Brazilian Journal of Anesthesiology 2023;73(4): 441—445

Sociedade Brasileira de Anestesiologia

Brazilian Journal of ANESTHESIOLOGY

EXPERIMENTAL TRIALS

Morphine promotes migration and lung metastasis of mouse melanoma cells

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Received 31 January 2021; accepted 24 October 2021
Available online 1 February 2022

KEYWORDS
Morphine;
toll-like receptor-4;
melanoma;
metastasis

Abstract

Background: Morphine is an analgesic agent used for cancer pain management. There have been recent concerns that the immunosuppressant properties of morphine can also promote cancer metastasis. Morphine is an agonist for toll like receptor 4 (TLR4) that has a dual role in cancer development. The promotor or inhibitor role of morphine in cancer progression remains controversial. We investigated the effects of morphine on migration and metastasis of melanoma cells through TLR4 activation.

Methods: Mouse melanoma cells (B16F10) were treated with only morphine (0, 0.1, 1, and 10 μM) or in combination with a TLR4 inhibitor (morphine10 μM +CLI-095 1μM) for either 12 or 24 hours. Migration of cells was analyzed by transwell migration assays. Twenty C57BL/6 male mice were inoculated with B16F10 cells via the left ventricle of the heart and then randomly divided into two groups (n = 10 each) that received either morphine (10 mg.kg^{-1}, sub-q) or PBS injection for 21 days (control group). Animals were euthanized and their lungs removed for evaluation of metastatic nodules.

Results: Morphine (0.1, 1, and 10 μM) increased cell migration after 12 hours ($p < 0.001$) and after 24 hours of treatment with morphine (10 μM) ($p < 0.001$). Treatment with CLI-095 suppressed migration compared to cells treated with morphine alone ($p < 0.001$). Metastatic nodules in the morphine-treated group (64 nodules) were significantly higher than in the control group (40 nodules) ($p < 0.05$).

Conclusion: Morphine increases the migration and metastasis of mouse melanoma cells by activating TLR4.

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https://doi.org/10.1016/j.bjane.2021.10.019

Introduction

Pain associated with cancer severely impacts the patient's quality of life and treatment protocols.[1] Opioids such as morphine are frequently applied in surgery for tumor removal in patients with cancer.[2,3] However, the use of morphine can have immunosuppressant effects which can paradoxically promote cancer progression and metastasis,[4,5] although other studies have reported a protective role of morphine against cancer spread.[6-8] Numerous studies conducted *in vivo* and *in vitro* demonstrate dual effects of morphine on cancer cell proliferation, survival and migration, which are related to the dose, duration of drug use and receptor subtypes activated by morphine.[9-15] The μ-opioid receptor subtype is the primary target for the analgesic effects of morphine, although recent studies suggest opioid-receptor independent effects mediated by other pathways such as the activation of toll-like receptor 4 (TLR4).[16-18]

Morphine and its metabolites activate TLR4 during innate immunity responses.[19] TLR4 is expressed in various immune and non-immune cell types,[20] and also in malignant cells and cells in the tumor microenvironment that can influence metastasis.[21] Morphine activates TLR4 but suppresses lipopolysaccharide (LPS)-induced TLR4 activation.[22,23]

TLR4 is overexpressed during the cellular transformation of some cancers.[24,25] An overexpression of TLR4 is associated with a poor prognosis of tumor size, invasion and metastasis.[26] We hypothesized that using morphine in cancer pain management, increases cancer cell metastasis by activating TLR4 signaling pathways. We recently reported that the overexpression of TLR4 in melanoma and breast cancer cells increased cell proliferation and tumor size, and that inhibition of TLR4 suppressed melanomas *in vitro* and *in vivo*.[27-29] Our previous investigation showed that the effects of morphine on TLR4 expression in breast cancer cells were time and concentration dependent.[30] The current study further investigates the role of TLR4 in morphine induced migration and metastasis of melanoma cells *in vitro* and *in vivo*.

Methods

Cells and reagents

Mouse melanoma cells (B16F10) were obtained from the National Cell Bank of Iran (affiliated to the Pasteur Institute, Tehran, Iran). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). CLI-095 [resatorvid, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate] was provided by InvivoGen (San Diego, CA, USA). Morphine sulfate was purchased from Temad (Temad Co, Tehran, Iran).

Cell culture

Mouse melanoma cancer cells (B16F10) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Cells were sub-cultured in fresh media when they reached 80% confluence.

Trans-well migration assay

To estimate the effects of morphine on melanoma cell migration and its interaction with TLR4, B16F10 cells were cultured in T-25 flasks and treated with morphine (0.1, 1, and 10 μM) for either 12 or 24 hours. Untreated B16F10 cells (no morphine) were used as controls. The interaction of morphine with TLR4 was examined in other groups of B16F10 cells cultured in T-25 flasks and treated with CLI-095 (a TLR4 inhibitor, 1 μM) with or without morphine (10 μM) for 12 or 24 hours. The culture medium was discarded after incubation and cells were washed three times with phosphate-buffered saline (PBS).

Cell migration was assayed using transwell chambers with 8 μm pore size inserts (BD Biosciences, USA). In brief, 1×10^3 serum-starved cells from each group were suspended in serum free medium and transferred to the upper chambers of each transwell plate. The lower chambers contained fresh medium either with or without FBS (10%) as a chemo-attractant. The cells remaining on the upper surface of the membrane were removed with a swab after 24 hours, while those cells that migrated to the lower membrane surface were fixed with 100% methanol (5 minutes) and stained with 0.5% crystal violet (5 minutes). The number of cells that migrated through the filter were photographed and counted using a Leica microscope equipped with a Leica camera (DFC450 C) at a magnification of 200 x. Pictures of five randomly chosen visual fields were taken and the number of cells that migrated were counted using ImageJ 1.8.0 software (National Institutes of Health).[31] The average percent of migrating cells was calculated.

Experimental metastasis by intra-cardiac injections

All animal experiments followed the ARRIVE (Animals in Research: Reporting In vivo Experiments) guidelines and ethical standards of the Iran National Committee for Ethics in Biomedical Research. The project was approved by the Ethics Committee of Isfahan University of Medical sciences (approval ID: IR.MUI.MED.REC.1398.118). In total, twenty male C57BL/6 mice (eight weeks old, weighing 23 $\pm$ 2 g) were purchased from the Pasteur Institute of Iran (Tehran). The mice were housed using a 12/12 hour light/dark cycle at 25 $\pm$ 2°C for one week before starting the experiments. The mouse model of experimental metastasis was created using an intracardiac injection of tumor cells after they were anesthetized (100 mg.kg^{-1} ketamine and 10 mg.kg^{-1} xylazine). Mice were restrained on their backs, shaved, and disinfected with antiseptic solution. A single-cell suspension of B16F10 cells (3×10^5/100 μL PBS) was then introduced into the left ventricle of the heart.[30] Mice were then randomly divided into two groups (n = 10 per group) after three days: mice in the control group received only vehicle while those in the treated group received daily injections of morphine (10 mg.kg^{-1}, subcutaneous) for 21 days.[32] Animals were euthanized at the end of the treatments and their lungs removed for evaluation of metastatic nodules.

Counting of lung metastatic nodules

Lungs were removed from the mice after 21 days and tumor nodules on the lung surface were counted using a light

microscope. The combined sum of the gross and microscopic counts was taken as the final count of metastatic lung nodules; a false count was defined as no metastatic nodules reported under gross or microscopic observations. All experiments were done in a blind manner.

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Changes observed in treated groups compared with the control group were analyzed with a one-way ANOVA followed by Bonferroni's post-test and Student's *t*-test. Statistical significance was set for *p*-values of < 0.05.

Results

Morphine enhances the *in vitro* migration of melanoma cells

The migration of B16F10 mouse cancer cells increased after 12 hours treatment with morphine, 0.1 (25.43 ± 0.87%), 1 (25.12 ± 0.26%) and 10 μM (27.2 ± 0.92%), compared with 19.57 ± 0.67% in the control group ($p < 0.001$) (Fig. 1). Treatment with different doses of morphine indicated that only morphine (10 μM) increased cell migration (27.8 ± 1.07%) compared to the control group (19.36 ± 0.39%) ($p < 0.001$) (Fig. 2).

Effects of CLI-095, an inhibitor of TLR4, on morphine induced cell migration

Mouse melanoma cells were pre-incubated with CLI-095 (1 μM) for 1 hour at 37°C to examine the role of TLR4 in morphine-induced migration of melanoma cells. Migration of melanoma cells induced by morphine (10 μM) was significantly decreased after pretreatment with CLI-095 for either 12 or 24 hours, ($p < 0.001$) (Figs. 1 and 2).

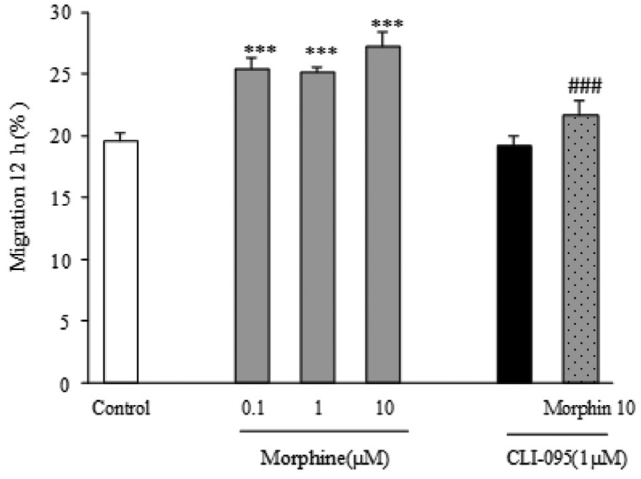

Figure 1 Effect of morphine on B16F10 cell migration after 12 h treatment. B16F10 cells were incubated with morphine (0, 0.1, 1, 10 μM) for 12 h. In the other experiment the cells were treated with morphine (10 μM) with or without CLI-095. After incubation a transwell migration assay was done. *$p < 0.001$ compared to the negative control, #$p < 0.001$ compared to morphine 10 μM. One representative experiment of three is depicted. Each graph has been represented as mean ± SEM.

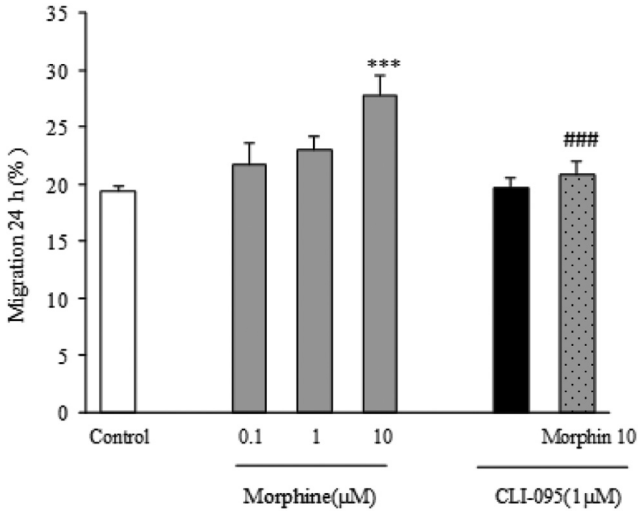

Figure 2 Effect of morphine on B16F10 cell migration after 24 h. B16F10 cells were incubated with morphine (0, 0.1, 1, 10 μM) for 24 h. In the other experiment the cells were treated with morphine (10 μM) with or without CLI-095. After incubation a transwell migration assay was done. *$p < 0.001$ compared to the negative control, $^{\#}p < 0.001$ compared to morphine 10 μM. One representative experiment of three is depicted. Each graph has been represented as mean ± SEM.

Effect of morphine on lung metastasis induced by B16F10

The mouse model of experimental metastasis was created using an intracardiac injection of B16F10 cells. None of the mice died before the end of the study and there were no significant differences in body weight between the two groups. Mice were sacrificed 21 days after treatment with morphine (10 mg.kg^{-1}) and metastasis nodules in their lungs counted. The lungs of mice injected with morphine contained more metastatic nodules ($p < 0.05$) (Fig. 3).

Discussion

We examined the ability of morphine to stimulate melanoma metastasis by activation of TLR4. Our findings indicate that morphine increased the number of migrating B16F10 cells *in vitro* and promoted pulmonary metastasis *in vivo*.

Melanoma is an aggressive form of skin cancer characterized by rapid growth and early metastasis to other organs such as the lungs, liver, bone or brain.[33-35] Activation of inflammation promotes melanomas,[36] with increases in the inflammation level related to the over-expression of TLR.[37,38] Morphine can both inhibit or stimulate immune cell function to affect cancer progression.[39] Morphine binds to myeloid differentiation protein 2 (MD-2), a TLR4 accessory protein, and activates TLR4 to increase cancer metastasis.[40]

The effect of morphine on cell migration is time and dose dependent.[41] Our results indicate that morphine (0.1, 1, 10 μM) increases the migration of B16F10 cells after 12 hours, or after treatment with morphine (10 μM) 24 hours. Treating

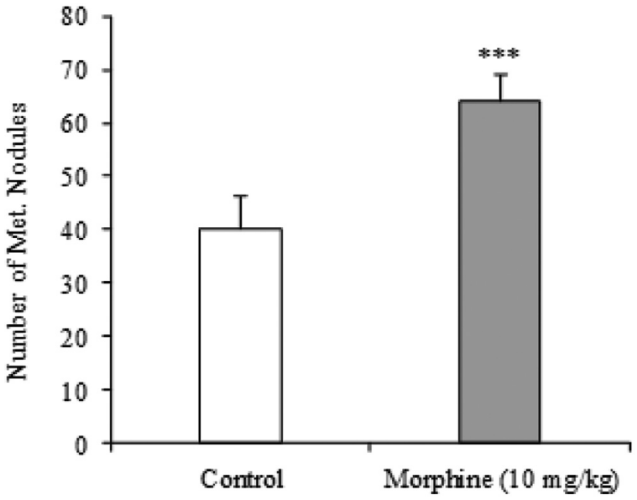

Figure 3 The number of tumor nodules on the lung. The C57BL/6 mice received left heart ventricle injection of B16/F10 melanoma cells and were treated with morphine (10 mg.kg^{-1}) or PBS for 21 days. ***$p < 0.001$ compared to the negative control, each graph has been represented as mean ± SEM.

cells with low concentrations of morphine for 24 hours mimicked the effects of a single dose of morphine,[6] as shown in Figures 1 and 2. The underlying mechanism has not been understood yet, the nature of opioid receptor may be the key to this mechanism.[42] Morphine increased the number of lung metastatic nodules compared to the control group.

Reports on the effects of morphine on metastasis are contradictory, as both inhibitory and stimulatory effects have been observed. For example, some studies suggested that morphine inhibited metastasis in animal models of cancer,[7,8] while other studies report that clinically relevant doses of morphine increased tumor growth and angiogenesis in a mouse model of breast cancer,[43,44] and tumor growth and sarcoma in a mouse model of leukemia.[14] These contradictory results are likely due to differences in the concentration, type and time of administration of morphine.[13] Administration of low daily doses or a single dose of morphine enhances tumor growth,[45] while high doses of morphine inhibit tumor progression.[7,8,12] Generally, the effect of morphine on cancer progression is dependent on the cancer type because, different cancer cells express different opioid receptors[42]; we show that TLR4 may be one of key receptors involved in morphine effects.

This study has some limitations. First is that we did not evaluate the *in vivo* effect of the TLR4 inhibitor in the presence of morphine. Second, we did not evaluate the dose related effects of the TLR4 inhibitor. Third, we did not measure activation of the downstream targets of TLR4 activation. Next, we did not evaluate the activity of TLR4 after its increase in expression. Last, we did not evaluate cancer pain of mice in this study.

Conclusion

Our results suggest that morphine increases melanoma cell migration by activating TLR4. Overexpression of TLR4 is associated with tumor metastasis. Further studies are needed to determine the role of TLR4 in the management of cancer pain with morphine. However, we still need to fully understand the adequate dose of morphine required to reduce cancer pain in patients.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This article was part of a PhD thesis submitted at the Isfahan University of Medical Sciences with a grant number of No. 397808 and project number 194230.

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