Activation of Mu Opioid Receptors Inhibits Microglial Cell Chemotaxis

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ABSTRACT

Opiates modulate many macrophage functions. Microglia, the resident macrophages of the brain, migrate to sites of inflammation within the CNS. Using primer sets designed to span the entire open reading frame of the human brain mu opioid receptor (MOR), we found that microglial cells constitutively expressed MOR mRNA. The cDNA sequences of the MOR open reading frame in microglia were identical to those of human brain tissue. Using enriched human fetal microglial cell cultures, we found that morphine potently inhibited the directed migration (chemotaxis) of microglial cells toward C5a in a dose-dependent manner with an IC50 value of 1 fM morphine. We also found that DAMGO, a selective MOR ligand, dose-dependently suppressed microglial cell chemotaxis with an IC50 value of 1 nM, which was significantly attenuated by 10 nM β-funaltrexamine. Taken together, these findings suggest that activation of constitutively expressed MOR inhibits microglial cell chemotaxis and support the notion of an anti-inflammatory role of MOR within the brain.

Microglia, the resident macrophages of the brain, are derived from monocytes arising from the bone marrow during fetal development (Perry and Gordon, 1988). These cells are believed to be functionally equivalent to monocytes or tissue macrophages of the somatic immune system (Guilian, 1987; Gehrmann et al., 1995). It has long been recognized that microglia migrate to, differentiate and proliferate at sites of brain injury and inflammation (del Río-Hortega, 1932). Recent in vitro studies have indicated that activated microglia can migrate toward the complement component C5a (Yao et al., 1990). Although directed migration (i.e., chemotaxis) of microglia may play a beneficial role in the elimination of damaged neurons or invading microorganisms (Brockhaus et al., 1996), activated microglia can also be injurious to neighboring neurons (Chao et al., 1992a). Thus down-regulation of the chemotactic ability of activated microglia could prevent potential neuronal damage in areas of brain injury.

Opiates have been demonstrated to have a broad range of immunomodulatory activities, including a variety of effects on macrophages (Eisenstein et al., 1995). In the CNS, MOR have been shown to be associated with neuronal plasma membranes of dendrites and cell bodies visualized by confocal microscopy (Elde et al., 1995). Anatomic mapping of the MOR in rat brain has been extensively investigated (George et al., 1994; Delfs et al., 1994; Mansour et al., 1995). Cloning of the MOR was reported for rat brain (Chen et al., 1993; Fukuda et al., 1993; Thompson et al., 1993; Wang et al., 1993) and the cloning of human brain MOR followed soon (Wang et al., 1994). Recently, expression of MOR mRNA has been observed in human immune cells, including monocytes (Chuang et al., 1995). Expression of MOR mRNA in human microglia, however, has not been reported.

Activation of opioid receptors within the CNS may result in alterations of the functional activities of immunocytes within the periphery. For example, morphine has been shown to induce suppression of natural killer cell activity via a primary effect in the CNS (Shavit et al., 1986), and the periaqueductal gray matter has been identified as the site of morphine-induced immunosuppression (Weber and Pert, 1989). Although the mechanism of morphine-induced immunomodulation has been associated with indirect effects operating via the hypothalamo-pituitary-adrenal axis, direct effects on immunocytes also have been described. Morphine, at concentrations in the micromolar range, inhibits human monocyte (Perez-Castrillon et al., 1992; Stefano et al., 1993) and granulocyte (Makman et al., 1995) chemotactic activity. In i.v. drug abusers, a defect in monocyte chemotaxis has been reported (Poli et al., 1985). Little is known, however, about the role of MOR in the regulation of microglial cell chemotactic activity. In the present study, we investigated the

ABBREVIATIONS: β-FNA, β-funaltrexamine; hpf, high power fields; MOR, mu opioid receptor(s); ORF, open reading frame; RT-PCR, reverse transcriptase-polymerase chain reaction.
expression of MOR in human fetal microglia and the effect of the activation of MOR in microglial cell chemotaxis.

Materials and Methods

Reagents. The following drugs and chemicals were kindly provided by or obtained from the sources indicated: morphine sulfate (Hennepin County Medical Center pharmacy), MOR antagonist β-FNA (Dr. P. S. Portoghese, University of Minnesota, Minneapolis, MN), MOR selective ligand DAMGO (Research Biochemicals International, Natick, MA), anti-MOR antibodies that recognize protein C-terminal intracellular sequences and synthetic peptides (Dr. R. Elde, University of Minnesota, Minneapolis, MN), anti-CD68 antibodies (a marker of human macrophages) and antiglial fibrillary acid protein antibodies (an astrocyte marker) (Dako, Carpinteria, CA), the complement component C5a, trypsin, penicillin, streptomycin and Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO), heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), Oligo (dT)12-18 primer (Clontech, Palo Alto, CA), Taq DNA polymerase, spermidine, avian myeloblastosis virus reverse transcriptase (RT), and PolyATtract mRNA Isolation System III (Promega, Madison, WI), deoxynucleotide triphosphate mixture containing dATP, dTTP, dGTP and dCTP (Boehringer Mannheim, Indianapolis, IN) and RNase inhibitor (Pharmacia, Piscataway, NJ).

Microglial cell cultures. Human fetal brain tissues were obtained from aborted fetuses under a protocol approved by the Human Subjects Research Committee at our institution. The microglial cell cultures were prepared using a previously described technique (Chao et al., 1994). Briefly, brain tissues from 16- to 22-week-old abortuses were dissociated after a 30-min trypsinization (0.25%) and were plated in 75 cm² Falcon culture flasks in medium containing 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). On day 14 of culture, plates were gently shaken, and harvested cells were plated onto wells of 24-well culture plates for 60 min before washing. Purified microglia were composed of a cell population >98% of which stained with CD68 antibodies and <2% of which stained with antibodies specific to glial fibrillary acid protein. After treatment with either opiates or C5a, microglial cell cultures were >99% viable as assessed by trypan blue exclusion assay.

Expression of MOR mRNA and sequencing analysis. To determine whether microglial cells constitutively express MOR, we used a RT-PCR technique to detect expression of the MOR gene in untreated microglial cell cultures. Initially, one pair of oligonucleotide primers, M1+ and M1− (fig. 1), was selected on the basis of the sequences obtained from adult human caudate nucleus from Gen Bank accession number L29301 (Mestek et al., 1995). These primers amplify a 441-bp cDNA fragment corresponding to the region that encompasses the third extracellular loop of the human brain MOR chain (Chuang et al., 1995). This domain of the human MOR cDNA was chosen because of recent findings that characterized the third extracellular loop of the brain MOR as important for selectively binding MOR agonists such as morphine (Xue et al., 1995). For sequence analysis of the entire MOR ORF, three additional sets of oligonucleotide primers M2+ and M2− were used (fig. 1). These primers were designed to amplify a 158-bp cDNA fragment corresponding to the 3′ untranslated region of the human brain MOR cDNA. For sequencing of the entire MOR ORF, three additional sets of oligonucleotide primers M3+ and M3−were used (fig. 1).

Fig. 1. cDNA sequence of MOR from human brain (Gen Bank accession number L29301), showing the location of primers (M1–M4) used for PCR amplification of human microglial cell cDNA. Primer sequences are designated as sense (+) or antisense (−). Start and stop codons are expressed in lower-case letters.
primers (M2+/M2−, M3+/M3− and M4+/M4−) were selected (fig. 1).

Total RNA was isolated as previously described (Chao et al., 1996). Reverse transcription of 1 μg total RNA from resting microglial cell cultures was performed using the oligo (dT)$_{12-18}$ primer and avian myeloblastosis virus RT. Amplification of the MOR cDNA was performed in a final reaction volume of 50 μl consisting of 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100), 3 μl of 25 mM MgCl$_2$, 1 μl of deoxynucleotide triphosphate mixture (10 mM), 1 μl each of sense and antisense primer (from a 25 μM stock), 2 μl of cDNA and 2 units of Taq DNA polymerase. The mixture was subjected to 35 amplification cycles with each cycle as follows: 94°C for 45 sec, 65°C for 45 sec and 72°C for 90 sec. Originally, the amplified cDNA fragment (M1) was analyzed and visualized by 1.5% agarose gel electrophoresis, cloned into Bluescript plasmid and sequenced using a Sequenase version 2.0 sequencing kit (USB, Cleveland, OH). After sequence analysis confirmed that the PCR product of the M1 segment was indeed MOR cDNA, the entire ORF was amplified in segments (M2–M4) and sequenced. In order to facilitate sequencing of the ORF, total RNA was further purified to poly A+ mRNA using a Poly A Ttract mRNA Isolation kit (Promega, Madison, WI). cDNA was synthesized as described above, using approximately 40 ng of mRNA per reaction. The cDNA was amplified using the remaining primer pairs, and PCR products were sequenced directly by an automated fluorescent method (Applied Biosystems, Inc., DNA Sequencing Facility, Iowa State University, Ames, IA).

**Immunocytochemical staining.** To verify the constitutive expression of the MOR protein at a cellular level, immunocytochemical staining using an ABC kit (Vector Laboratories, Inc., Burlingame, CA) was performed as previously described (Elde et al., 1995). In brief, microglial cell cultures were fixed with 4% paraformaldehyde for 20 min before treatment for 10 min with 3% H$_2$O$_2$, to eliminate endogenous cellular hydrogen peroxidase. Microglial cell cultures were then treated with 10% goat serum for 1 h, followed by the primary anti-MOR antibodies (1:6000 dilution) in the absence or presence of 100 μl of synthetic peptides (10$^{-6}$ M) overnight at 4°C. After washing, cell cultures were exposed to goat anti-rabbit IgG for 60 min at room temperature, followed by the ABC complex for enhanced binding and DAB for color development. Control cultures were incubated with anti-MOR antibodies without the goat anti-rabbit IgG.

**Effects of MOR activation on microglial cell chemotaxis.** To establish a chemotaxis assay, the complement component C5a was selected as a chemottractant, as previously reported (Yao et al., 1990). The dose–response curve of C5a ranging from 10$^{-12}$ to 10$^{-6}$ M was selected to assess the chemotactic activity of human microglial cells. Once the chemotactic activity of microglial cells was established, we evaluated the dose–response curves of morphine (ranging from 10$^{-18}$ to 10$^{-6}$ M) and DAMGO (between 10$^{-12}$ and 10$^{-6}$ M). Direct chemotactic effects of morphine and DAMGO also were explored when these MOR ligands were used in the lower chamber as a chemottractant. To investigate the specificity of MOR activation, microglial cells were pretreated with the MOR-selective antagonist β-FNA (at an equal to 100-fold higher concentrations) for 30 min before the addition of DAMGO or morphine.

**Chemotaxis assay.** A 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD) was used to measure the migration of microglia toward assay medium (random migration) or the chemottractant C5a. Chemotaxis was measured using a previously described technique (Yao et al., 1990) with minor modifications. The upper and lower compartments of the chamber were separated by a 5-μm polycarbonate filter. Microglial cells were added to the upper chamber (2 × 10$^5$ cells/well), and after a 3-h incubation period, the nonmigrating cells were gently scraped from the upper surface of the filter. In a preliminary experiment, it was found that microglial cell chemotactic activity reached a maximum after a 3-h incubation in the chemotaxis chamber. Thus all subsequent experiments were performed using 3-h incubation as an end point to assess microglial cell chemotaxis. Cells on the lower surface were fixed in methanol and stained with Diff-Quik (Baxter, McGaw Park, IL). The number of cells migrating to the underside of the filter were counted microscopically by an investigator blind to the experimental conditions. Five hpfwell of triplicate wells were examined at 400×, and cell numbers were averaged.

**Statistical analysis.** Where appropriate, data were expressed as mean ± S.E.M. To compare means of two groups, Student’s t test was used.

**Results**

**Constitutive expression of the MOR in microglia.** Using RT-PCR to detect the expression of MOR mRNA in microglial cells, we produced bands of the predicted size with all four primer sets (fig. 2). To characterize further the coding region of microglial MOR, each of these PCR products was purified and sequenced. DNA sequence data obtained from these four overlapping segments proved to be identical to those of previously identified human brain MOR cDNA (fig. 1).

**Immunohistochemical staining of the MOR protein on microglia.** To investigate whether MOR protein is present in microglial cells, we selected an immunohistochemical staining technique that employs antibodies specific for the intracellular segment of the rat MOR, which is identical to corresponding human sequences (Elde et al., 1995). We found that >50% of microglial cells stained positively for the presence of MOR protein (fig. 3B). Nonspecific staining was assessed in microglial cell cultures in which the second antibody was omitted (fig. 3A). Neutralization of MOR antibody staining with synthetic peptides, which were used to generate MOR antibodies, markedly attenuated the positive staining (fig. 3C), a result that supports the specificity of MOR staining.

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**Fig. 2.** Constitutive expression of MOR mRNA. Total RNA was harvested from microglial cell cultures, and RT-PCR was performed to evaluate constitutive expression of MOR mRNA. RT-PCR products (M1–M4) were loaded onto a 2% agarose gel followed by electrophoresis. Right lane: 100-bp molecular weight marker. The second lane (M1 no RT) represents a negative control for DNA contamination (RT-PCR in the absence of RT).
Chemotactic activity. To determine the functional activity of MOR on microglial cells, we investigated the migratory activity of these cells. Random migration of microglial cells toward culture medium was minimal (28 ± 4 cells, n = 5). However, microglial cells migrated in a directed manner toward the chemotractant C5a. This effect was dose-dependent with maximal stimulation by 10^{-8} M C5a (fig. 4). Thus microglial cell migratory activity toward 10^{-8} M C5a was selected in the following experiments to assess the effects of activation of MOR.

Treatment of microglial cells with morphine (ranging from 10^{-18} to 10^{-6} M) alone did not stimulate chemotactic activity when morphine was added to the lower compartment of the chemotaxis chambers. Treatment of microglial cells with morphine for 30 min before loading cells into the chemotaxis chamber resulted in a marked suppression of chemotactic activity, an opiate-mediated effect that was dose-dependent with an IC_{50} value of 1 fM morphine (fig. 5). Morphine, however, did not affect random migration of the microglial cells (fig. 5). To assess whether the MOR is associated with morphine-induced suppression of microglial cell chemotaxis, we used the selective ligand DAMGO. Treatment of microglial cells for 30 min induced a dose-dependent inhibition of microglial cell chemotaxis with an IC_{50} value of 1 nM DAMGO (fig. 6). Pretreatment of microglial cells for 30 min with β-FNA (10^{-8} M) markedly attenuated (P < .01) the suppressive effects of 10^{-8} M DAMGO by 52% and 10^{-10} M morphine by 77% (fig. 7), a result that suggests the involvement of the MOR.

Discussion

In the present study, we found that human fetal microglial cells constitutively express a MOR identical to the previously reported brain MOR cDNA sequence (Wang et al., 1994; Mestek et al., 1995). Anti-MOR antibodies stained approximately 50% of microglial cells, which suggests the presence of the MOR protein at the plasma membrane level of a majority of these brain macrophages. Activation of the MOR by either morphine or DAMGO profoundly suppressed microglial cell migratory activity toward the chemotractant C5a with an IC_{50} value of 1 fM and 1 nM, respectively. Because the specific MOR antagonist β-FNA attenuated the morphine-induced suppression of microglial cell chemotaxis, we used the selective ligand DAMGO. Treatment of microglial cells for 30 min induced a dose-dependent inhibition of microglial cell chemotaxis with an IC_{50} value of 1 nM DAMGO (fig. 6). Pretreatment of microglial cells for 30 min with β-FNA (10^{-8} M) markedly attenuated (P < .01) the suppressive effects of 10^{-8} M DAMGO by 52% and 10^{-10} M morphine by 77% (fig. 7), a result that suggests the involvement of the MOR.

Fig. 3. Immunocytochemical staining of MOR on microglia. Microglial cells were incubated with anti-MOR antibodies (1:6000) overnight. The control cultures (panel A) were omitted for the goat anti-rabbit IgG. The goat anti-rabbit IgG were added to the positive staining group (panel B) and the neutralizing group (panel C), i.e., anti-MOR antibodies plus 100 µl of synthetic peptides (10^{-4} M) for an additional 30 min. Color development followed. Photomicrographs are representative of findings using four separate brain preparations. (×400)

Fig. 4. Dose-response relationship of C5a on microglial cell chemotaxis. Microglial cells were loaded into chemotaxis chambers that contained either medium to assess random migration (control) or indicated concentrations of C5a. Microglial cell migration was terminated after a 3-h incubation. Data are expressed as microglia/5 hpf. Values are mean ± S.E.M. of duplicates and are representative of three separate experiments.
and DAMGO-induced inhibition of microglial cell chemotaxis, these findings suggest that activation of MOR down-regulates the chemotactic responsiveness of microglia.

Early in this century, histopathological evidence suggested that microglial cells migrate to sites of injury and inflammation within the brain (del Rio-Hortega, 1932). Thus modulation of microglial cell chemotaxis could play an anti-inflammatory role in neurodegenerative diseases in which activated microglia have been implicated (Chao et al., 1995a). Previously, it has been reported that activation of delta opioid receptors in vitro (Ruff et al., 1995; Milligan et al., 1995) or injection of ligands selective for delta opioid receptors into cerebral ventricles in vivo (van Epps and Saland, 1984; Saland et al., 1988) was associated with up-regulation of rat peritoneal macrophage or monocyte chemotactic activity, results that suggest a proinflammatory role for endogenous opioid peptides of this class. In contrast, we demonstrated in the present study that activation of the MOR by either morphine or DAMGO suppressed microglial cell chemotaxis. Further studies seem warranted to assess the potential biological consequences of MOR activation in protection of neurons in diseases that involve reactive microglial cells, such as acquired immunodeficiency syndrome dementia (Yoshioka et al., 1995) and Alzheimer’s disease (McGee and McGee, 1995).

The finding that DAMGO exerted its immunosuppressive effect at a nanomolar concentration is consistent with the pharmacological (Makman, 1994) and neurobiological (Schoffelmeer et al., 1992) reported $K_d$ value for the MOR ligands (Chen et al., 1993; Makman, 1994) and supports a regulatory role of the classical MOR in microglial cell chemotaxis. Recently, Dobrenis et al. (1995) have reported a $\mu$ subtype of the MOR on cat microglia with a $K_d$ value of 14 nM using a $^3$H-morphine binding assay. All $\mu$ subtypes have a pharmacological $K_d$ value in the nanomolar range for morphine (Makman, 1994). This high-affinity binding site is consistent with dose-response studies using certain biological assays. For example, morphine has been shown to stimulate human microglial cell phagocytosis of Mycobacterium tuberculosis at an opiate concentration of 10 nM (Peterson et al., 1995). In studies of human peripheral blood mononuclear cell cultures, morphine dose-dependently enhanced mitogen-stimulated release of transforming growth factor-$\beta$ (Chao et al., 1992b) or inhibited tumor necrosis factor-$\alpha$ (Chao et al., 1993) and respiratory burst activity (Peterson et al., 1989) with a maximal potentiating effect at concentrations between 10 nM and 1 $\mu$M. In the present study, morphine at concentrations between 1 nM and 1 $\mu$M also inhibited microglial cell chemotaxis, which suggests that morphine could mediate an immunomodulatory effect via the classical MOR. Unlike the $\mu$ selective agonist DAMGO, however, the suppressive effect of morphine was sustained at extraordinarily low concentrations, i.e., in the femtomolar range.

Morphine appears to mediate some of its biological effects in a biphasic mode: opposite effects are observed at low and high concentrations.
high concentrations. For example, we have found that the immunomodulatory effects of morphine in microglial cell cultures are often bell-shaped with a maximal effect observed in the picomolar range, whereas at higher concentrations (between nM and μM), morphine has the opposite or little effect (Chao et al., 1994; Peterson et al., 1994; Chao et al., 1995b; Peterson et al., 1995). This bell-shaped dose-response curve of morphine has also been observed in vitro studies using human peripheral blood mononuclear cell cultures (Peterson et al., 1990; Peterson et al., 1991). It is possible that, depending on the immune cell, the type of cellular function and the stimuli used, morphine exerts its biological effects through either a classical (high-affinity) or an unusual (ultra-high-affinity) receptor.

The finding in the present study that morphine exerts a potent anti-inflammatory effect at such low concentrations is evidently not explained by what is known about the classical MOR or the μ3 subtype receptor (Makman, 1994), both of which appear to require manomolar concentrations of morphine to see their biological activities. The findings in this study could be explained by a multiplicity of morphine recognition sites on a single MOR, by multiple-affinity states of the receptor or by different populations of MOR. However, there is currently no evidence to support any of these interpretations. Alternatively, it is possible that a separate receptor distinct from the classical MOR exists for morphine on microglia. We have observed in other systems that morphine at pM concentrations potentiated human (Peterson et al., 1994) or murine (Chao et al., 1994) microglial cell tumor necrosis factor-α release upon stimulation with the bacterial cell wall glycoprotein lipopolysaccharide, a finding consistent with an ultra-high-affinity receptor site on these cells for morphine. Finally, the findings in this study beg the question of whether morphine could play a physiological role in the CNS, because endogenous morphine has been reported to be present in brain tissue at concentrations in the femtomolar to picomolar range (Horak et al., 1993).

In summary, the findings in this in vitro study suggest a potent anti-inflammatory role for morphine in the brain. The appearance of activated microglial cells has been a hallmark of several neurodegenerative diseases, such as Alzheimer’s disease (McGeer and McGeer, 1995) and acquired immunodeficiency syndrome dementia (Yoshioka et al., 1995). Because of the extremely potent inhibitory effect of morphine on microglial cell chemotaxis, it is possible that opiates of the mu class could be therapeutic in diseases where migration of microglial cells is neuropathogenic. This hypothesis could be explored in animal models of inflammatory CNS diseases.

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References
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