; table 2). END- and ENK-IR immune cells were absent in non-inflamed tissue (fig 2C). In both inflamed and non-inflamed tissue, ENK (but not END) was occasionally detected singly or in bundles of nerve fibres (fig 2A).

Preabsorption of END or ENK antibodies with their respective antigens or omission of primary or secondary antibodies completely abolished immunostaining (data not shown). Preincubation of END antiserum with purified ENK or ENK antiserum with purified END had no effect on END or ENK immunoreactivity, respectively, excluding cross reactivity between END or ENK antisera (data not shown). Omission of either the primary or the secondary antibody did not produce the first or second (double) colour, respectively (data not shown).

Localisation of ORs

μ OR- or δ OR-IR nerve fibres were identified within the sublining tissue of all patients. These consisted partly of rather thick bundles of fibres and partly of thin varicose structures

Figure 1 Localisation of endorphin (END) (A, B) in patients with joint trauma (JT), (C, D) in those with osteoarthritis (OA) and (E, F) in those with rheumatoid arthritis (RA). (A) Most macrophages (M), fibroblast-like cells (arrows) in the lining layer (LL) contain END in patients with JT. (B) Sublining tissue from patients with JT contains many granulocytes (G), and few macrophages/monocytes (M) and lymphocytes (L) stained for END. (C, E) Macrophages/monocytes (M) and fibroblast-like cells (arrows) in the lining layer (LL), in patients with OA (C) and in those with RA (E) contain END. (D, F) Sublining layers of OA (D) and RA (F) synovium contain END-IR cells including macrophages/monocytes (M), lymphocytes (L) and plasma cells (PC) and very few granulocytes (G). Bar = 20 μ m.

Figure 2 Localisation of enkephalin (ENK; A–D) and μ opioid receptors (μ ORs; E) in synovium of patients with non-inflamed (C) rheumatoid arthritis (RA; A, B, E) and osteoarthritis (OA; D). (A) ENK-immunoreactive (IR) cells in sublining layers of RA include predominantly lymphocytes (L), macrophages/monocytes (M) and plasma cells (PC), with very few granulocytes (G). ENK was also expressed in nerve fibres (arrow). (B) An occasional aggregation of plasma cells containing ENK in RA. (C) IR cells are scarce (arrow) within lining epithelium (LL) and absent in sublining tissue of non-inflamed synovium. (D) In some OA samples, ENK-IR cells are predominantly fibroblasts (arrowheads). (E) Sublining layers of RA synovium with μ OR-IR cells including macrophages/monocytes (M), granulocytes (G) and lymphocytes (L). Bar = 20 μ m.

that may correspond to single nerve fibres or endings (fig 4A–F). The density of μ OR- and δ OR-IR nerve fibres was significantly greater in RA than in OA and JT (fig 4A–C; $p < 0.05$; table 2). μ ORs and δ ORs were also expressed on immune cells (fig 2D) and on fibroblasts infiltrating the lining and sublining layers in all diseases. The number of OR-IR immune cells was greater in RA (μ OR: 5.1 (1.6); δ OR: 5.9 (2.0)/

384 μ m²) than in OA (μ OR: 3.2 (1.0); δ OR: 3.1 (1.1)/384 μ m²) or in JT (μ OR: 2.8 (1.1); δ OR: 3.0 (1.4)/384 μ m²; $p < 0.05$; Kruskal–Wallis test followed by Dunn's test). All μ OR- and δ OR-IR nerve fibres coexpressed CGRP. Some fibres expressed CGRP alone (fig 4I D–F). In contrast, close proximity of OR- and TH-IR fibres, but no colocalisation, was observed in all diseases (fig 4II A, B). Preabsorption of OR antibodies with their

Table 2 Number of endorphin- and enkephalin-immunoreactive (IR) immune cells (per 384 μ m²) and of μ opioid receptor (μ OR)- and δ OR-IR nerve fibres (per 38.4 mm²) in the sublining layers and the lining layers of patients with joint trauma, osteoarthritis and rheumatoid arthritis

Subject	END		ENK		μ OR SL	δ OR SL
	LL	SL	LL	SL		
JT	3.1 (0.4)	4.2 (0.8)	2.8 (0.4)	3.9 (0.5)	2.9 (0.2)	2.6 (0.2)
OA	6.0 (0.6)*	5.5 (0.5)	5.9 (0.4)*	4.7 (0.3)	3.1 (0.2)	2.7 (0.1)
RA	6.7 (0.5)*	9.9 (0.3)†	6.9 (0.5)*	9.1 (0.5)†	4.8 (0.2)†	4.1 (0.2)†

END, endorphin; ENK, enkephalin; JT, joint trauma, LL, lining layers; OA, osteoarthritis; OR, opioid receptors; RA, rheumatoid arthritis; SL, sublining layers.

Values are means (SEM) obtained from eight patients per disease group and are expressed as the number of positive immune cells/384 μ m² and the number of positive nerve fibres/38.4 mm².

*Mean was significantly higher than that of JT ($p < 0.05$, analysis of variance (ANOVA) followed by Student–Newman–Keuls test).

†Mean was significantly higher than that of JT and OA ($p < 0.05$, ANOVA followed by Student–Newman–Keuls test).

Figure 3 Synovial tissue from patients with joint trauma (JT; A, B), osteoarthritis (OA; C, D) and rheumatoid arthritis (RA; E-H) double immunostained for endorphin (END)/CD15, enkephalin (ENK)/CD15 (A, B), END/CD68 (C, E), ENK/CD68 (D, F), END/CD3 (G) and ENK/CD3 (H). (A, B) In sublining layers of those with JT, most END- and ENK-immunoreactive (IR) cells express CD15 (granulocytes; double arrow), some cells contain only END or ENK (brown; arrow) or CD15 (green; arrowhead). (C, D) In sublining layers of those with OA, most END- and ENK-IR cells express CD68 (macrophages; double arrow); some cells contain only END or ENK (brown; arrow) or CD68 (green; arrowhead). (E-H) In sublining layers of RA, a large population of END- or ENK-IR cells express CD68 (macrophages) (E, F) or CD3 (T-lymphocytes) (G, H) (double arrow); some cells contain only END or ENK (brown; arrow), CD68 or CD3 (green; arrowhead). Some nerve fibres were immunostained with ENK (thick arrows) (B, D, H). Bar = 20 μ m.

respective antigens or omission of primary or secondary antibodies completely abolished immunostaining (data not shown).

DISCUSSION

We found the highest amount of synovial lining cells, vascularity and infiltrating immune cells in patients with RA compared with those with JT and OA. END and ENK were expressed predominantly in granulocytes in patients with JT, and in macrophages, lymphocytes and plasma cells in those with OA and RA. Overall, END- and ENK-IR cells were significantly more abundant in patients with RA than in those with JT and OA. μ ORs and δ ORs were localised on immune

cells and on sensory nerve fibres but not on sympathetic nerve fibres, and were significantly more abundant in patients with RA than in those with JT and OA.

The lining layer of the synovial membrane contains macrophage-like (type A cells) and fibroblast-like cells (type B cells).³³ Our double staining demonstrated that most of the lining layer cells containing END or ENK were positive for CD68 (macrophages). Relatively few END- and ENK-IR cells had dendritic-shaped processes, consistent with fibroblast-like synoviocytes (type B).³⁴ In the sublining layers, we found different patterns of opioid peptide-containing leucocyte subsets in patients with JT, OA and RA. END and ENK were expressed predominantly in granulocytes in patients with JT, and in macrophages,

Figure 4 (I) Localisation of opioid receptors (μ OR; D, E) and δ OR (A–C, F) in joint trauma (JT; A), osteoarthritis (OA; B, D) and rheumatoid arthritis (RA; C, E, F). δ ORs are localised on nerve fibres in JT (A), OA (B) and RA (C). Tissue from patients with OA (D) and RA (E, F) was double immunostained for μ OR/calcitonin gene-related peptide (CGRP; D, E) and δ OR/CGRP (F). μ OR- or δ OR-immunoreactive nerve fibres express CGRP (black; double arrows), with some nerve fibres containing only CGRP (brown; arrow). (II) Double immunofluorescence staining of μ ORs (A; red) and δ ORs (B; red) with tyrosine hydroxylase (TH; green; A, B) in RA (A) and OA (B). μ ORs and δ ORs immunoreactivity (red; arrow) did not colocalise with TH (green; arrowhead). Bar = 20 (I) and 10 (II) μ m.

lymphocytes and plasma cells in those with OA and RA. These results extend previous reports of END and ENK expression in humans^{14–25} and in dogs³⁵ that did not differentiate the synovial cell types. The pattern of opioid-expressing cells described here is similar to the known distribution of cell types in patients with JT, OA and RA.^{36–39} Occasionally, ENK immunoreactivity

was also found in nerve fibres, consistent with previous studies on animals and humans.^{40–43}

Both END- and ENK-IR cells were significantly more abundant in patients with RA than in those with JT and OA, in parallel with the highest degree of inflammatory parameters in patients with RA. These findings suggest that increases of

opioid peptide-containing cells are correlated with the inflammatory activity in the joint. Consistently, we have shown that ongoing Freund's adjuvant-induced inflammation stimulates a progressively increasing recruitment of opioid peptide-containing immune cells to the site of injury in the rat paw.⁴⁴ In this model, opioid-containing granulocytes predominate at early stages, whereas opioid-producing monocytes/macrophages, T lymphocytes and B lymphocytes follow at later stages.^{8, 45–47} In addition to their main role as producers of antibodies and cytokines, B lymphocytes also play a role in the synthesis and release of opioid peptides.^{8, 45, 48, 49} The recruitment of opioid-containing leucocytes from the circulation to the site of inflammation is orchestrated by a successive expression of chemokines and adhesion molecules, primarily CXCR2 ligands, L and P selectins, α_4/β_2 integrins and intercellular adhesion molecule 1.^{21, 50–52}

We next examined the localisation of ORs. Previous studies have shown such receptors in human,¹⁴ canine¹² and equine¹¹ synovium based on radioligand-binding assays and autoradiography. We have now extended these studies by use of specific antibodies against the human μ OR and δ OR to identify the exact anatomical structures and possible differences in the expression of ORs between the three diseases. Our results show that μ ORs and δ ORs are expressed on nerve fibres and on various types of immune cells in all disorders. The latter findings are in agreement with previous studies that detected ORs in human immune and synovial cells.^{9, 10, 53–55} Such ORs have been reported to modulate various functions such as proliferation, antibody production, chemotaxis, phagocytosis, tumoricidal activity and gene expression in vitro, but their roles in vivo remain to be investigated in more detail.^{10, 56}

Since a large number of animal studies have shown that inflammatory pain can be inhibited by activation of ORs on peripheral sensory neurones (reviewed in Stein *et al.*), we investigated these ORs in more detail. Using immunohistochemical analysis on floating sections, we were able clearly to define the anatomical localisation and appearance of nerve fibres expressing OR in the sublining synovial layers. The number of μ OR- and δ OR-IR fibres was significantly higher in patients with RA than in those with JT and OA. Again, this suggests a correlation with inflammatory activity in the joint and is in agreement with animal studies that have revealed an upregulation of μ ORs and δ ORs in sensory neurones in models of Freund's adjuvant-induced inflammation,^{31, 57–59} and with a higher number of sensory nerve fibres in synovial tissue of patients with RA than in those with OA.²⁸ Double staining for μ ORs and δ ORs with CGRP disclosed coexpression in both cases. In contrast, neither μ ORs nor δ ORs colocalised with TH. Since CGRP and TH are specific markers of primary afferent C fibres and sympathetic efferent fibres, respectively,³⁰ our findings agree with animal studies suggesting that μ ORs and δ ORs are localised on nociceptive C fibres but not on sympathetic fibres.^{30, 60–62} However, since there is a loss of sympathetic neurones in synovial tissue in patients with RA, a definitive conclusion could not be drawn from our experiments at this time.²⁸ Together, these findings suggest that neuronal ORs most probably modulate sensory afferent functions rather than sympathetic efferent functions within inflamed joints and provide an anatomical correlate to clinical studies demonstrating that IA opioid agonists (eg, morphine) inhibit postsurgical and chronic arthritic pain.^{14, 15, 17, 63–66} Moreover, the greater increase of sensory nerve endings expressing ORs in RA than in JT and OA is consistent with reports that IA morphine produces a more pronounced analgesic effect in patients with RA than in those with OA.¹⁸

In summary, our findings extend recent concepts regarding the interaction of ORs and opioid peptides in inflamed tissues.

Animal studies have shown that cytokines, hormones and chemokines can stimulate the release of immune cell-derived opioid peptides, and that the activation of neuronal ORs by these peptides results in inhibition of inflammatory pain.^{19, 21, 22, 45, 58, 67, 68} Importantly, clinical studies have shown that the IA administration of an OR antagonist exacerbates pain in JT,²⁵ indicating that opioid peptides are not only secreted on artificial stimulation but are tonically released from inflammatory cells and from sensory nerves and, thus, maintain a certain level of intrinsic pain control. We have now provided the anatomical correlates in human tissue by demonstrating that two different opioid peptides are expressed in granulocytes, monocytes/macrophages and lymphocytes in synovium and that the corresponding ORs are expressed on sensory neurones and immune cells. Both peptides were significantly more abundant in patients with RA than in those with JT and OA, and μ OR- and δ OR-expressing sensory nerve endings were increased more in patients with RA than in those with JT and OA. Although the functional role of opioids and neuronal ORs in the control of pain has been established, it remains to be clarified which clinical outcome parameters are modulated by ORs on inflammatory cells.

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