Preliminary report

Long-term intrathecal morphine and bupivacaine upregulate MOR gene expression in lymphocytes

Gabriele Campana a, Donatella Sarti b, Santi Spampinato a,⁎, William Raffaelli b,c

a Department of Pharmacology, University of Bologna, Italy
b Pain Therapy and Palliative Care Unit, “Infermi” Hospital, Rimini, Italy
c Department of Anaesthesiology, University of Parma, Italy

Abstract

Background: Several studies have shown that opioids may suppress the immune system either by binding to mu-opioid receptors (MOR) expressed in immune cells or by activating these receptors within the central nervous system.

Objective: To assess the level of lymphocyte expression of MOR mRNA in patients with chronic non-cancer pain who were treated with intrathecal morphine or with morphine plus bupivacaine via an intrathecal drug delivery system, and to investigate whether intrathecal morphine and the associated local anesthetic administration influences lymphocyte subpopulations.

Methods: In total, 29 people [10 controls (age range 59–85 years) and 19 patients (age range 47–89 years) with various chronic non-malignant pain conditions] were enrolled in the study. MOR mRNA levels were evaluated in peripheral lymphocytes, and lymphocyte subsets were determined by direct immunofluorescence using flow cytometry.

Results: After 12 months of treatment with intrathecal morphine (1.5–4 mg/day), there was an increase in MOR mRNA levels in lymphocytes of 65% compared with controls and 47% with pretreatment values. Even higher levels (increase of 142% compared with controls and 135% with pretreatment values) were observed in the patients treated with morphine plus bupivacaine (0.2–0.4 mg/day). Elevation of MOR mRNA levels was confirmed in patients after 24 months of treatment. At this time point, the percentage of natural killer cells was significantly decreased.

Conclusion: This preliminary study suggests that opioids must be used with care in patients who are already immunosuppressed by disease or by other, concurrently administered drugs.

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1. Introduction

Morphine is frequently used for control of chronic cancer and non-cancer pain [1]. The action of morphine is elicited by its binding to G protein-linked receptors with seven transmembrane domains, such as mu-opioid receptor (MOR), kappa-opioid receptor and delta-opioid receptor subtypes [2]. Each receptor subtype is expressed in neurons implicated in several nociceptive circuits. At analgesic doses, morphine binds mainly to MOR [3]. In particular, MOR has been shown to mediate morphine analgesia after spinal and supraspinal administration [4].

Recently, there has been increasing use of intrathecal (IT) morphine for pain control in patients who do not respond to traditional methods of drug administration or who cannot tolerate high doses of orally administered opioids because of the onset of systemic side effects [5].

Pharmacological therapy via intrathecal drug delivery systems (IDDS) has been successfully used for the treatment of pain resulting from cancer or non-cancer conditions [6]. The use of IT bupivacaine for the treatment of non-cancer chronic pain is also widely acknowledged because it has no bone marrow toxicity and has positive synergy with opioids, acting on different nociceptive circuits [5]. Moreover, the association of bupivacaine with morphine in IT therapy allows lower doses of morphine to be used, thereby reducing the incidence of opioid-related side effects [7,8].

Several studies on humans and animals have shown that opioid administration has effects on the immune system, which can be adverse; in particular, morphine has an antiproliferative effect on blood lymphocytes [9]. Raffaelli and colleagues studied the opioid system and the interactions between chronic use of morphine/heroin and endogenous release of opioid peptides. They showed that opioids reduced the endogenous cytotoxic activity of natural killer (NK) cells while increasing the development of lymphokine activated killer (LAK) cell cytotoxicity after short-term and long-term administration. These effects on the immune system were dependent on the administration route, with IT administration being a stronger inhibitor of LAK cytotoxicity [10].

⁎ Corresponding author. Dept. of Pharmacology, University of Bologna, Imerini 48, 40126 Bologna, Italy. Tel.: +39 051 2091451; fax: +39 051 248862.
E-mail address: santi.spampinato@unibo.it (S. Spampinato).
Moreover, Raffaei et al. demonstrated that IT morphine administration leads to interactions with neurohormones of clinical significance [11–14].

It has been shown that peripheral lymphocytes express MOR mRNA and MOR receptors [15], and that the effect of peripherally administered morphine on the immune system is mediated by a direct action on immune cells [16]. Interestingly, the cytolytic activity of NK cells and the proliferative responses of lymphocytes to mitogens appear to be modulated predominantly through activation of opioid receptors within the central nervous system [17].

The aim of this study was to assess the levels of expression of peripheral lymphocyte MOR mRNA in patients with chronic non-cancer pain who were received long-term (>24 months) treatment with IT morphine or with morphine plus bupivacaine via an IDDS system, and to associate these expression levels with the lymphocyte subset profile.

2. Materials and methods

2.1. Ethics

The procedure was approved by the hospital ethics committee and conducted according to the principles on human clinical studies of the Helsinki Declaration. All the patients, after a thorough explanation of the procedure and the study, gave written informed consent.

2.2. Study population

This study population comprised 29 people: 10 controls (age range 59–85 years) and 19 patients (age range 47–89 years) with various chronic non-malignant pain conditions. The 19 patients were implanted with IDDS systems for IT opioid delivery (Table 1).

The IDDS patients were enrolled at the Pain Therapy and Palliative care Unit of the Infermi Hospital (Rimini, Italy) and were treated with IT infusion of morphine hydrochloride (1.25–3.75 mg/day; n=9) or of morphine hydrochloride (as before) combined with bupivacaine (0.2–0.4 mg/day) (n=10). The control group were 10 healthy volunteers who were recruited from the local blood donor center.

The inclusion criteria were: age older than 18 years, and presence of chronic non-cancer pain (Table 2). Patients had to be on treatment with Dnase I (Intrigren, Milan, Italy), and contaminating DNA was removed by treatment with DNase 1 (Intrigren). Samples of messenger (m)RNAs (1 mg) were reverse transcribed and amplified by a semiquantitative reverse transcriptase (RT)-PCR method (One Step RT-PCR beads; GE Healthcare, Milan, Italy) with ribosomal protein L19 as internal standard. For MOR, a 440 bp fragment was amplified (forward and reverse primers, corresponding to bases 921–950 and 1332–1361 of the human MOR sequence; GenBank accession no. L29301). For L19, the amplicon was 168 bp long (forward and reverse primer corresponding to bases 97–115 and 247–265 of the L19 human sequence; GenBank accession no. NM_000981). Each sample was run in triplicate, and the mean value of the three results was calculated.

2.4. RNA extraction and reverse transcriptase PCR

Total RNA from lymphocytes was extracted using Trizol (Invitrogen, Milan, Italy), and contaminating DNA was removed by treatment with DNase I (Invitrogen). Samples of messenger (m)RNAs (1 mg) were reverse transcribed and amplified by a semiquantitative reverse transcriptase (RT)-PCR method (One Step RT-PCR beads; GE Healthcare, Milan, Italy) with ribosomal protein L19 as internal standard. For MOR, a 440 bp fragment was amplified (forward and reverse primers, corresponding to bases 921–950 and 1332–1361 of the human MOR sequence; GenBank accession no. L29301). For L19, the amplicon was 168 bp long (forward and reverse primer corresponding to bases 97–115 and 247–265 of the L19 human sequence; GenBank accession no. NM_000981). Each sample was run in triplicate, and the mean value of the three results was calculated.

2.5. Assessment of lymphocyte subpopulations by flow cytometry

Blood samples were collected within 1 month of the last evaluation of lymphocyte MOR mRNA levels from patients chronically treated with IT morphine or morphine plus bupivacaine. Peripheral blood mononuclear cells (PBMCs) were isolated as previously reported, using a commercial preparation (Lymphoprep). Lymphocyte subsets were analyzed by flow cytometry. The surface phenotype of the separated PBMCs was analyzed by double immunofluorescence using two subsets of antibodies (Cyto-Stat Tetrachrome, Beckman Coulter, Milan, Italy): phycoerythrin (PE)-conjugated monoclonal antibodies to CD4 and estrogen–dendrimer conjugate (EDC)–conjugated monoclonal antibodies to CD8 lymphocytes and CD19 (B cells). NK cells were analyzed using a CD56 PE-conjugated monoclonal antibody. The total lymphocyte number was measured by PE cyanin (PC)5-conjugated monoclonal antibodies to CD3. Stained lymphocytes were analyzed by flow cytometry (Cytomics™ FC 500 Flow Cytometry System, Beckman Coulter). Results are presented as the percentage of total lymphocytes present in each sample.

2.6. Statistical analysis

The Friedman test, followed by the Dunn test, was used to analyze changes between pretreatment and post-treatment levels within each group. The Kruskal–Wallis test was performed to compare ≥3 unpaired groups. Data are presented as mean±S.E.M. P<0.05 was deemed significant.

3. Results

Constant IT administration of morphine or morphine plus bupivacaine for 12 months produced an increase in MOR mRNA levels. Morphine alone (1.5–4 mg/day) increased MOR mRNA expression by 65% versus controls and by 47% versus pretreatment values. Higher mRNA levels were observed in the morphine (1.25–3.75 mg/day) plus bupivacaine (0.2–0.4 mg/day) patient group, with increases of 142% versus controls and 135% versus pretreatment values (Fig 1). Lymphocyte MOR

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of the sample.</th>
<th>N(%)</th>
<th>Average age (Range) Males Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDDS group</td>
<td>19 (65.5%)</td>
<td>47–89</td>
</tr>
<tr>
<td>Controls</td>
<td>10 (34.5%)</td>
<td>59–85</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>74.04±1</td>
</tr>
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</table>

### Table 2

<table>
<thead>
<tr>
<th>Pain categories</th>
<th>Gender (M/F)</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoporosis-related back pain</td>
<td>0/3</td>
<td>80±1</td>
</tr>
<tr>
<td>Failed back surgery syndrome</td>
<td>0/3</td>
<td>70±5</td>
</tr>
<tr>
<td>Brachial neuritis</td>
<td>0/3</td>
<td>66±10</td>
</tr>
<tr>
<td>Atherosclerosis of native arteries</td>
<td>3/1</td>
<td>79±3</td>
</tr>
<tr>
<td>Others</td>
<td>3/3</td>
<td>80±3</td>
</tr>
</tbody>
</table>

*Data are presented as numbers (n) or mean values±S.E.M.*

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mRNA levels measured in patients before IDDS implantation were similar to controls (Fig. 1). Elevation of MOR mRNA expression was confirmed in a second set of experiments performed after a further 12 months of treatment (Fig. 1).

Analysis of lymphocyte subsets was carried out in patients on long-term IT treatment with morphine or morphine plus bupivacaine within 1 month of the last lymphocyte MOR mRNA evaluation. As shown in Table 3, the percentage of NK cells was modestly decreased in treated patients compared with controls. Conversely, total number of lymphocytes and the relative percentage of CD8+, CD4+ and B cells was found to be similar in controls and IT-treated patients.

4. Discussion

Previously, we have showed that IT opioids suppress the immune system [18]. This observation was also supported by the study of Yokota et al. [19], who reported that IT morphine reduces NK cell activity. Opioids may suppress the immune system either by binding to MOR receptors expressed in immune cells (T lymphocytes, B lymphocytes, macrophages) or by activating MOR within the central nervous system [20].

Shavit et al. [21] demonstrated that systemic administration of morphine, but not of N-methylmorphine (a form of morphine that does not penetrate the blood–brain barrier), produces suppression of splenic NK cell activity in the rat. Nelson et al. [22] found that centrally administered opioids activate the hypothalamic–pituitary–adrenal (HPA) axis, which elicits the production of immunosuppressive glucocorticoids [23]. Thus, it could be hypothesized that IT morphine may diffuse via cerebrospinal fluid within the central nervous system, and may cause prolonged activation of opioid receptors that are chronically exposed to low morphine concentrations, which could have consequences for modulation of the immune system.

Another hypothesis suggests activation of the sympathetic nervous system by morphine that elicits the release of catecholamines into primary and secondary lymphoid organs [24], which have been demonstrated to modulate lymphocyte, NK cell and macrophage functions (reviewed by Hall et al. [25]). However, a direct peripheral action of IT morphine on lymphocytes cannot be ruled out. In fact, opioids that are commonly administered spinally may pass into systemic circulation with subsequent redistribution to brainstem opioid receptors [26].

Interestingly, we found that the combination of morphine plus bupivacaine, produces a more marked upregulation of MOR transcripts in peripheral lymphocytes. The mechanisms of this additive effect are not known. However, it has been proposed that local anesthetics, including bupivacaine, enhance the transcription of the pro-opiomelanocortin gene in anterior pituitary cells [27]. As mentioned previously, this effect is also elicited by opioids. Therefore, both morphine and bupivacaine may exert an additional effect on the HPA axis, which could influence the immune system [28].

We found that elevated lymphocyte MOR mRNA levels were associated with a lower number of NK cells. This observation is in accordance with previous reports showing that opioids suppress NK cell number [10] and activity [19–22]. However, the combination of morphine plus bupivacaine did not cause any further reduction of NK cells number compared with patients treated with morphine alone. We plan to better address this point in future investigations aimed at evaluating NK cell functioning in both groups of patients.

The functional significance and the consequence of MOR mRNA upregulation on immune system responses in the lymphocytes of patients given long-term treatment with IT morphine plus bupivacaine are not clearly known. Assuming that a correlation exists between transcription and translation, considerably higher amounts of MOR receptors should be expected in the lymphocytes of these patients and consequently, activation of these receptors by exogenous and endogenous opioids may induce the production of cytokines [29] and exert a number of immunomodulatory effects influencing the development and function of T lymphocytes in the thymus [30]. Several studies have shown that cytokines may mediate morphine-induced upregulation of MOR in immune cells [31]. Moreover, immune cells containing and releasing opioid peptides can accumulate in chronically inflamed tissue and act as cytokines, and might activate an autocrine/paracrine immune response binding to MOR expressed on lymphocytes [32].

Further studies are necessary to confirm our preliminary observations of the additive effect of bupivacaine on morphine-induced lymphocyte MOR mRNA upregulation and any possible clinical consequences. Although it remains unclear whether this finding is clinically relevant in patients chronically treated with opioids, it has been suggested previously that such agents should be used with care in patients already immunosuppressed by disease or by other concurrently administered drugs [33].

Acknowledgments

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References


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