Anesthesiology 2001; 95:500–8

Opioid Peptide–expressing Leukocytes

Identification, Recruitment, and Simultaneously Increasing Inhibition of Inflammatory Pain

Heike L. Rittner, M.D.,* Alexander Brack, M.D.,* Halina Machelska, Ph.D.,† Shaaban A. Mousa, Ph.D.,† Monika Bauer, Ph.D.,† Michael Schäfer, M.D.,‡ Christoph Stein, M.D.§

Background: **Inflammatory pain can be effectively controlled by an interaction of opioid receptors on peripheral sensory nerve terminals with opioid peptides released from immune cells upon stressful stimulation. To define the source of opioid peptide production, we sought to identify and quantify populations of opioid-containing cells during the course of Freund's complete adjuvant–induced hind paw inflammation in the rat. In parallel, we examined the development of stress-induced local analgesia in the paw.**

Methods: **At 2, 6, and 96 h after Freund's complete adjuvant inoculation, cells were characterized by flow cytometry using a monoclonal pan-opioid antibody (3E7) and antibodies against cell surface antigens and by immunohistochemistry using a polyclonal antibody to β-endorphin. After magnetic cell sorting, the β-endorphin content was quantified by radioimmunoassay. Pain responses before and after cold water swim stress were evaluated by paw pressure thresholds.**

Results: **In early inflammation, 66% of opioid peptide–producing ($3E7^+$) leukocytes were $HIS48^+$ granulocytes. In contrast, at later stages (96 h), the majority of $3E7^+$ immune cells were $ED1^+$ monocytes or macrophages (73%). During the 4 days after Freund's complete adjuvant inoculation, the number of $3E7^+$ cells increased 5.6-fold ($P < 0.001$, Kruskal–Wallis test) and the β-endorphin content in the paw multiplied 3.9-fold ($P < 0.05$, Kruskal–Wallis test). In parallel, cold water swim stress–induced analgesia increased by 160% ($P < 0.01$, analysis of variance).**

Conclusions: **The degree of endogenous pain inhibition is proportional to the number of opioid peptide–producing cells, and distinct leukocyte lineages contribute to this function at different stages of inflammation. These mechanisms may be important for understanding pain in immunosuppressed states such as cancer, diabetes, or AIDS and for the design of novel therapeutic strategies in inflammatory diseases.**

PAIN can be relieved by systemic administration of opioids acting on specific opioid receptors within the central nervous system. Although centrally acting opioids are effective, they have substantial side effects including nausea, vomiting, respiratory depression, and even respiratory arrest. However, opioid receptors are expressed not only within the central nervous system but also on peripheral sensory nerve terminals.[1] Intraplantar inoculation of Freund's complete adjuvant (FCA) induces a localized painful inflammation in the rat paw. Opioid receptors are up-regulated and opioid peptide–containing immune cells are recruited to the inflamed site.[2] Upon stressful stimulation (cold water swim [CWS]), these cells can locally secrete opioid peptides that bind to opioid receptors on sensory neurons leading to analgesia.[3] This peripheral opioid mechanism is highly potent in controlling inflammatory pain and, importantly, is devoid of any central side effects.

Two endogenous opioid peptides, β-endorphin (END) and enkephalin (ENK), seem to be primarily responsible for this intrinsic analgesia. END and ENK are detectable by immunohistochemistry in various immune cells of the inflamed paw including monocytes, lymphocytes, and granulocytes.[4] Opioid peptides have been quantified in immune cells by radioimmunoassay,[5–9] and their opioid peptide precursor messenger RNAs (mRNAs), proopiomelanocortin and proenkephalin mRNA, are detectable in inflamed tissue.[5] The relevance of immune cells for the generation of peripheral stress-induced analgesia is supported by studies using total-body irradiation and cyclosporine A. Both treatments induce immunosuppression and abolish stress-induced endogenous opioid analgesia.[5,9]

To date, trafficking of END-containing lymphocytes has been studied in the peripheral blood and the lymph nodes.[9] However, these experiments provide only indirect evidence for the role of opioid peptide–containing immune cells at the site of inflammation. Quantification of the major opioid peptide–containing cell subpopulations in the inflamed tissue itself is necessary to characterize cell recruitment. The detailed examination of such basic mechanisms of endogenous analgesia may have important implications for understanding pain in diseases with impaired immune function such as cancer, sepsis, or AIDS and for the design of novel therapeutic strategies.

To identify the main target cell population for the generation of analgesia, we established a triple-color intracellular stain for flow cytometry to simultaneously detect intracellular opioid peptides and hematopoetic

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

* Resident, † Postdoctoral Fellow, ‡ Assistant Professor, § Professor and Chairman.

Received from Klinik für Anaesthesiologie und operative Intensivmedizin, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, D-12200 Berlin, Germany. Submitted for publication November 10, 2000. Accepted for publication February 21, 2001. Supported by Deutsche Forschungsgemeinschaft, Bonn, Germany. Presented in part at the annual meeting of the German Society of Anesthesiology (Deutscher Anaesthesiekongress), Munich, Germany, May 6–9, 2000, and the annual meeting of the American Society of Anesthesiologists, San Francisco, California, October 13–18, 2000.

Address reprint requests to Dr. Rittner: Klinik für Anaesthesiologie und operative Intensivmedizin Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, D-12200 Berlin, Germany. Address electronic mail to: rittner@medizin.fu-berlin.de. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

lineage markers in immune cells of the inflamed rat paw. In parallel, we measured CWS-induced analgesia at different stages of the inflammatory reaction. The aims of the present study were (1) to characterize subpopulations of opioid peptide–expressing immune cells at different stages of paw inflammation, (2) to quantify opioid peptide–containing immune cells and peptide content at these different stages, and (3) to examine the relation of the number of opioid-expressing immune cells with the potency of endogenous peripheral analgesia.

Material and Methods

Animal Experiments

Animal protocols were approved by the Animal Care Committee of the State of Berlin (Landesamt für Arbeitsschutz, Gesundheitsschutz und Technische Sicherheit (Berlin, Germany). Male Wistar rats weighing 150–200 g were injected with 150 μl FCA (Calbiochem, La Jolla, CA) in the right-side hind paw under brief halothane anesthesia. Rats developed an inflammation confined to the inoculated paw.

Tissue and Cell Preparation

Tissue samples were harvested 2, 6, and 96 h after injection of FCA. Animals were killed and popliteal lymph nodes were dissected. Subcutaneous paw tissue was obtained from the plantar surface leaving the deep flexor tendon *in situ*. To obtain a single cell suspension for fluorescence activated cell staining (FACS) analysis, magnetic cell separation, and radioimmunoassay (RIA), lymph nodes and subcutaneous paw tissue were cut into 1- to 2-mm pieces. Fragments were digested for 1 h at 37°C with 10 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Paisley, Scotland)/1 g tissue containing 30 mg collagenase (Sigma, Deisenhofen, Germany), 10 mg hyaluronidase (Sigma), and 0.5 ml HEPES, 1 M (Sigma). The digested fragments were pressed through a 70-μm nylon filter (Pharmingen/Becton Dickinson, Heidelberg, Germany) to remove particles.

Fluorescence Activated Cell Staining

Single cell suspensions obtained from one or two paws were centrifuged, decanted, and fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and washed with PBS. Cells were permeabilized with saponin buffer (0.5% saponin, 0.5% bovine serum albumin, 0.05% NaN_3 [all from Sigma]) and subsequently stained with 20 μg/ml 3E7 mouse monoclonal immunoglobulin G_{2a} (IgG_{2a}) antibody in saponin buffer for 30 min at room temperature. This antibody recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe-Met at the N-terminus of opioid peptides.[10] Afterwards, cells were incubated with the secondary rat anti-mouse IgG_{2a+b} phytoerythrin (PE)–conjugated monoclonal antibody (1.5 μg/ml) for 15 min at room temperature. Negative controls included the replacement of the primary antibody with an isotype matched irrelevant antibody (mouse IgG_{2a}) and omission of the secondary PE–conjugated antibody at the same concentration.

Subsequently, cells were labeled with fluorescein isocyanate (FITC) or Cy Chrome–conjugated surface markers to differentiate subpopulations of monocytes or macrophages, granulocytes, and T cells and then fixed in 1% paraformaldehyde in PBS. To calculate absolute numbers of cells per paw, the stained cell suspension was transferred to a TruCOUNT® tube (Pharmingen/Becton Dickinson) containing a known number of fluorescent beads. FACS events from the fluorescent TruCOUNT® beads and stained cells were collected simultaneously using the FACScan (Pharmingen/Becton Dickinson). Numbers of $CD45^+$ cells per tube were calculated in relation to the known number of fluorescent TruCOUNT® beads and extrapolated for the whole paw. At least 10,000 FACS events were collected in the lymph node, and 30,000 FACS events in the paw. Data were analyzed using CellQuest software (Pharmingen/Becton Dickinson).

Preabsorption

To verify the specificity of staining, exogenous END or a control peptide (adrenocorticotropic hormone [ACTH]) (both from Peninsula, Merseyside, United Kingdom) was added to the lymphocytes from an inflamed lymph node after 96 h of FCA treatment before staining with 20 μg/ml 3E7. Each peptide was added at four concentrations: equimolar to 3E7 concentration, 0.5, 1.5, and 2.0 M excess of the peptide. Amounts were calculated as follows: (1) 3E7 monoclonal antibody (IgG, MW = 150,000 Da), final concentration, 20 μg/ml = 0.132 nmol; (2) END peptide (MW = 3,464), equimolar 0.132 nmol × 3,464 = 462 ng/ml; and (3) ACTH peptide (MW = 4,579): equimolar 0.132 nmol × 4,579 = 610.8 ng/ml.

Antibodies

The following antibodies were used: mouse monoclonal anti–pan-opioid 3E7 (subtype IgG_{2a}; Gramsch Laboratories, Schwabhausen, Germany); mouse IgG_{2a} and rat anti-mouse IgG_{2a+b} PE-conjugated (both from Pharmingen/Becton Dickinson); mouse anti-rat CD3 FITC/PE (T-cell marker), mouse anti-rat granulocyte FITC (HIS48), mouse anti-rat CD45 Cy Chrome and streptavidin–conjugated mouse anti-rat CD45 (all hematopoetic cells); mouse anti-rat ED1 FITC (monocyte/macrophage marker) (Pharmingen/Becton Dickinson and Serotec, Oxford, United Kingdom).

$CD45^+$ Purification by Magnetic Cell Separation

To obtain a sufficient number of $CD45^+$ cells to measure END by RIA, cells from several paws had to be

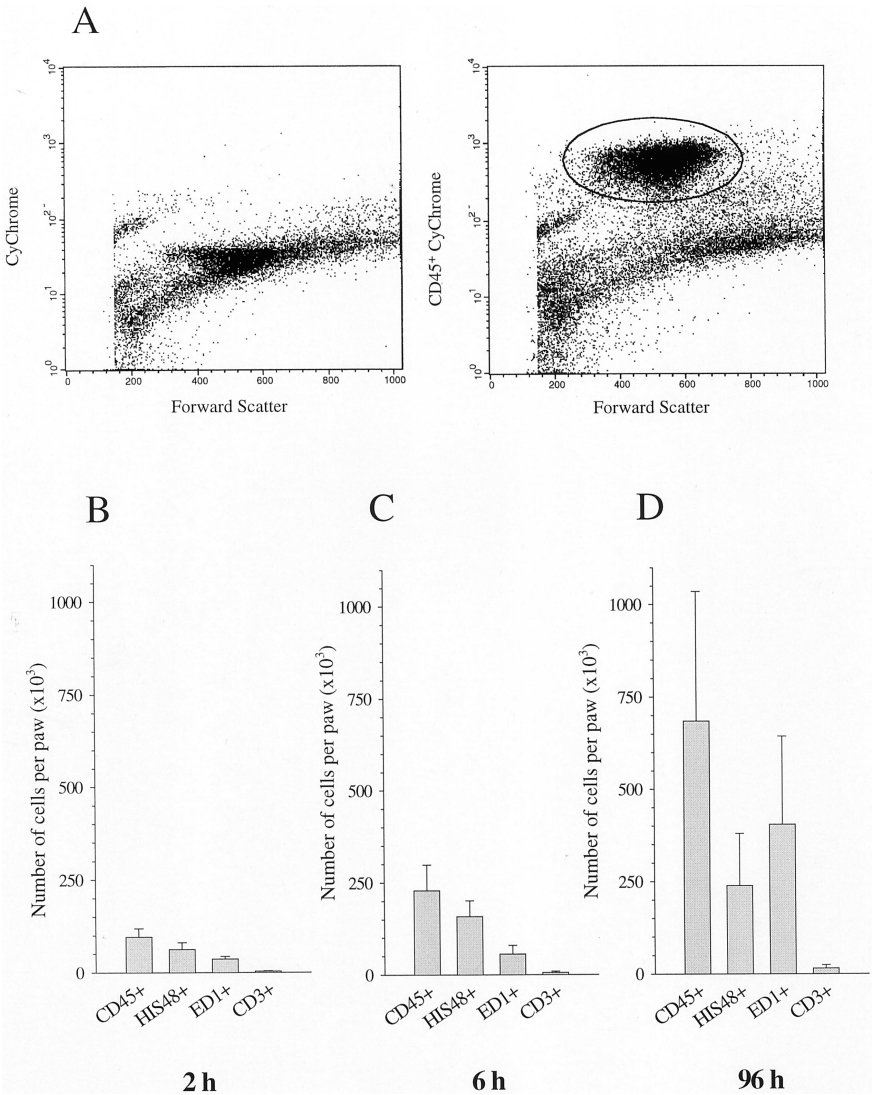

Fig. 1. Cellular composition of infiltrating immune cells in the paw tissue. Single cell suspensions from the inflamed paw were stained with Cy Chrome only (control; *A, left panel*) or with CD45 Cy Chrome (*A, right panel*). CD45$^+$ cells were pregated and examined for expression of anti-HIS48 fluorescein isocyanate (FITC) monoclonal antibody (mAb) (granulocytes), anti-ED1 FITC mAb (monocytes or macrophages) and anti-CD3 phytoerythrin (PE) mAb (T cells). Subpopulations of infiltrating inflammatory cells are analyzed in early (2 [*B*] and 6 h [*C*]) and late inflammation (96 h [*D*], mean ± SD, n = 6 per group). Total cell numbers increased significantly with ongoing inflammation (CD45$^+$ cells, $P < 0.001$; HIS48$^+$ cells, $P < 0.01$; and ED1$^+$ cells, $P < 0.01$, all by analysis of variance on ranks).

pooled. After 2 h of inflammation, 16 paws; after 4 h, 6 paws; and after 96 h, 3 paws were processed and measured together. Cell suspensions were stained with streptavidin-conjugated mouse anti-rat CD45 monoclonal antibody and subsequently labeled with avidin-coupled magnetic beads (Milteny Biotec, Bergisch-Gladbach, Germany) in PBS (0.5% bovine serum albumin [BSA], 2 mM EDTA). CD45$^+$ cells were isolated using a VS$^+$ column (Milteny Biotec). Purified CD45$^+$ cells were pelleted, lysed in 250 μl RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.01 M NaN_3, 0.1% BSA, and 0.1% Triton X-100) containing aprotinin (all from Sigma), and shock-frozen until further used. Purity of the cells was greater than 95% as measured by FACS after appropriate staining.

Radioimmunoassay of β-Endorphin

After the above-described cell separation, END peptide content in CD45$^+$ cells was measured by rat END RIA (Peninsula) as described previously.[9]

Immunohistochemistry

Rats were anesthetized with halothane 2, 6, and 96 h after FCA inoculation and perfused through the ascending aorta with 50 ml PBS and 400 ml Zamboni fixative (4% paraformaldehyde in PBS and 14% picric acid). Subcutaneous paw tissue was stained as previously described using a primary rabbit anti-rat END (1:1,000 dilution, Peninsula) and secondary biotinylated anti-rabbit antibody, Vectastain avidin-biotin-peroxidase complex, and 3,3'-diaminobenzidine as a substrate (all from Vector Laboratories, Burlingave, CA).[2]

Algesiometry

Paw pressure testing was performed 2, 6, and 96 h after FCA injection as described previously.[4] In brief, rats were handled before testing and gently restrained under paper wadding, and pressure was applied onto the dorsal surface of each hind paw. The pressure threshold (cut-off at 250 g) for paw withdrawal was determined using an algesiometer (Ugo Basile, Comerio, Italy) in

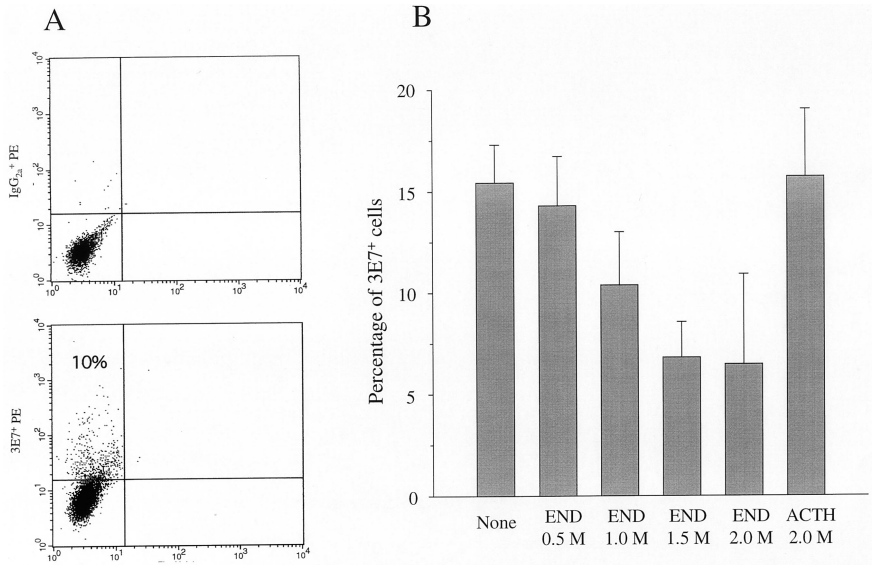

Fig. 2. Fluorescence activated cell staining for intracellular opioid peptides in lymphocytes. Cells from an inflamed lymph node 96 h after Freund complete adjuvant injection were stained with a control mAb (anti-mouse immunoglobulin G_{2a} [IgG_{2a}], upper panel) or anti–pan-opioid 3E7 (*lower panel*), and both were subsequently incubated with PE-conjugated anti-mouse IgG_{2a} mAb. Ten percent of the cells contained opioid peptides (*A*). To test for specificity of staining, lymphocytes were preincubated with increasing concentrations of β-endorphin (END) or a control peptide (adrenocorticotropic hormone [ACTH]) (*B*). Staining was decreased in a dose-dependent manner by END but not by ACTH (mean ± SD, n = 3 per dose, $P < 0.001$ by analysis of variance).

three consecutive measurements separated by 10-s intervals to calculate mean baseline values. To test stress-induced opioid analgesia, rats were exposed to ice-cold water for 1 min (CWS) and gently dried, and pressure threshold values were obtained after 1 min as described previously.[4]

Statistics

Data obtained at three time points (2, 6 and 96 h) were compared by analysis of variance (ANOVA) if the normality test and the equal variance test were passed. Otherwise, the Kruskal–Wallis ANOVA on ranks was used. *Post hoc* multiple pairwise comparisons were performed by the Student–Newman–Keuls method. If group sizes were unequal, *post hoc* comparisons were performed by Dunn test. Data of two cellular subpopulations (granulocytes *vs.* monocytes) were compared by the *t* test. If data were not normally distributed, the Mann–Whitney U test was used. A value of $P < 0.05$ was considered significant.

Results

Pattern of Inflammatory Cell Subtypes

Immune cells isolated from the hind paw were pregated by staining with anti-CD45 monoclonal antibody to exclude debris and nonhematopoietic cells such as fibroblasts or lipocytes (fig. 1A). $CD45^+$ Cells were further differentiated into hematopoetic lineages. In early FCA inflammation (2 and 6 h) the majority of infiltrating cells were $HIS48^+$ granulocytes, whereas $ED1^+$ monocytes or macrophages were significantly less abundant ($HIS48^+$ granulocytes, 65–70%; $ED1^+$ monocytes, 24–38% [$P < 0.05$]) (figs. 1B and C). At 96 h of FCA inflammation, the percentage of $HIS48^+$ granulocytes decreased and $ED1^+$ monocytes or macrophages were the prominent cell type (35% $HIS48^+$ cells *vs.* 60% $ED1^+$ cells [$P > 0.05$, *t* test]) (fig. 1D). The percentage of $CD3^+$ lymphocytes remained unchanged and relatively low (2–4%). The total number of infiltrating $CD45^+$ cells increased significantly over time (2.5-fold, 2 *vs.* 6 h, and threefold, 6 *vs.* 96 h [$P < 0.001$, Kruskal–Wallis; $P < 0.05$ at all pairwise comparisons by Student–Newman–Keuls]) (figs. 1B–D).

Specificity of 3E7 Staining in Opioid Peptide–Expressing Lymphocytes

An intracellular stain for opioid peptides was established. Lymphocytes obtained from an inflamed lymph node were stained with 3E7 or an isotype matched control monoclonal antibody and quantified by flow cytometry (fig. 2A). Specificity of the staining was demonstrated by dose-dependent and significant inhibition of 3E7 staining by preincubation with END but not with ACTH ($P < 0.001$, ANOVA; *post hoc* multiple comparisons by Student–Newman–Keuls: $P < 0.05$ for 1.5 and 2 M END *vs.* without peptide competition [NONE] and 1.5 and 2 M END *vs.* 2 M ACTH; $P > 0.05$ for 2 M ACTH 2 *vs.* NONE) (fig. 2B). The 3E7 staining was reduced by 60% at 2 M excess of exogenous END.

Opioid Peptide–producing $CD45^+$ Cells in Inflamed Paw

Single cell suspensions from paws obtained 2, 6, and 96 h after FCA-induced inflammation were double-stained for 3E7 and CD45 and analyzed by flow cytometry (fig. 3A). The absolute number of 3E7-positive $CD45^+$ cells increased significantly from 18,200 ± 4,000 cells at 2 h to 40,900 ± 8,100 cells at 6 h and to 102,300 ± 35,100 cells at 96 h after injection of FCA ($P < 0.001$ Kruskal–Wallis, $P < 0.05$ for all pairwise comparisons by Student–Newman–Keuls). The noninflamed paw was not analyzed because of the absence of $CD45^+$ cells in the FACS analysis.

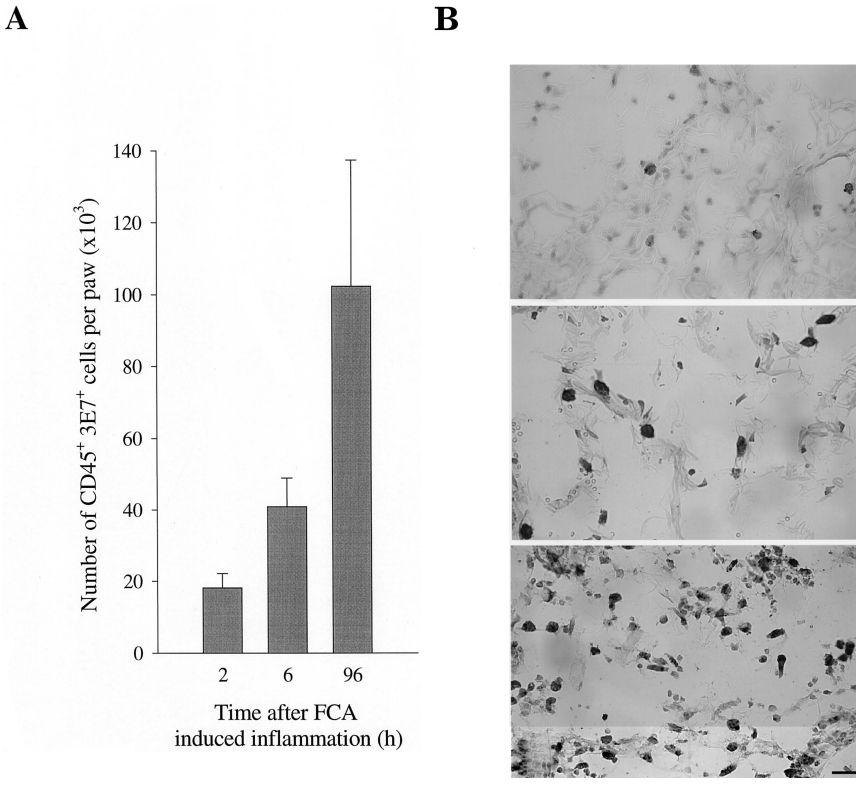

Fig. 3. Quantification of opioid peptide–containing immune cells in the paw during Freund's complete adjuvant (FCA)-induced hind paw inflammation. Absolute numbers of $CD45^+3E7^+$ cells per paw were calculated by adding TruCOUNT® fluorescent beads (mean ± SD, n = 6 per group [*A*]). The number of $CD45^+3E7^+$ cells increased significantly over time ($P < 0.001$ by analysis of variance on ranks). Histologic sections from the inflamed hindpaw showed increasing numbers of END-positive cells (*upper panel*, 2 h; *middle panel*, 6 h; and *lower panel*, 96 h [*B*]) (bar = 20 μm).

Immunohistochemistry

To confirm these findings, histologic sections from the inflamed hindpaw were stained with polyclonal anti-END at 2, 6, and 96 h after FCA injection. Specificity of staining was demonstrated by preabsorption of the primary antibody with excess of rat END peptide (data not shown). The number of END-positive immune cells increased substantially with the duration of inflammation (fig. 3B).

Radioimmunoassay

To quantify the total END content in $CD45^+$ cells per paw at various intervals after FCA injection, cells were purified by magnetic beads and END content was measured by RIA. Before selection, $CD45^+$ cells accounted for 36% of total FACS events collected at 96 h (fig. 4A). After magnetic cell separation, positively selected cells were greater than 95% pure. The END content in purified $CD45^+$ cells increased in a time-dependent manner during the course of inflammation from 9.3 ± 2.1 pg/paw at 2 h to 17.3 ± 4.1 pg/paw at 6 h and to 36.9 ± 17.1 pg/paw at 96 h, confirming the results obtained by flow cytometry ($P < 0.05$, Kruskal–Wallis ANOVA on ranks, *post hoc* pairwise comparison by Dunn test: $P < 0.05$ for 2 *vs.* 96 h, $P > 0.05$ for 2 *vs.* 6 h and 6 *vs.* 96 h) (fig. 4B).

Characterization of Subpopulations of Opioid Peptide-producing $CD45^+$ Cells

To assess hematopoietic origin of opioid peptide–producing immune cells, triple-color flow cytometry was performed. All samples were stained with the CD45 and 3E7 monoclonal antibodies. For lineage assignment, cells were additionally labeled with HIS48 or ED1 monoclonal antibody, respectively. Stains were pregated on $CD45^+$ cells as shown in figure 2A and subsequently analyzed for 3E7 expression and for hematopoietic lineage markers (fig. 5A). The $3E7^+HIS48^+$ granulocytes increased significantly during early inflammation, whereas minor and nonsignificant changes occurred between 6 and 96 h (fig. 5B) ($P < 0.01$, Kruskal–Wallis; *post hoc* pairwise comparison by Student–Newman–Keuls: $P < 0.05$ for 2 *vs.* 96 h and 2 *vs.* 6 h, $P > 0.05$ for 6 *vs.* 96 h). In contrast, $3E7^+ED1^+$ cells increased significantly between all time points ($P < 0.001$, Kruskal–Wallis; $P < 0.05$ at all pairwise multiple comparisons comparison by Student–Newman–Keuls). However, although the initial increase in recruitment of monocytes or macrophages was small (1.8-fold, 2 *vs.* 6 h), a dramatic 4.4-fold increase in recruitment occurred later (6 *vs.* 96 h) (fig. 5C). Opioid peptide–producing cells were predominantly $HIS48^+$ granulocytes in early inflammation (at 2 h, 66% of total $3E7^+$ cells, $P > 0.05$ by *t* test [$HIS48^+$ *vs.* $ED1^+$]; at 6 h, 63%, $P < 0.05$ by *t* test [$HIS48^+$ *vs.* $ED1^+$]). At later stages of inflammation the pattern changed, and the majority of $3E7^+$ cells were $ED1^+$ monocytes or macrophages (at 96 h, 73% of total $3E7^+$ cells, $P < 0.05$ by *t* test [$HIS48^+$ *vs.* $ED1^+$]).

Endogenous Analgesia

To examine the functional relevance of the increase in the number of opioid peptide–producing immune cells

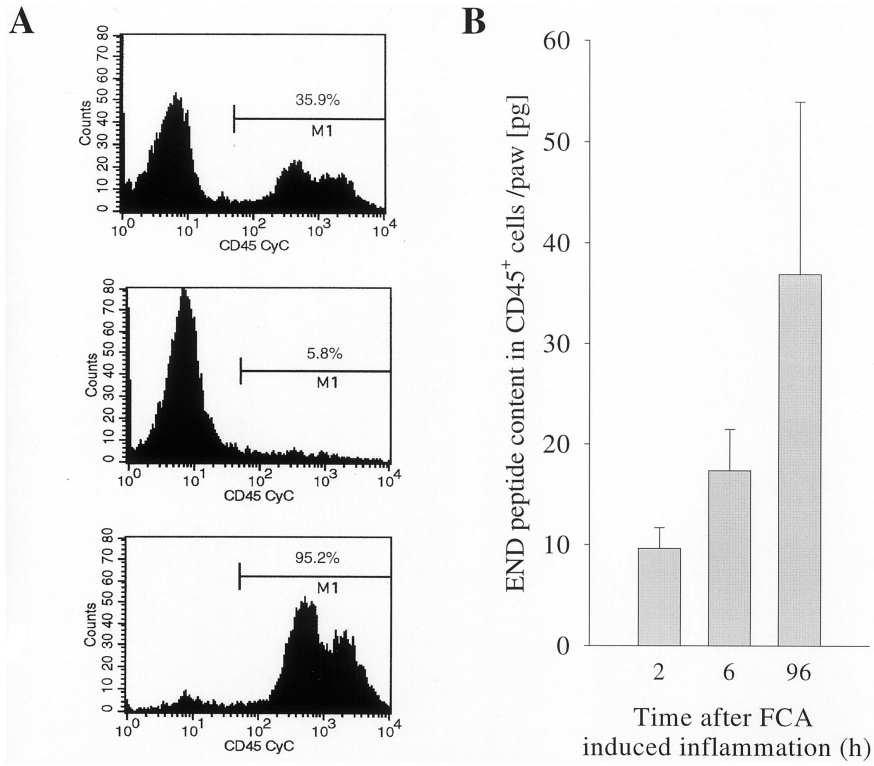

Fig. 4. β-Endorphin content in CD45$^+$ immune cells by radioimmunoassay. The CD45$^+$ cells were positively selected by magnetic cell separation (*A*), and END content was determined by radioimmunoassay (*B*). Efficacy of CD45 cell separation by magnetic beads was verified by flow cytometry (representative sample at 96 h after injection of Freund's complete adjuvant (FCA); (*upper panel*, unselected; *middle panel*, negative selection; *lower panel*, positive selection [*A*]). The amount of END in CD45$^+$ cells, calculated per one paw, increased substantially during the course of inflammation (mean ± SD; 2 h, n = 2; 6 and 96 h, n = 3; $P < 0.05$ by analysis of variance on ranks [*B*]).

and in END content, endogenous analgesia was tested by CWS. The CWS-induced pressure threshold elevation increased significantly with the duration of inflammation ($P < 0.01$, ANOVA; *post hoc* pairwise comparisons by Student–Newman–Keuls: $P < 0.05$ for 2 *vs.* 96 h and 6 *vs.* 96 h; $P > 0.05$ for 2 *vs.* 6 h (figs. 6A–C). Consistent with the absence of opioid-containing cells in noninflamed tissue,[4] CWS-induced analgesia was never observed in the contralateral paw.

Discussion

Our findings show that (1) the cell pattern after FCA inoculation of the rat paw is consistent with a typical nonspecific inflammatory reaction of the innate immune system; (2) the monoclonal antibody 3E7 produces specific intracellular staining and allows the quantification of opioid-producing cells in flow cytometry; (3) the number of opioid-producing cells, determined both by FACS and immunohistochemistry, increases in parallel with the END content; (4) opioid-containing cells are predominantly granulocytes during early and monocytes or macrophages during later stages; and (5) endogenous opioid pain control increases in parallel with cell recruitment and inflammation.

Characterization of Immune Cell Subpopulations Expressing Opioid Peptides

At early stages of inflammation (2 and 6 h), opioid peptides were mainly produced by HIS48$^+$ granulocytes, whereas ED1$^+$ monocytes or macrophages became the predominant opioid-containing cell type at later stages (96 h). CD3$^+$ T cells were detected in low numbers in the paw tissue during the first 4 days of this inflammation. This time-dependent pattern of immune cell recruitment to subcutaneous tissue after FCA injection is similar to the emergence of cells observed in other types of inflammation including infection by pathogens or wound healing.[11]

Importantly, our studies expand this concept in that granulocytes and monocytes are not only members of the frontline innate immunity, but they also produce and secrete opioid peptides to inhibit pain. Our findings are in agreement with prior studies examining opioid peptide production in immune cells. ENK production has been demonstrated in granulocytes,[12] mononuclear cells, and T cells.[8] END is detectable in a variety of cell types including ED1$^+$ monocytes or macrophages,[5] lymphocytes,[9] and mast cells.[4] Furthermore, precursor mRNAs of END and ENK can be found within inflamed tissue by *in situ* hybridization.[5] Our previous study examined trafficking of END$^+$ immune cells in the circulating blood and the regional lymph nodes.[9] It suggested that END$^+$ cells are of lymphocytic origin, migrate from the peripheral blood to the inflamed hind paw where they secrete opioid peptides, and, finally, leave the site of inflammation to the draining and inflamed lymph node. In contrast to the lymph node and the blood, opioid peptide–expressing cells in the paw are mainly of granulocytic or monocytic origin. Nonetheless, CD3$^+$

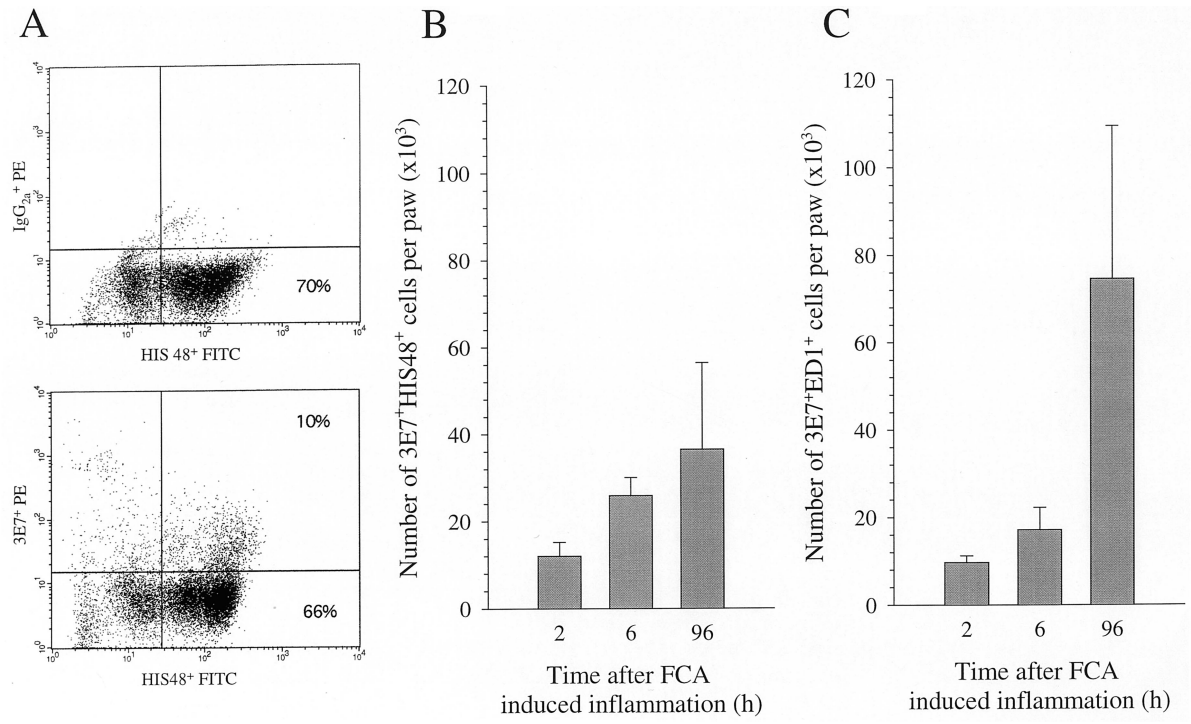

Fig. 5. Hematopoietic lineage of 3E7$^+$ cells in the inflamed paw. The CD45$^+$ cells were pregated and analyzed for expression of 3E7 (PE-labeled on y-axis) and a granulocyte surface marker (HIS48-FITC on the x-axis). The upper panel shows HIS48$^+$ staining in the presence of an isotype matched control antibody, whereas the lower panel demonstrates 3E7 staining (*A*). Absolute number of cells expressing 3E7 and HIS48 (or ED1) were calculated using known numbers of TruCOUNT® fluorescent beads added after staining (mean ± SD; n = 6 per group). The number of 3E7$^+$HIS48$^+$ granulocytes steadily increased during the course of inflammation ($P < 0.01$ by analysis of variance on ranks, n = 6 per group [*B*]). Likewise, a significant rise of 3E7$^+$ED1$^+$ monocytes or macrophages was observed ($P < 0.001$ by analysis of variance on ranks, n = 6 per group [*C*]). FCA = Freund's complete adjuvant.

lymphocytes also contain opioid peptides and might be of importance in truly chronic inflammation.

Enhanced Endogenous Opioid Peptide Production Results in More Endogenous Opioid Analgesia during Inflammation

To examine the functional relevance of opioid peptide–producing immune cells in pain control, we tested antinociceptive effects after a stressful stimulus (CWS). Previously, we extensively characterized this paradigm and showed that, on CWS, opioid peptides are released within the paw, then occupy opioid receptors on sensory neurons and thereby produce analgesia.[2,4,5] In this study we found that intrinsic peripheral analgesia triggered by CWS correlated tightly with the number of 3E7$^+$ CD45$^+$ cells. To substantiate our newly developed triple-color flow cytometry, we showed that not only the number of 3E7$^+$ CD45$^+$ cells but also the END content in the inflamed paw increased in a time-dependent manner. The latter was quantified by radioimmunoassay, similar to previous studies.[6,9,13] It seems that the END content in the inflamed paw mainly reflects the degree of migration of immune cells. At present, it is unknown whether immune cells increase their production of opioid peptides already in the circulation and then migrate to the site of inflammation or whether opioid peptide production in immune cells is primarily induced at the site of the inflammation.

Is pain control determined by the amount of opioid peptide at the site of inflammation or rather by opioid receptor expression? This question is particularly important because future therapeutic strategies in pain inhibition can be directed toward either opioid peptide–containing immune cells or neuronal opioid receptor expression. Earlier studies showed that opioid receptors are transported from the dorsal root ganglion to peripheral terminals of sensory neurons and that receptor transport is up-regulated by inflammation.[1] In the current study we show that recruitment of more opioid peptide–containing immune cells led to more profound analgesia. This view is supported by a prior study in which recruitment of opioid peptide–producing immune cells was blocked by the selectin blocker fucoidin, resulting in substantially reduced analgesia. Importantly, intraplantar injection of the synthetic opioid agonist fentanyl induced an identical degree of analgesia in both control and fucoidin-treated rats, suggesting the presence of similar numbers of receptors in both cases.[2] Therefore, we conclude that at the beginning of an inflammatory process intrinsic opioid analgesia is primarily dependent on the number of opioid-containing leukocytes rather than on the number of receptors.

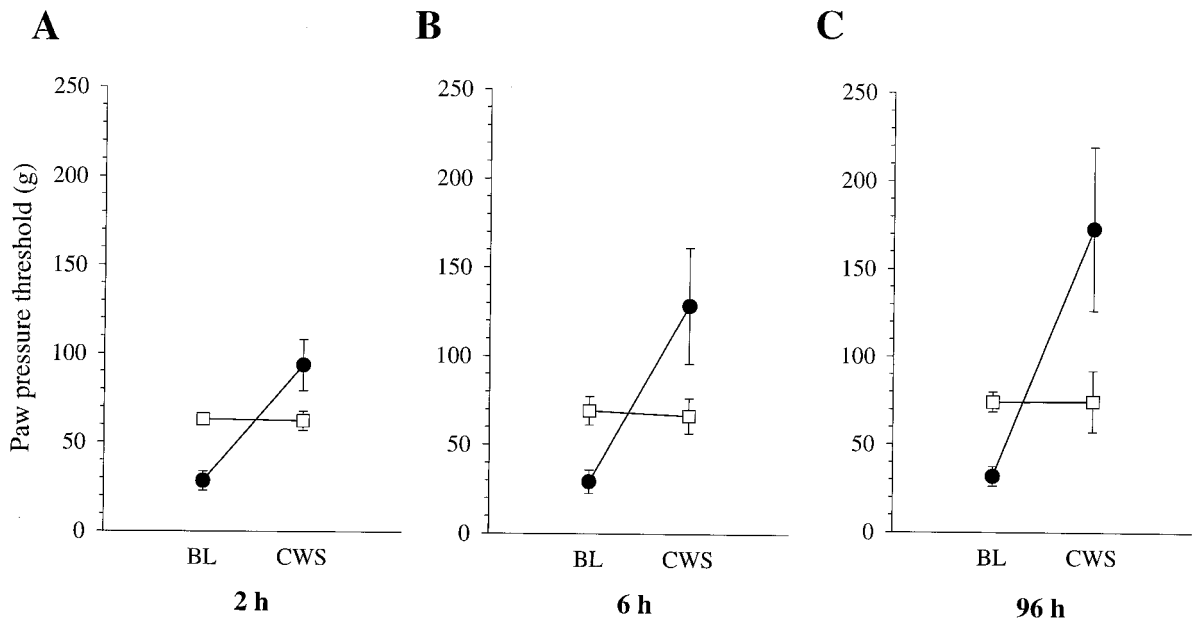

Fig. 6. Endogenous analgesia during inflammation. Paw pressure thresholds are given as raw values (n = 6 per group, mean ± SD). Values in inflamed (filled circle) and noninflamed paw (open square) before (baseline [BL]) and 1 min after cold water swim stress (CWS) are shown. CWS-induced endogenous analgesia increased steadily during the course of inflammation ($P < 0.01$ by analysis of variance).

Role of Different Immune Cells in the Control of Pain in Inflammatory Disease

In the current study we demonstrated that opioid-containing immune cells are HIS48$^+$ granulocytes during the early stages of inflammation, whereas ED1$^+$ monocytes or macrophages predominate later. These results predict that immunosuppression targeting granulocytes or monocytes should lead to a reduction of analgesia. Indeed, immunosuppression by whole-body irradiation[5] or cyclosporine[4] abolishes CWS-induced analgesia in FCA-treated rats. Similarly, immunomodulation can be achieved by selective blockage of cell migration.[2] These three examples demonstrate that both suppression and modulation of the immune system have profound consequences for endogenous pain control. This is particularly important because manipulation of the immune system is a prime target in various diseases including sepsis, rheumatoid arthritis, and chemotherapy for cancer. Immunosuppression with inhibition of the function of macrophages in autoimmune disease or agranulocytosis induced by chemotherapy could have the unanticipated side effect of abolishment of endogenous pain control.

These considerations have clinical consequences: Peripheral opioid analgesia has been conclusively demonstrated in surgical patients and in patients with arthritis.[14] Evidence for endogenous peripheral opioid peptide release under stressful conditions such as surgery was presented in a study showing that injection of naloxone after knee arthroscopy exacerbates pain and increases pain medication consumption.[3,15] Our studies in the rat model suggest that opioid peptide-containing immune cells are recruited to sites of injury and inflammation as part of the innate immune reaction. These opioid peptides apparently mount a parallel defense against the accompanying pain response. Thus, it is conceivable that modulation of this peripheral opioid mechanism (*e.g.,* by gene transfer of opioid peptide DNA) could be used in future therapeutic strategies to inhibit pain. Such strategies would have the advantage of being devoid of central opioid side effects such as sedation, nausea, dependence, or respiratory depression.

In summary, tissue insults result in the immediate recruitment of granulocytes and some monocytes that not only attempt to limit the extent of tissue destruction but also allow endogenous analgesia to occur. During the ongoing inflammation other cells such as monocytes, and probably T cells at later stages, are recruited that ensure ongoing endogenous opioid-mediated analgesia. Endogenous local analgesia after tissue destruction could be a principle of fundamental importance: On the one hand, pain at the site of damage signals the degree of injury and limits unnecessary usage. On the other hand, potent analgesia is mounted simultaneously so that the handicap of the injury can be temporarily ignored in periods of danger.

The authors thank Andreas Thiel, Ph.D., and Andreas Radbruch, Ph.D., Deutsches Rheumaforschungszentrum, Berlin, Germany, for technical advice regarding intracellular staining for flow cytometry.

References

1. Hassan AH, Ableitner A, Stein C, Herz A: Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. Neuroscience 1993; 55:185–95

2. Machelska H, Cabot PJ, Mousa SA, Zhang Q, Stein C: Pain control in inflammation governed by selectins. Nat Med 1998; 4:1425–8

3. Stein C, Hassan AH, Lehrberger K, Giefing J, Yassouridis A: Local analgesic effect of endogenous opioid peptides. Lancet 1993; 342:321–4

4. 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 U S A 1990; 87:5935–9

5. 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

6. Lyons PD, Blalock JE: Pro-opiomelanocortin gene expression and protein processing in rat mononuclear leukocytes. J Neuroimmunol 1997; 78:47–56

7. Saravia F, Ase A, Aloyz R, Kleid MC, Ines M, Vida R, Nahmod VE, Vindrola O: Differential posttranslational processing of proenkephalin in rat bone marrow and spleen mononuclear cells: Evidence for synenkephalin cleavage. Endocrinology 1993; 132:1431–7

8. Kuis W, Villiger PM, Leser HG, Lotz M: Differential processing of proenkephalin-A by human peripheral blood monocytes and T lymphocytes. J Clin Invest 1991; 88:817–24

9. Cabot PJ, Carter L, Gaiddon C, Zhang Q, Schafer M, Loeffler JP, Stein C: Immune cell-derived beta-endorphin: Production, release, and control of inflammatory pain in rats. J Clin Invest 1997; 100:142–8

10. Gramsch C, Meo T, Riethmuller G, Herz A: Binding characteristics of a monoclonal beta-endorphin antibody recognizing the N-terminus of opioid peptides. J Neurochem 1983; 40:1220–6

11. Singer AJ, Clark RA: Cutaneous wound healing. N Engl J Med 1999; 341:738–46

12. Vindrola O, Padros MR, Sterin-Prync A, Ase A, Finkielman S, Nahmod V: Proenkephalin system in human polymorphonuclear cells: production and release of a novel 1.0-kD peptide derived from synenkephalin. J Clin Invest 1990; 86:531–7

13. Sacerdote P, Bianchi M, Manfredi B, Panerai AE: Intracerebroventricular interleukin-1 alpha increases immunocyte beta-endorphin concentrations in the rat: Involvement of corticotropin-releasing hormone, catecholamines, and serotonin. Endocrinology 1994; 135:1346–52

14. Schafer M: Peripheral opioid analgesia: From experimental to clinical studies. Curr Opin Anaesthesiol 1999: 12:603–7

15. Stein C, Comisel K, Haimerl E, Yassouridis A, Lehrberger K, Herz A, Peter K: Analgesic effect of intraarticular morphine after arthroscopic knee surgery. N Engl J Med 1991; 325:1123–6