Morphine alters the circulating proteolytic profile in mice: functional consequences on cellular migration and invasion

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ABSTRACT: Opioids modulate the tumor microenvironment with potential functional consequences for tumor growth and metastasis. We evaluated the effects of morphine administration on the circulating proteolytic profile of tumor-free mice. Serum from morphine-treated (1 or 10 mg/kg, i.p. every 12 h) or saline-treated mice was collected at different time points and tested ex vivo in endothelial, lymphatic endothelial, and breast cancer cell migration assays. Serum from mice that were treated with 10 mg/kg morphine for 3 d displayed reduced chemotactic potential for endothelial and breast cancer cells, and elicited reduced cancer cell invasion through reconstituted basement membrane compared with serum from saline controls. This was associated with decreased circulating matrix metalloproteinase 9 (MMP-9) and increased circulating tissue inhibitor of metalloproteinase 1 (TIMP-1) and TIMP-3/4 as assessed by zymography and reverse zymography. By using quantitative RT-PCR, we confirmed morphine-induced alterations in MMP-9 and TIMP expression and identified organs, including the liver and spleen, in which these changes originated. Pharmacologic inhibition of MMP-9 abrogated the difference in chemotactic attraction between serum from saline-treated and morphine-treated mice, which indicated that reduced proteolytic ability mediated the decreased migration toward serum from morphine-treated mice. This novel mechanism may enable morphine administration to promote an environment that is less conducive to tumor growth, invasion, and metastasis.—Xie, N., Khabbazi, S., Nassar, Z. D., Gregory, K., Vithanage, T., Anand-Apte, B., Cabot, P. J., Sturgess, D., Shaw, P. N., Parat, M.-O. Morphine alters the circulating proteolytic profile in mice: functional consequences on cellular migration and invasion. FASEB J. 31, 5208–5216 (2017). www.fasebj.org

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There is disagreement in the literature with regard to the ability of opioids, especially morphine, to affect tumor growth and metastasis. We reviewed previously (1–3). These discrepancies relate to multiple facets of tumor biology, including antitumor immunity, angiogenesis, inflammation, or cancer cell proliferation, apoptosis, migration, and invasion. Matrix-degrading enzymes modulate several of these processes, and their expression and/or activity have been documented to be altered by morphine; matrix metalloprotease 2 (MMP-2) and MMP-9 mRNA and protein levels were reported to be decreased in cultured breast cancer cells that were incubated with nanomolar to micromolar concentrations of morphine (4), and the activity of MMP-2 and MMP-9 in conditioned media of colon cancer cells exposed to morphine was reduced in a concentration-dependent manner (5). In contrast, acute morphine exposure increased MMP-9 expression and activity in dorsal root ganglia (DRG) both in vivo and in vitro (6). We have previously shown that morphine decreased MMP-9 expression in a model of TGF-β–induced breast cancer cell epithelial to mesenchymal transition (7). Morphine also decreased MMP-9 in cocultures of breast cancer cells with accessory cells (either endothelial cells of macrophages), whereas MMP-2 was unaffected (8, 9). The decrease in MMP-9 was associated with an increased production of the MMP inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1), in these coculture experiments (8). Our results further demonstrated that morphine affected MMP-9 production in cocultured cells but not in
cells grown individually, and this involved the regulation by morphine of the paracrine interaction between cancer cells and stromal/accessory cells (8, 9). Production of MMP-9 contributed by both cancer cells and accessory cells in coculture was reduced by morphine (9). In a model of IL-4-induced alternate polarization of macrophages in vitro, morphine decreased MMP-9 production without affecting MMP-2 (9). Lastly, in preclinical experiments, we demonstrated that morphine administration reduced the tumor load in mice injected in the tail vein with syngeneic breast cancer cells, and this was associated with decreased levels of circulating MMP-9 (8). In the present study, we evaluated the effect of morphine administration to tumor-free mice on the circulating proteolytic potential and its functional consequences on the behavior of cancer and cancer-associated cell types ex vivo.

MATERIALS AND METHODS

Materials

DMEM, RPMI-1640, DMEM/F-12, trypsin-EDTA, penicillin/streptomycin, and t-glutamine solutions were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Nonessential amino acids were from Lonza (Mount Waverly, VIC, Australia). Morphine sulfate DBL was purchased from Hospira (Mulgrave, VIC, Australia). Coomassie blue was from Bio-Rad (Coomassie Brilliant Blue R-250; Gladesville, NSW, Australia). Polycarbonate membranes were purchased from Neuro Probe (Gaithersburg, MD, USA). CultureCoat 24-well plates with basement membrane extract–coated inserts were from Bio Scientific Pty (Sydney, NSW, Australia). MMP-9 inhibitor I (CAS 1177749-58-4) and real-time PCR reagents were from Thermo Fisher Scientific. The following reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): DMSO, crystal violet, di styrene, a plasticizer, and xylene (DPX) mounting medium for histology, MT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], gelatin from bovine skin (type B), rat tail collagen, Tween 20, and fetal bovine serum (FBS).

Cell culture

Bovine aortic endothelial cells (BAECs) and murine mammary breast carcinoma cells (line 4T1) were cultured in DMEM/F-12 and RPMI-1640 medium, respectively, that was supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C in a 5% CO2 atmosphere. Lympohtic endothelial cells (LEC) that were isolated from mice that express temperature-sensitive SV40 large T (H-2Kb-tsAS8) (10) were maintained in DMEM medium that was supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 1% t-glutamine and grown in an 8% CO2 atmosphere at 35°C. LECs were preincubated at 37°C with 5% CO2 for 72 h before each experiment.

Mice and morphine treatment

Six-week-old female BALB/c mice were maintained under 12-h light/dark cycles and controlled temperature and humidity with free access to standard food and water. Mice were administered an intraperitoneal injection of 1 or 10 mg/kg morphine or an equivalent volume of saline at 12-h intervals for 3 d. Blood was collected 10 min and 12 h after the first (d 1) and last (d 3) injection as previously described (11).

Cell migration assay

Cell migration assays were performed in a 48-well modified microchemotaxis Boyden chamber using an 8-µm-diameter pore size polycarbonate membrane that was precoated with rat tail collagen type 1 (100 µg/ml in 0.2 N acetic acid). Medium (28.5 µl) that contained chemoattractant—serum from morphine-treated or saline-treated mice, blank serum spiked with drugs, or drugs only—was added in the lower chamber. Cells in suspension (50 µl; 15 × 10⁶ cells/ml of BAECs, 30 × 10⁶ cells/ml of LECs or 4T1) in serum-free medium were placed in the upper compartment of the chamber. After a 4-h incubation (37°C, 5% CO2), nonmigrated cells on the upper side of the membrane were wiped and the membrane was fixed with 4% (w/v) formalin in PBS for 1 h, stained with 0.5 g/L crystal violet solution in 25% (v/v) methanol overnight, and mounted using DPX mounting medium. Migrated cells on the lower membrane surface were counted in microscopic fields that covered the whole well. The effect of MMP-9 inhibitor I was tested by adding it to both the upper and lower wells of the chamber.

Invasion assay

Invasion assays were conducted by using a 24-well transwell system with basement membrane extract–coated polycarbonate membrane filter (8-µm pore size) insert. After 24 h of serum deprivation, 4T1 cells were harvested and resuspended in serum-free medium. A volume of 100 µl that contained 2 × 10⁵ cells was added to each insert. Serum from morphine-treated or saline-treated mice (2% v/v) was used as chemoattractant. In some experiments, MMP-9 inhibitor I was added at indicated concentrations to both the upper insert and lower well media. After 24 h of incubation, inserts were fixed with 4% (w/v) formalin in PBS for 1 h, stained with 0.5 g/L crystal violet solution in 25% (v/v) methanol overnight, and noninvaded cells that remained on the upper side of the membranes were carefully removed with cotton swabs. Stained inserts were imaged in microscopic fields that covered the entire surface of the insert and all invaded cells for each insert were counted.

Zymography and reverse zymography

Sera from morphine-treated or saline-treated mice were analyzed for MMPs and TIMPs by using zymography and reverse zymography, respectively. As previously described (8, 9), a polycrylamide gel [11% (w/v)] that contained 1% (w/v) gelatin was used to quantitate gelatinases (MMP-2 and MMP-9), and a 12% (w/v) polycrylamide gel that contained 1% (w/v) gelatin and 20% (v/v) NIH3T3-conditioned medium as a source of gelatinase was prepared for the purpose of quantifying the level of TIMPs. In this study, equal amounts of protein from samples were separated by using SDS-PAGE. Gels were incubated and rinsed with the following solutions: renaturing solution [5 mM CaCl₂, 50 mM Tris and 2.5% (v/v) Triton X-100] overnight, solution that contained 50 mM Tris–HCl and 5 mM CaCl₂ at 37°C for 3 h, and staining solution [0.25% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid] overnight. Gels were destained by using an aqueous solution of 25% (v/v) methanol and 10% (v/v) glacial acetic acid. Hydrolysis of gelatin by proteinases is reflected as white bands on a blue background of undegraded gelatin after destaining, whereas TIMP activity inhibits the proteolytic activity of MMPs, which results in dark blue bands against the region of gelatin degradation after Coomassie Blue staining. Gels were scanned by using high-resolution flatbed scanning and band intensity was measured by using ImageJ (National Institutes of Health, Bethesda, MD, USA).
Real-time quantitative PCR

Total RNA was isolated and purified by using a Purelink RNA mini kit (Thermo Fisher Scientific) from the brain, spleen, liver, lung, and heart tissues of morphine-treated or saline-treated mice. RNA was reverse-transcribed to complementary DNA by using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) and cDNA was quantified by using TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific) with AmpliTaq Gold DNA Polymerase and TaqMan Gene Expression Assay for mouse Mmp9 (Mm00442991-m1), Timp1 (Mm00341361-m1), Timp2 (Mm00441825-m1), Timp3 (Mm00441826-m1), and Timp4 (Mm01184417-m1) in a StepOnePlus 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative quantification was performed by reference to 18S rRNA and analyzed by using the Ct method (12).

RESULTS

Morphine treatment alters ex vivo chemotactic potential of mouse serum

To assess whether morphine modulates angiogenesis, lymphangiogenesis, or cancer cell metastasis by altering the production of chemotactic factors in vivo, we tested the ability of serum that was isolated from morphine-treated or saline-treated mice to act as a chemoattractant for endothelial cells, LECs, and 4T1 breast cancer cells in a modified Boyden chamber assay. We used serum concentrations that elicited optimal chemotaxis for each cell type, namely, 0.2% (v/v) for BAECs and 2% (v/v) for LECs and 4T1 cells. Results demonstrated that serum samples from mice treated with 10 mg/kg morphine for 3 d—collected either 10 min or 12 h after the last injection—attracted significantly less migrated BAECs or 4T1 cells than did serum samples from saline-treated controls (Fig. 1A, B). This was not observed with LECs, which instead migrated more toward serum from 1 mg/kg morphine-treated mice collected 10 min after injection at either d 1 or 3 (Fig. 1C), with statistical significance only for serum isolated at the d 1 10-min time point. To examine the possibility that the effects on migration could be a result of the presence of morphine or its quantitatively important metabolite, morphine-3-glucuronide (M3G), in serum from morphine-treated mice, we tested the migration of cells toward serum to which morphine, M3G, or an equivalent volume of their respective solvent had been added (Supplemental Fig. 1). Concentrations of morphine or M3G were chosen on the basis of the average concentration observed in the serum of mice treated with 10 mg/kg morphine (11). This experiment demonstrated that morphine or M3G did not alter the chemotaxis or random migration of any of the 3 cell types and ruled out that the effects observed in Fig. 1 could be a result of the drug or its metabolite, M3G, present in the circulation at the time of blood collection.

Morphine treatment alters the ex vivo ability of mouse serum to elicit cancer cell invasion

We assessed the invasiveness of 4T1 cells through Matrigel-coated transwells toward medium that contained 2% (v/v) serum from saline-treated or morphine-treated mice collected at d 3 (Fig. 2). Invasion toward serum from mice treated with 10 mg/kg morphine was less than that observed toward serum from saline-treated mice. There was no difference between the invasion elicited by serum from mice treated with 1 mg/kg morphine and that elicited by serum from saline-treated control mice.

Morphine administration alters the circulating proteolytic profile

To test the hypothesis that decreased migration and invasion were a result of decreased matrix protease levels in the circulation, we quantified the activity of gelatinases
MMP-9 and MMP-2 in samples that were collected at d 3 by using in-gel gelatin zymography. Whereas MMP2 activity was unaltered by morphine administration at either 1 or 10 mg/kg, the intensity of the bands corresponding to MMP-9 (pro-MMP-9 + active MMP-9) was significantly lower in serum samples from mice treated with 10 mg/kg morphine, and densitometric quantitation revealed statistically significant reductions at both the d 3 10-min and 12-h time points compared with samples from control mice (Fig. 3A). We also tested the levels of circulating TIMPs using reverse zymography. Results showed that TIMP-1 was increased to different extents—from 2- to 7-fold—in several organs, with all 3 increased in the spleen, although not statistically significant, heart (Fig. 4A). In contrast, brain levels of MMP-9 mRNA were unaltered by morphine treatment. TIMPs 1–3 were increased to different extents—from 2- to 7-fold—in several organs, with all 3 increased in the spleen, although statistical significance was not reached for TIMP-2 or TIMP-3—both increased ~3-fold, a result that is likely to have biologic significance—as a result of interindividual variability. TIMP mRNA levels were unaltered in the brains.

**Morphine modulates tissue mRNA expression of MMP-9 and TIMPs**

In an attempt to elucidate the origin of circulating MMP-9 or TIMPs that were decreased or increased, respectively, by morphine administration, we isolated organs—brain, liver, lungs, spleen, and heart—from mice treated with 10 mg/kg morphine for 3 d, the tissues being harvested 12 h after the last injection. Quantitative RT-PCR revealed that the mRNA level of MMP-9 was decreased in liver, lungs, spleen, and, although not statistically significant, heart (Fig. 4A). In contrast, brain levels of MMP-9 mRNA were unaltered by morphine treatment. TIMPs 1–3 were increased to different extents—from 2- to 7-fold—in several organs, with all 3 increased in the spleen, although statistical significance was not reached for TIMP-2 or TIMP-3—both increased ~3-fold, a result that is likely to have biologic significance—as a result of interindividual variability. TIMP mRNA levels were unaltered in the brains.
Figure 3. Effect of morphine administration on circulating gelatinases and TIMPs. A) MMP activity in serum from saline-treated or morphine-treated mice was analyzed using gelatin zymography. Zymograms were scanned and used for densitometric quantitation. B) TIMP activity was assessed using reverse zymography with gels that contained both gelatin and gelatinase. Reverse zymograms were scanned and used for densitometric quantitation. Means ± SEM are shown (n = 6 individual mouse samples). *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student’s t test).
of morphine-treated mice compared with saline-treated controls. Overall, these results correlate with the decrease in MMP-9, and the increase in TIMPs observed in the circulation indicate that the intermediate-size TIMP that seemed to be increased in reverse zymograms is TIMP-3 rather than TIMP-4, and suggest that multiple organs contribute to the change in proteolytic profile observed in morphine-treated mice, with the spleen being a major contributor.

**DISCUSSION**

Our data demonstrate that after 3 d of 10 mg/kg, i.p. administration of morphine every 12 h, the amount of circulating MMP-9 is significantly decreased, whereas the amount of MMP-2 is unaffected. This decrease in MMP-9 is accompanied by an increase in circulating TIMP-1 and TIMP-3/4, and the overall functional consequence of these changes is an environment that is less conducive to tumor cell invasion and tumor and endothelial cell migration.

Our previous work had shown that administration of morphine 10 mg/kg, i.p. every 12 h for a 3-d period after tail vein inoculation of tumor cells resulted—18 d after inoculation—in a decrease in both tumor load in the lungs and matrix metalloproteinases in the circulation (8). These results could suggest: 1) that morphine decreases tumor growth,
which results in fewer circulating proteases; 2) that morphine reduces the proteolytic potential at the extravasation site and in the tumor microenvironment, which, in turn, reduces tumor growth; or 3) that tumor load and circulating proteolytic profile are both regulated by morphine without mediating one another. We now show that morphine decreases circulating matrix proteolytic potential irrespective of the presence of tumors.

The literature on the opioid regulation of MMP-9 production in nontumor contexts indicates that increased MMP-9 mediates undesirable effects of opioid administration: an increase in spinal cord and DRG MMP-9 activity and expression was observed with remifentanil administration in a rat model of postincisional hyperalgesia (13); µ-opioid receptor–dependent up-regulation and increase in activity of MMP-9 by morphine in DRG in vitro and in vivo was shown to mask morphine analgesia (6). In a mouse model of morphine tolerance in which mice were administered 10 mg/kg morphine daily for 5 d, MMP-9 expression and activity were increased in the midbrain and the development of tolerance was attenuated by MMP-9 pharmacologic inhibition or gene disruption (14). In similar studies, increased spinal MMP-9 production was also reported to mediate dependence on morphine in mice that were subjected to 7 escalating doses of morphine (20–100 mg/kg) administered every 8 h (15). Our results show that morphine administration (10 mg/kg every 12 h for 3 d) results in diminished, rather than increased, MMP-9 and further identifies some organs that contribute to this change at the transcriptional level. Although we did not examine spinal or DRG expression of MMP-9 mRNA, our data demonstrate that it is unchanged in the brain in our experimental conditions. In agreement with our results, endorphin and enkephalin prevented MMP-9 expression by synovial cells that were isolated from patients with rheumatoid arthritis and this effect was reversed by naloxone (16).

The mechanisms by which morphine administration results in decreased production of MMP-9 in vivo are not known and could be direct or indirect in nature. Regulation of MMP-9 expression involves a number of transcription factors that bind to the promoter region, and down-regulation of MMP-9 transcription often results from interference with the binding of AP-1 and NF-κB (17). Morphine has been shown to negatively regulate NF-κB upon long exposure (18–20) and to inhibit AP-1 (18, 21). Furthermore, transcriptional regulation of MMP-9 has been shown to be modulated by MAPK signaling (17), which can also be inhibited (18, 22) or stimulated (23, 24) by morphine. Lastly, NO could mediate the effects of morphine on MMP-9 expression in vivo, as morphine can increase NO production (21, 25, 26) and NO decreases MMP-9 (27–29). In addition, indirect effects of morphine administration on MMP-9 production can be envisioned [e.g., MMP-9 expression is regulated by cytokines (17), many of which are altered upon morphine administration (30)].

Figure 5. Effect of MMP-9 inhibition on saline-treated and morphine-treated mouse serum-induced 4T1 cell migration (A) and invasion (B). MMP-9 inhibitor I was added at 500 nM (migration) or 1 μM (invasion) to Boyden chamber or transwell experimental systems that contained serum that was collected from saline-treated mice or mice treated with 10 mg/kg morphine at d 3, 12 h. Results are shown as means ± SEM (n = 6 individual mouse samples per group). **P < 0.05, ***P < 0.01 (unpaired Student’s t test).
variability, and resulted in significant activity alterations in the reverse zymograms for TIMP-1 and TIMP-3/4. Our results are consistent with a study that has shown a 3-fold increase in TIMP-1 mRNA in DRG neurons of morphine-treated rats, although that same study unveiled a 2-fold increase, not decrease, in MMP-9 mRNA (6). Of importance, a decrease in MMP-9 associated with an increased in TIMP generates an environment of lower proteolytic power, which corresponds to a dramatically decreased MMP-9-to-TIMP ratio (8) and likely explains, at least in part, the functional consequences of invasion. Involvement of the spleen in the response of mice to intermittent morphine administration is reminiscent of our previous observations that involved the spleen as the source of increased TNF-α (11), and suggests that immune cells may be responding to this paradigm of morphine administration by increasing TIMP production. In contrast, multiple tissues seemed to contribute to the decrease in circulating MMP-9.

Overall, these results demonstrate that morphine administration modulates the circulating proteolytic profile and suggest that this may impact processes that rely on matrix degradation. In the context of cancer, this novel mechanism may enable morphine administration to promote an environment that is less conducive to tumor growth, invasion, and metastasis. Clinical studies that correlate the administration of morphine with circulating gelatinase and TIMP levels are needed to confirm our preclinical findings.

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AUTHOR CONTRIBUTIONS

N. Xie, B. Anand-Apte, and M.-O. Parat designed research studies and interpreted results; N. Xie, S. Khambazi, Z. D. Nassar, and M.-O. Parat established/optimized analytical methods; N. Xie conducted experiments; N. Xie and M.-O. Parat analyzed data; N. Xie and M.-O. Parat wrote the manuscript; and S. Khambazi, Z. D. Nassar, K. Gregory, T. Vithanage, B. Anand-Apte, P. J. Cabot, D. Sturgess, and P. N. Shaw critically edited the manuscript.

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Figure S1. Effect of morphine and M3G on the migration of BAEC, 4T1 and LEC cells. Migration of BAEC (A), 4T1 (B) and LEC (C) toward medium with indicated concentrations of morphine, M3G or their respective solvent in the absence/presence of untreated mouse serum was determined. Results are expressed as the percent of migration towards medium containing serum only. Mean ± SEM is shown, n=3 independent experiments.
Figure S2. Lack of effect of MMP-9 inhibitor I on the viability of 4T1 cells. 4T1 cells were exposed to MMP-9 inhibitor I at indicated concentrations. All wells contained 0.1% (V/V) DMSO and 2% (V/V) serum, (consistent with migration and invasion assays) for either 4 h (A) or 24 h (B). Viability was assessed with the MTT assay. Results are expressed as the percent of control and shown as mean ± SEM with triplicate determination for each group.