Morphine Enhances Macrophage Apoptosis

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Morphine Enhances Macrophage Apoptosis1,2

Pravin C. Singhal,3 Puneet Sharma, Aditi A. Kapasi, Krishna Reddy, Nicholas Franki, and Nora Gibbons

Laboratory data indicate that morphine decreases the number of peritoneal and alveolar macrophages (Mφ) and compromises their phagocytic capability for immune complexes and bacteria. We hypothesize that morphine decreases the number of, as well as compromises the phagocytic capability of, Mφ by programming their death. We studied the effect of morphine on Mφ apoptosis in vivo as well as in vitro. Peritoneal Mφ harvested from morphine-treated rats showed DNA fragmentation. Morphine enhanced murine Mφ (J 774.16) apoptosis in a dose-dependent manner. Human monocytes treated with morphine showed a classic ladder pattern in gel electrophoretic and end-labeling studies. Morphine promoted nitric oxide (NO) production both under basal and LPS-activated states. Nω-nitro-L-arginine methyl ester (l-NAME) and Nω-monomethyl-l-arginine monoacetate (l-NMMA), inhibitors of NO synthase, attenuated the morphine-induced generation of NO by Mφ. Morphine also enhanced Mφ mRNA expression of inducible NO synthase (iNOS). Since morphine-induced Mφ apoptosis was inhibited by L-NAME and L-NMMA, it appears that morphine-induced Mφ apoptosis may be mediated through the generation of NO. Morphine promoted the synthesis of Bax and p53 proteins by Mφ. Moreover, IL-converting enzyme (ICE)-1 inhibitor attenuated morphine-induced Mφ apoptosis. These studies suggest that morphine activates the induction phase of the apoptotic pathway through accumulation of p53. The effector phase of morphine-induced apoptosis appears to proceed through the accumulation of Bax and activation of ICE-1. The present study provides a basis for a hypothesis that morphine may be directly compromising immune function by promoting Mφ apoptosis in patients with opiate addiction. The Journal of Immunology, 1998, 160: 1886–1893.

Clinical evidence indicates that heroin addicts are prone to infections (1–4). Besides the sharing of unsterilized, contaminated needles, the occurrence of infections in these patients has been attributed to the immune modulatory effect of morphine. Morphine inhibits T cell proliferation (5) and suppresses concanavalin A-stimulated IFN-γ production from these cells (6). Opiates suppress primary and secondary Ab response at the cellular level and in the whole animal (7). Polymorphonuclear cells and monocytes from patients subjected to morphine treatment show decreased phagocytic and bacterial killing properties, as well as modulation of the generation of reactive oxygen species (8, 9).

The mononuclear phagocyte system plays an important role in the host defense against microorganisms (10, 11). Previously, we and other investigators demonstrated that morphine attenuated macrophage (Mφ)1 phagocytosis of microbial organisms and immune complexes (12–14). Morphine has been reported to decrease the number of murine peritoneal and rabbit alveolar Mφ (12). However, the mechanism of morphine-induced decreased Mφ was not examined in these studies (12). We hypothesize that morphine may be promoting Mφ apoptosis. This will provide an explanation for morphine-induced decreased peritoneal and alveolar Mφ and decreased phagocytosis of immune complexes by Mφ (in vitro studies).

In the present study, we evaluated the effect of morphine on apoptosis of Mφ. We also evaluated the effect of morphine on the apoptotic pathway of Mφ. Recently, Meßmer et al. reported that endogenously generated or exogenously supplied nitric oxide (NO) promoted apoptosis in the mouse Mφ cell line RAW 264.7 (15). In these studies, apoptotic signaling was an early accumulation of p53 before DNA fragmentation. Therefore, we hypothesized that morphine-induced Mφ apoptosis may be mediated through the generation of NO and may also be associated with the accumulation of p53. To study whether the effect of morphine is species specific, experiments were performed on Mφ harvested from different species (i.e., rats, mice and humans). To determine the direct effect of morphine on Mφ apoptosis, we conducted the majority of studies in vitro.

Materials and Methods

Eighteen Sprague Dawley rats, weighing 100 g each, were administered (i.p.) either normal saline 0.5 ml (control, nine rats) or normal saline containing morphine sulfate (40 mg/kg body weight, experimental, nine rats) daily for 3, 5, and 10 days. At the end of each scheduled period, three control and three experimental rats were killed, and peritoneal Mφ were harvested (16).

Four mice weighing 50 g were used for the isolation of mouse peritoneal Mφ.

Murine Mφ culture

To determine whether morphine has any species-specific effect, we also studied the effect of morphine on murine Mφ (Mφ cell line J 774.16, American Type Culture Collection, Rockville, MD). Mφ were grown in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Life

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2 This work was presented at the 59th Annual Scientific Meeting of the College on Problems of Drug Dependence on June 18, 1997 in Nashville, TN.
3 Address correspondence and reprint requests to Dr. Pravin C. Singhal, Nephrology Division, Room 228, Long Island Jewish Medical Center, New Hyde Park, NY 11040.
4 Abbreviations used in this paper: Mφ, macrophage; NO, nitric oxide; DPDPPE, [d-Pen2, d-Pen5]enkephalin; DADLE, [d-Ala2, d-Leu5]enkephalin; DAGO, [d-Ala2, N-Me-Phe4, Gly5-ol]enkephalin; H, Hoechst; l-NAME, Nω-nitro-l-arginine methyl ester; l-NMMA, Nω-monomethyl-l-arginine monoacetate; ICE, IL-converting enzyme; iNOS, inducible NO synthase; GADPH, glyceraldehyde-3-phosphate dehydrogenase.
Isolation of human monocytes

One hundred milliliters of blood was collected in 25-ml tubes containing EDTA from healthy volunteers. Monocytes were isolated with the use of a monocyte-separating solution (Accurate Chemical and Scientific, Westbury, NY).

Confirmation of peritoneal Mφ by anti-CD14 Abs

Isolated mouse peritoneal Mφ were labeled with mouse anti-CD14 Abs (Becton Dickinson, San Jose, CA) and examined under immunofluorescence microscope.

Apoptosis studies

Hoechst (H)-33342 (Molecular Probes, Eugene, OR) stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence whereas propidium iodide (Sigma) stains the necrosed cells. Double staining by these two agents enables us to obtain the percentage of live, apoptotic, and necrosed cells (17).

Equal numbers (10,000 cells/well) of J774.16 Mφ were plated in 24-well plates containing DMEM + 10% FCS. Forty-eight hours later, cells were washed twice with PBS and incubated in media containing either buffer (control), morphine (10−6 M), or morphine (10−8 M) for 48 h. Three sets of experiments were conducted, each in triplicate.

To determine the time course effect of morphine on murine peritoneal Mφ, equal numbers of subconfluent Mφ were incubated in media containing either buffer (control) or variable concentrations of morphine (10−6 to 10−8 M) for 24 h. Three sets of experiments were performed.

To determine whether morphine-induced Mφ apoptosis may be mediated through opiate receptors, we evaluated the effect of morphine on Mφ apoptosis with or without naloxone (NIDA), an opiate receptor antagonist. In brief, equal numbers of Mφ (10,000 cells/well) were seeded in 24-well plates containing DMEM + 10% FCS containing either buffer or morphine (10−8 M) for variable periods (12, 24, and 48 h) at 37°C. Three sets of experiments were performed, each in triplicate.

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Table 1. Effect of morphine on macrophage apoptosis

<table>
<thead>
<tr>
<th>Morphine</th>
<th>10−10 M</th>
<th>10−8 M</th>
<th>10−6 M</th>
<th>10−4 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3 ± 0.2</td>
<td>5.2 ± 1.0</td>
<td>13.0 ± 1.2</td>
<td>27.3 ± 4.1</td>
<td>44.3 ± 5.9</td>
</tr>
</tbody>
</table>

*a Equal numbers of J774.16 Mφ were plated in 24-well plates containing DMEM + 10% FCS for 48 h. Subsequently, Mφ were incubated in media containing either buffer (control) or variable concentrations of morphine (10−6 to 10−8 M) for 48 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide and examined under an inverted microscope with UV light. Results (means ± SEM % apoptotic cells/field) are from three sets of experiments, each carried out in triplicate. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value.

To determine whether other NO-releasing agents can also modulate Mφ apoptosis, equal numbers of subconfluent Mφ were incubated in media containing either buffer (control), morphine (10−6 M), or morphine (10−8 M) for 24 h. Twelve sets of experiments were conducted.

To determine whether morphine-induced Mφ apoptosis is mediated through the activation of IL-converting enzyme (ICE)-1, we evaluated the effect of ICE-1 inhibitor (Calbioche) on morphine-induced Mφ apoptosis. Equal numbers of subconfluent Mφ were incubated in media containing either buffer (control), morphine (10−6 M), or morphine (10−8 M) + ICE-1 inhibitor (200 μM) for 24 h. Twelve sets of experiments were conducted.

To determine whether morphine-induced Mφ apoptosis may be mediated through the activation of IL-converting enzyme (ICE)-1, we evaluated the effect of ICE-1 inhibitor (Calbioche) on morphine-induced Mφ apoptosis. Equal numbers of subconfluent Mφ were incubated in media containing either buffer (control), morphine (10−6 M), or morphine (10−8 M) + ICE-1 inhibitor (200 μM) for 24 h. Twelve sets of experiments were conducted.

To determine the effect of L-NMMA, another inhibitor of NOS, on morphine-induced Mφ NO production, equal numbers of Mφ (10,000 cells/well) were seeded in 24-well plates containing DMEM + 10% FCS for 48 h. Subsequently, cells were washed twice with PBS and incubated in media containing either buffer (control), morphine (10−8 M), naloxone (10−6 M), or morphine (10−8 M) + naloxone (10−6 M) for 48 h. Three sets of experiments were conducted.

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To determine the effect of sulfo-NONOate-disodium salt (negative control; produces nitrous oxide but no nitric oxide at physiologic pH; Alexis Corp. San Diego, CA) on Mφ production, equal numbers of subconfluent Mφ were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of sulfo-NONOate-disodium salt (10^{-6} M to 10^{-3} M) for 24 h. At the end of the incubation period, cells were stained with H-33342 and examined under inverted microscope with ultraviolet light. A, Control Mφ showed nuclei with moderate fluorescence. B, A significant number of Mφ in the morphine-treated group showed nuclei with bright fluorescence (apoptotic) (magnification ×150).

Table II. Dose response of morphine on murine peritoneal macrophages

<table>
<thead>
<tr>
<th>Morphine</th>
<th>Control</th>
<th>10^{-12} M</th>
<th>10^{-10} M</th>
<th>10^{-8} M</th>
<th>10^{-6} M</th>
<th>10^{-8} M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.2 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>^*</td>
<td>5.7 ± 0.1</td>
<td>^*</td>
</tr>
</tbody>
</table>

* Equal numbers of mouse peritoneal Mφ were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of morphine (10^{-12} to 10^{-3} M) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. Percentages of live, apoptotic, and necrosed cells were examined in eight random fields. Results (mean ± SEM) are from three sets of experiments, each carried out in triplicate. *p < 0.001 compared with control; †p < 0.001 compared with morphine, 10^{-12} M; ¶p < 0.01 compared with morphine, 10^{-12} M; ¶p < 0.01 compared with morphine, 10^{-10} M; ¶p < 0.01 compared with morphine, 10^{-8} M; ¶p < 0.01 compared with morphine, 10^{-6} M; ¶p < 0.01 compared with morphine, 10^{-8} M.
Detection of rat peritoneal Mφ/human monocyte apoptosis by gel electrophoresis

Gel electrophoresis is a simple method for detection of apoptosis. Briefly, equal numbers of rat peritoneal Mφ (harvested from control and morphine-treated rats) were plated on 100-mm petri dishes containing media for 24 h. In parallel experiments, human monocytes were plated on 100-mm petri dishes containing either media alone (control) or media + morphine (10^{-6} M) for 24 h. At the end of the incubation period, cells were lysed in DNA lysis buffer, and DNA was extracted (21). DNA was run on a 1.8% agarose gel electrophoresis at 5 V/cm in 0.5 × TE buffer (Tris 10 mM; EDTA 1 mM, pH 8.0) containing 10 μg/ml ethidium bromide.

Detection of rat peritoneal Mφ/human monocyte apoptosis by using DNA end-labeling technique

DNA end-labeling is a more sensitive method for detection of apoptosis (21). In brief, 5 μg of isolated peritoneal Mφ DNA from control and morphine-treated rats, as well as from control and morphine-treated human monocytes (as described above), were end labeled (21). The end-labeled DNA were electrophoresed on a 1.8% agarose gel, and radiolabeled fragments were visualized by exposure to Kodak (Rochester, NY) x-ray film at −70°C for 30 min to 3 h.

Protein extraction

Equal numbers (10,000) of J774.16 Mφ were seeded in 100-mm petri dishes and grown to subconfluence. Subsequently, Mφ were washed twice with PBS and incubated in medium containing either buffer (control) or morphine (10^{-8} M to 10^{-6} M) for 4 h. At the end of the incubation periods, cells were washed twice with PBS and incubated with 600 μl of PBSTDS lysis buffer (Calbiochem; with 2 μg/ml leupeptin, 1 mM EDTA, 1 μg/ml pepstatin, and 100 μg/ml PMSF) for 30 min at room temperature. Cells were scraped and protein was assayed using a BCA kit (Pierce, Rockford, IL).

Western blotting

The proteins (20 μg/lane) extracted from Mφ lysates were separated on a 4 to 20% gradient polyacrylamide gel and transferred onto a nitrocellulose membrane using a Bio-Rad (Hercules, CA) Western blotting apparatus. Nitrocellulose membranes were then processed further for p53 and Bax using either mouse anti-p53 (Zymed, San Francisco, CA) and Bax (Santa Cruz, Santa Cruz, CA) antibodies.

Table III. Effect of opiate agonists and antagonists on macrophage apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10^{-8} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>2.3 ± 0.02</td>
<td>13.0 ± 1.2</td>
<td>27.3 ± 5.9</td>
</tr>
<tr>
<td>β-Endorphin</td>
<td>3.5 ± 0.5</td>
<td>8.3 ± 1.6</td>
<td>13.6 ± 0.5</td>
</tr>
<tr>
<td>DADLE</td>
<td>1.4 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>DPDPE</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Naloxone</td>
<td>1.4 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>2.1 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Equal numbers of Mφ were seeded in 24-well plates containing DMEM + 10% FCS for 48 h. Subsequently, Mφ were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of agonists and antagonists (10^{-8} M to 10^{-6} M) for 48 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide and examined under UV light. Results (means ± SEM % apoptotic cells/field) are from three sets of experiments, each carried out in triplicate.
or rabbit anti-Bax (PharMingen, San Diego, CA) at a 1 μg/ml concentration; at room temperature using HRP (horseradish peroxidase)-labeled secondary goat anti-mouse Ab or goat anti-rabbit (Pierce); blots were developed using enhanced chemiluminescence (ECL, Amersham).

**Northern blotting**

Equal numbers of subconfluent Mφ grown in 100-mm petri dishes were incubated in media (DMEM + 1% FCS) containing either buffer (control) or morphine (M, 10^-8 M), l-NMMA (10^-6 M), or morphine (10^-8 M)+l-NMMA (10^-6 M) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide and examined under UV light. Results (means ± SEM) are from three sets of experiments, each carried out in triplicate. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. *p < 0.001 compared with control, l-NMMA (10^-6 M), and morphine (10^-8 M)+l-NMMA (10^-6 M).

**Statistical analysis**

For comparison of mean values between two groups, the unpaired t test was used. To compare values between multiple groups, analysis of variance (ANOVA) was applied and a Newman-Keuls multiple range test was used to calculate a q value. All values are means ± SE except where otherwise indicated. Statistical significance was defined as p < 0.05.

**Results**

**Morphine-enhanced Mφ apoptosis**

As shown in Table I, morphine in concentrations of 10^-8 M to 10^-6 M promoted murine Mφ apoptosis. Representative photographs of control and morphine (10^-6 M)-treated Mφ are shown in Figure 1. This effect of morphine (10^-8 M) was time dependent (12 h, control 2.3 ± 0.2% and morphine 3.6 ± 0.2%;* apoptotic cells/field; 24 h, control 2.6 ± 0.1% and morphine 4.8 ± 0.1%;* apoptotic cells/field; 48 h, control 2.5 ± 0.1% and morphine-7.4 ± 0.6%;*** apoptotic cells/field; n = 3; *p = 0.05 compared with control, 12 h; **p < 0.001 compared with control, 24 h; ***p < 0.001 compared with control.

**Table IV. Effect of l-NMMA on morphine-induced Mφ apoptosis**

<table>
<thead>
<tr>
<th>% Apoptotic cells/field</th>
<th>Control</th>
<th>M (10^-6 M)</th>
<th>M (10^-6 M) + l-NMMA (10^-6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.04 ± 0.24</td>
<td>7.90* ± 1.40</td>
<td>1.9 ± 0.06</td>
</tr>
<tr>
<td>M (10^-6 M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-NMMA (10^-6 M)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Equal numbers of subconfluent macrophages were incubated in media (DMEM + 1% FCS) containing either buffer (control) or morphine (M, 10^-6 M), l-NMMA (10^-6 M), or morphine (10^-6 M) + l-NMMA (10^-6 M) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide and examined under UV light. Results (means ± SEM) are from three sets of experiments, each carried out in triplicate. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. *p < 0.001 compared with control, l-NMMA (10^-6 M), and morphine (10^-8 M)+l-NMMA (10^-6 M).

**DNA end-labeling of control and morphine-treated human monocytes.** M: molecular marker; C: control monocytes. Morphine (10^-6 M and 10^-4 M)-treated monocytes showed a classic ladder pattern.

**Equal numbers of subconfluent J774.16 Mφ were incubated in media (DMEM + 1% FCS) containing either buffer (control), morphine (10^-8 M), l-NAME (1 mM), or morphine (10^-8 M) + l-NAME (1 mM) for 48 h. At the end of the incubation period, cells were stained with H-33342, and the percentage of apoptotic cells was counted under an inverted microscope with ultraviolet rays. Results (means ± SEM) are from three sets of experiments, each carried out in triplicate. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. *p < 0.001 compared with control, l-NAME (10^-6 M), and morphine (10^-8 M)+l-NAME (10^-6 M).**
apoptosis. ICE-1 inhibitor attenuated (\( p < 0.001 \)) morphine-induced M\( \Phi \) apoptosis (control, 1.9 ± 0.1%; morphine, 10\(^{-6}\) M, 6.8 ± 0.2%; ICE-1 inhibitor, 200 pM, 3.0 ± 0.1%; morphine + ICE-1 inhibitor, 3.1 ± 0.1% apoptotic cells/field; \( n = 12 \)). These results indicate that morphine may be promoting an apoptotic pathway through the activation of ICE-1.

### Role of NOS and NO in morphine-induced M\( \Phi \) apoptosis

To determine whether morphine-induced M\( \Phi \) apoptosis is mediated through activation of NOS, we evaluated the effect of L-NAME and L-NMMA, inhibitors of NOS on morphine-induced M\( \Phi \) apoptosis. The effect of L-NAME is shown in Figure 6. L-NAME attenuated (\( p < 0.05 \)) morphine (10\(^{-8}\) M)-induced M\( \Phi \) apoptosis (control, 2.35 ± 0.45%; morphine, 7.67 ± 2.1%; morphine + L-NAME, 3.86 ± 0.88% apoptotic cells/field; \( n = 5 \)). As shown in Table IV, L-NMMA also attenuated (\( p < 0.001 \)) morphine-induced M\( \Phi \) apoptosis (morphine, 7.9 ± 1.40 vs morphine + L-NMMA, 2.20 ± 0.39% apoptotic cells/field). These results suggest that morphine-induced M\( \Phi \) apoptosis may be mediated through the activation of M\( \Phi \) NOS. To determine whether other NO-generating agents can also modulate M\( \Phi \) apoptosis, we evaluated the effect of sodium nitroprusside on M\( \Phi \) apoptosis. As shown in Table V, sodium nitroprusside promoted M\( \Phi \) apoptosis in a dose-dependent manner (control, 2.2 ± 0.6% vs sodium nitroprusside, 0.5 mM, 11.9 ± 2.4% apoptotic cells/field; \( p < 0.01 \)). These results suggest that other NO-generating agents may also induce M\( \Phi \) apoptosis.

### Effect of morphine and NOS inhibitors on M\( \Phi \) NO production

Since NO has been demonstrated to trigger M\( \Phi \) apoptosis (15), we evaluated the effect of morphine on M\( \Phi \) NO production. To study the role of NOS in morphine-induced NO production, we examined the effect of L-NAME and L-NMMA (NOS inhibitors) on morphine-induced M\( \Phi \) NO production. We also evaluated the effect of sodium nitroprusside (positive control) and sulfonamidoisulfonyl disodium salt (negative control) on M\( \Phi \) NO production. To evaluate whether this effect of morphine is mediated through opiate receptors, we examined the effect of naloxone (opiate receptor antagonist) on morphine-induced M\( \Phi \) NO production.

The effects of morphine on the production of NO under basal as well as LPS-activated states are shown in Tables VI and VII. Morphine (10\(^{-8}\) M) enhanced (\( p < 0.001 \)) production of NO by M\( \Phi \) under a basal state (control, 0.37 ± 0.02 vs morphine, 0.79 ± 0.02 nM; \( n = 4 \)). Morphine in concentrations of 10\(^{-10}\) M and 10\(^{-8}\) M increased (\( p < 0.001 \)) the synthesis of NO under an LPS-stimulated state. The production of morphine-induced NO by M\( \Phi \) was manyfold higher under LPS-activated states when compared with the basal state. As shown in Tables VI and VII, L-NAME attenuated morphine-induced M\( \Phi \) NO production under basal (Table VI) as well as LPS-activated (Table VII).

### Table V. Effect of sodium nitroprusside and morphine on M\( \Phi \) apoptosis

<table>
<thead>
<tr>
<th>Sodium nitroprusside (SNP)</th>
<th>% Apoptotic cells/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>0.125 mM</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>7.9* ± 1.0</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>11.9** ± 2.4</td>
</tr>
</tbody>
</table>

** Equal numbers of subconfluent M\( \Phi \) were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations (0.125, 0.25, and 0.5 mM) of sodium nitroprusside (SNP) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide and examined under light. Results (means ± SEM) are from three sets of experiments; each carried out in triplicate. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. * \( p < 0.05 \) compared with control, † \( p < 0.05 \) compared with SNP, 0.125 mM. ** \( p < 0.001 \) compared with morphine, 12 and 24 h. Morphine also enhanced apoptosis in mouse peritoneal M\( \Phi \) in a dose-dependent manner (Table II).

To determine whether the effect of morphine is mediated through \( \mu \) opiate receptors, we evaluated the effect of naloxone, an \( \mu \) opiate receptor antagonist, on morphine-induced M\( \Phi \) apoptosis. Naloxone attenuated (\( p < 0.01 \)) morphine (10\(^{-8}\) M)-induced M\( \Phi \) apoptosis (control, 2.3 ± 0.2%; morphine, 4.8 ± 0.5%; morphine + naloxone, 2.6 ± 0.4% apoptotic cells/field, \( n = 3 \)). To evaluate whether other \( \mu \) opiate agonists and antagonists also modulate M\( \Phi \) apoptosis, we determined the effect of agonists (\( \beta \)-endorphin, DAGO, DADLE and DPDE) and antagonists (naloxone and naltrexone) on M\( \Phi \) apoptosis. As shown in Table III, \( \mu \) receptor agonists (morphine, \( \beta \)-endorphin, and DAGO) promoted M\( \Phi \) apoptosis, whereas \( \delta \) receptor agonists showed only a mild alteration on M\( \Phi \) apoptosis. These results suggest that morphine-induced M\( \Phi \) apoptosis may be mediated through \( \mu \) opiate receptors. To determine whether this effect of morphine is species specific, we evaluated the effect of morphine on rat peritoneal M\( \Phi \) as well as monocytes isolated from healthy volunteers. Peritoneal M\( \Phi \) isolated from morphine-treated rats showed a greater percentage of apoptosis when compared with M\( \Phi \) isolated from control rats (control rats, 2.6 ± 0.8% vs morphine-treated rats, 13.5 ± 1.5% apoptotic M\( \Phi \)s/field). As shown in Figures 2 and 3, peritoneal M\( \Phi \) harvested from morphine-treated rats showed DNA fragmentation (integer multiples of 180 base pairs) in the form of a ladder pattern (at 3 and 10 days) when isolated DNA was run in agarose electrophoresis, as well as when end-labeled, whereas peritoneal M\( \Phi \) harvested from untreated rats (control) did not show DNA fragmentation. Similarly, morphine-treated human monocytes showed a classic ladder pattern characteristic of apoptosis (Figs. 4 and 5). These results suggest that the effect of morphine on M\( \Phi \) apoptosis is not species specific.

ICE-1 has been demonstrated to play an important role in the propagation of the effector phase of apoptosis to the irreversible commitment to death (22). To determine whether morphine-induced M\( \Phi \) apoptosis is mediated through activation of ICE-1, we evaluated the effect of ICE-1 inhibitor on morphine-induced M\( \Phi \) apoptosis. ICE-1 inhibitor attenuated (\( p < 0.001 \)) morphine-induced M\( \Phi \) apoptosis (control, 1.9 ± 0.1%; morphine, 10\(^{-6}\) M, 6.8 ± 0.2%; ICE-1 inhibitor, 200 pM, 3.0 ± 0.1%; morphine + ICE-1 inhibitor, 3.1 ± 0.1% apoptotic cells/field; \( n = 12 \)). These results indicate that morphine may be promoting an apoptotic pathway through the activation of ICE-1.

### Table VI. Effect of L-NAME on morphine (M)-induced NO production by M\( \Phi \)

<table>
<thead>
<tr>
<th>NO (nM)</th>
<th>M (10(^{-8}) M)</th>
<th>M (10(^{-10}) M)</th>
<th>L-NAME</th>
<th>M (10(^{-8}) M) + L-NAME</th>
<th>M (10(^{-10}) M) + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37 ± 0.02</td>
<td>0.79* ± 0.02</td>
<td>0.40† ± 0.01</td>
<td>0.16‡ ± 0.02</td>
<td>0.24 ± 0.03</td>
</tr>
</tbody>
</table>

* Equal numbers of subconfluent J774.16 M\( \Phi \) were incubated in media (DMEM + 1% FCS) containing either buffer (control), morphine (10\(^{-8}\) M), morphine (10\(^{-10}\) M), L-NAME (1 mM), morphine (10\(^{-8}\) M) + L-NAME (1 mM), or morphine (10\(^{-10}\) M) + L-NAME (1 mM) for 24 h. At the end of the incubation period, supernatants were collected and NO production was measured. Results (means ± SEM) are from four sets of experiments. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. * \( p < 0.01 \) compared with control, † \( p < 0.001 \) compared with M (10\(^{-10}\) M) and M (10\(^{-8}\) M) + L-NAME, ‡ \( p < 0.05 \) compared with control.
Table VII. Effect of L-NAME on morphine (M)-induced NO production by LPS-activated Mφ

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>M (10⁻⁸ M)</th>
<th>M (10⁻⁶ M)</th>
<th>L-NAME</th>
<th>M (10⁻⁶ M) + L-NAME</th>
<th>M (10⁻⁸ M) + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO (nM)</td>
<td>0.84 ± 0.08</td>
<td>2.07 ± 0.32</td>
<td>1.58 ± 0.28</td>
<td>0.65 ± 0.03</td>
<td>1.15 ± 0.04</td>
<td>0.83 ± 0.04</td>
</tr>
</tbody>
</table>

* Equal numbers of subconfluent J774.16 macrophages were incubated in media (DMEM + 1% FCS) containing either buffer (control), morphine (10⁻⁸ M), morphine (10⁻⁶ M), L-NAME (1 mM), morphine (10⁻⁸ M) + L-NAME (1 mM), or morphine (10⁻⁶ M) + L-NAME (1 mM) for 24 h. After 24 h of incubation, an aliquot of LPS (10 ng/ml) was added to each well. The incubation was continued for another 6 h. At the end of the incubation period, supernatants from each well were collected and NO content was measured. Results (means ± SEM) are from four sets of experiments. To compare values between multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a q value. *p < 0.001 compared with control and L-NAME; † p < 0.5 compared with control; ‡ p < 0.05 compared with M (10⁻⁸ M).

states. Similarly, L-NMMA, a specific inhibitor of iNOS, attenuated (p < 0.001) morphine-induced Mφ NO production (control, 1.1 ± 0.03; morphine, 10⁻⁶ M, 3.0 ± 0.16; L-NMMA, 1.3 ± 0.18; morphine + L-NMMA, 1.1 ± 0.09 nM nitrite). Sodium nitroprusside, another NO-releasing agent, enhanced (p < 0.001) Mφ NO production in a dose-dependent manner (control, 0.86 ± 0.16; sodium nitroprusside, 0.125 mM, 1.43 ± 0.16; sodium nitroprusside, 0.25 mM, 3.4 ± 0.13; sodium nitroprusside, 0.5 mM, 4.0 ± 0.15 nitrite nM; n = 3). On the contrary, sulfo-NONOate disodium salt did not alter Mφ NO production (control, 1.10 ± 0.10; sulfo-NONOate disodium salt, 10⁻⁶ M, 0.93 ± 0.03; sulfo-NONOate disodium salt, 10⁻³ M, 1.03 ± 0.09 nitrite nM). However, naloxone, an opiate receptor antagonist, attenuated (p < 0.01) morphine-induced Mφ NO production (control, 1.1 ± 0.03; morphine, 10⁻⁸ M, 2.3 ± 0.27; morphine + naloxone, 1.1 ± 0.14 nitrite nM). These results indicate that morphine promotes the generation of NO by Mφ. This effect of morphine seems to be mediated through the activation of NOS.

Since inhibition of NOS attenuated morphine-induced Mφ apoptosis, we studied the effect of morphine on Mφ mRNA expression of iNOS. As shown in Figure 7, morphine enhanced mRNA expression of iNOS by fivefold (control 0.15; morphine, 10⁻⁶ M, 0.84 iNOS/GAPDH ratios).

**Morphine promoted accumulation of p53 and Bax proteins by Mφ**

The induction of certain genes, such as p53, has been shown to be a requirement in apoptosis induced by DNA damage (23, 24); therefore, we studied the effect of morphine on the synthesis of p53 protein by Mφ. The effects of morphine on Mφ p53 accumulation is shown in Figure 8. Morphine promoted the accumulation of p53 protein when compared with untreated cells.

Survival of a cell has been considered to be dependent on the net dominance between cell survival genes and cell destructive genes (25–28). Based on this hypothesis, the expression of genes belonging to the Bcl-2 family may determine the fate of a cell. Therefore, we evaluated the effect of morphine on accumulation of Bax protein, which propagates cell death (26–28). As shown Figure 9, morphine promoted the accumulation of Bax protein by Mφ.

These results suggest that morphine promotes the effector phase of the apoptotic pathway through the generation of Bax protein by Mφ.

**Discussion**

The present study demonstrates that morphine directly promotes Mφ apoptosis. This effect on Mφ is not species specific. Since naloxone, an opiate receptor antagonist, attenuated morphine-induced Mφ apoptosis, this effect of morphine appears to be mediated through opiate receptors. Morphine promoted the production of NO both under basal and LPS-activated states. L-NAME and L-NMMA, inhibitors of NOS, attenuated the morphine-induced Mφ generation of NO. Since morphine-induced Mφ apoptosis was also inhibited by L-NAME and L-NMMA, it appears that morphine-induced Mφ apoptosis may be mediated through the generation of NO. It was further substantiated by morphine-induced enhanced Mφ mRNA expression of iNOS.

Opiate receptor binding sites have been previously described on human PBMC (29). Recently, these binding sites have been designated as µ₃ (30). Opiate alkaloid-selective µ₃ binding sites have also been shown to be present on murine J774.16 cells (31). These receptors have been postulated to mediate morphine-induced inhibition of monocyte/Mφ activation caused by IL-1α, TNF-α, IL-8, or N-FMLP. The µ₃ receptor has been suggested to function in response to both endogenously formed and exogenously administered opiate alkaloids (30). In the present study, µ receptor agonists promoted Mφ apoptosis, whereas δ receptor agonists showed only a mild alteration of Mφ apoptosis.

Tubaro et al. demonstrated that morphine can attenuate mouse peritoneal Mφ count in a dose-dependent manner (12). These investigators suggest that the morphine-induced decrease in Mφ count may contribute to morphine-associated immunomodulation. However, the mechanism of morphine-induced decreased Mφ count was not analyzed. The present study provides an explanation for morphine-induced decreased Mφ count.

Peterson et al. and Tubaro et al. also demonstrated that morphine decreases the respiratory burst in stimulated Mφ and PBMC.

**FIGURE 7.** Effect of morphine on Mφ mRNA expression of iNOS. The upper lane shows mRNA expression of iNOS by control (C) and morphine-treated Mφ. The lower lane shows Mφ mRNA expression of GAPDH.

**FIGURE 8.** Effect of morphine on Mφ p53 accumulation. Morphine (10⁻⁸ to 10⁻⁴ M)-treated cells show a greater accumulation of p53 protein when compared with control cells.
Bcl-w, Bfl-1, Brag-1, and A1) or death agonists (Bax, Bak, and Bax−/− and Bax−/− mice; Refs. 10 and 11).

We speculate that morphine-induced p53 contributes to morphine-induced immunomodulation. Recently, NO has been reported to promote Mφ apoptosis (15). This effect of NO was mediated through the generation of p53 (15). Expression of wild-type p53 appears to be linked to apoptosis (15). Frequently, p53 also signals apoptosis in the case of severe DNA damage. In the present study, morphine promoted the production of NO by Mφ. Inhibition of Mφ NO production by L-NAME attenuated morphine-induced Mφ apoptosis. Morphine also enhanced Mφ synthesis of p53. We speculate that morphine-induced Mφ apoptosis may be mediated through the generation of NO.

In the present study, morphine also promoted the accumulation of Bax protein, a member of the Bcl-2 family. Bcl-2 is reported to be a growing family of apoptosis regulatory gene products that may be either death antagonists (Bcl-2, Bcl-Xl, Bcl-w, Bfl-1, and A1) or death agonists (Bax, Bak, Bcl-Xl, Bad, Bid, Bik, and Hrk) (26–28). Bax, being a death agonist, may have contributed to the effector phase of the morphine-induced apoptotic pathway. In addition, morphine-induced activation of IEC may have pushed Mφ to an irreversible commitment of death.

While there is no controversy about morphine-induced immunomodulation, the direct effect of morphine on the mononuclear phagocyte system is far from clear. It has been suggested that morphine may be modulating the function of monocytes through opiate receptors present on lymphocytes (33, 34), indirectly via opiate receptors in the central nervous system, or by activating the hypothalamic-pituitary-adrenal axis (35) to secrete immunosuppressive glucocorticoids (36). The present study shows the direct effect of morphine on Mφ and thus provides a basis for a hypothesis that morphine can directly modulate immune function in patients with drug addiction.

References


