Exogenous morphine inhibits the growth of human gastric tumor *in vivo*

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Abstract: Morphine is commonly used to relieve severe pain that is often associated with cancer. Previous studies have found that morphine could affect cancer development; however, this effect is poorly understood. To further clarify the anti-cancer potential of morphine for the development of cancer *in vivo*, we observed how morphine affects the growth of human gastric tumor in a murine xenografting model and the expression of NF-κB and its downstream target genes (Bcl-2/Bax, cyclind1, and VEGF). The growth of the tumor was evaluated by its growth curves. The mRNA expression levels of NF-κB, Bcl-2/Bax, cyclind1, and VEGF were assessed by semi-quantitative polymerase chain reaction (qPCR). The protein expression of NF-κB, Bcl-2/Bax, cyclind1, and VEGF was detected by immunochemistry staining and western blot. Our data showed that morphine effectively inhibited the tumor growth in the nude mice. Morphine inhibits the expression of NF-κB, Bcl-2, cyclind1, and VEGF while enhancing the expression of Bax in the tumors. Furthermore, the anti-cancer effects of morphine could be reversed by naloxone. The mechanism might be associated with the action of opioid receptors that downregulate the expression of NF-κB leading to the regulation of the downstream target genes (Bcl-2/Bax, cylind1, and VEGF) in the tumors.

Keywords: Morphine; gastric cancer; nude mice; tumor growth; NF-κB

Introduction

Morphine is the most common analgesic used to relieve pain especially in patients with cancer. Since morphine has been widely used to alleviate severe cancer pain, there has been extensive research indicating that morphine can affect tumor development both *in vitro* and *in vivo*. For example, morphine promoted Jurkat cell apoptosis through FADD/p53 and NF-κB pathways (1), and morphine dose-dependently induced apoptosis in isolated cultured neuroblastoma cell (2). Our previous study also found that exogenous morphine could inhibit gastric cancer progression *in vitro* (3). Additionally, *in vivo* studies have demonstrated that morphine can inhibit tumor angiogenesis

in nude mice, thereby inhibiting tumor progression (4).

Although the effects of morphine and other opioids on tumor growth have been discussed in many studies, the specific mechanisms by which morphine influences tumor proliferation, invasion, and metastasis are far from being fully understood. Many researchers have observed that morphine inhibits tumor progression through some pathways in tumor cells. Tegeder *et al.* reported that morphine can regulate the cell cycle and apoptosis by activation of p53, inhibiting the cell cycle of tumor growth in adenocarcinoma cells (5). The attenuation of matrix metallopeptidases (MMP) secretion caused by opioids has been discovered to be under the control of the nitric oxide system (6). Moreover, morphine might alter the effect

Page 2 of 7

Li et al. Morphine inhibits the growth of gastric cancer

of tumor progression through c-Jun N-terminal kinase (JNK)/caspase pathway and HIF-1alpha/p38MAPK pathway (2,3). In our previous study, we proposed that the mechanism by which morphine inhibits the progression of gastric cancer *in vitro* could have associations with the triggering of apoptosis and the inhibition of NF-κB. NF-κB is an important nuclear transcription factor that severely affects the regulation of a numerous downstream target genes associated with apoptosis, tumorigenesis, immune diseases, and inflammation (7-9). There is also evidence that the inhibition of NF-κB might suppress tumor growth (10).

In the study presented here, we investigated the effects of exogenous morphine on gastric cancer progression *in vivo* and the expression of NF-κB and its downstream target genes, Bcl-2/Bax, cyclind1, and VEGF, which are related to apoptosis, cell cycle, and tumorigenesis, respectively, in a nude mice xenografting model using human gastric cancer MGC-803 cells. The goals of our research are to explore the further possible mechanism of morphine affecting tumor progression and to provide some practical suggestions for the treatment of cancer pain.

Methods

Cell culture

We obtained MGC-803 cells from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), which were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 75 U/mL streptomycin and 100 U/mL penicillin at 37 ℃ and 5% CO_2 in a standard humidified incubator.

Animals

We obtained 40 male Bagg Albino/c (BALB) nude mice with weight ranging between 20–25 g and the age ranging from 5–6 weeks from the Experimental Animal Center of Guangxi Medical University (Nanning, China). The environmental temperature and relative humidity were kept at 25 ℃ and 50%, respectively. The care and treatment of all animals were in keeping with the guidelines of the Experimental Animal Management Institution of Guangxi Medical University.

Nude mice xenograft model

To determine the effects of morphine on the development of human gastric cancer MGC-803 cell line *in vivo*, MGC-803 cells (2×10^6, 200 µL of PBS) were injected subcutaneously into the left flanks of the mice. The mice were randomly divided into 8 groups (n=5 each): group C (control group), group NS (normal saline group), group M (morphine group), group N (naloxone group), and group MN (morphine and naloxone group). Group C did not receive any treatment while the corresponding control group (group NS) was treated with normal saline. The mice in group M, M1, M2, M3, and M4 were injected with 5 mg/kg morphine, 10 mg/kg morphine, 20 mg/kg morphine, and 40 mg/kg morphine, respectively. Group N was treated with 1 mg/kg naloxone. Group MN was treated with 1 mg/kg naloxone 30 min prior to morphine injection (20 mg/kg). The solutions for all the groups were injected intraperitoneally using a microsyringe every 2 days.

Tumor measurement

The tumor volume was determined using a caliper and calculated by the following formula: length × width^2/2. All the groups were measured at days 2, 6, 10, and 14 to obtain tumor growth curves. Animals were sacrificed after the 14 days, and the tumors were extirpated and compared for volume and weight.

Immunochemistry staining

Paraffin tissue sections were deparaffinized using turpentine with rehydration occurring in a descending ethanol series following normal procedures. Heat-induced antigen retrieval was completed in 0.01 mol/L citrate buffer at 100 ℃ for 20 min. Antigen retrieval with enzyme digestion was completed in 0.1% trypsin at 37 ℃ for 25 min. The sections were subsequently blocked with serum for 30 min and incubated with primary antibodies (anti-NF-κB, anti-Bcl-2, anti-Bax, anti-cyclind1, or anti-VEGF) (CST, USA) for 1 to 2 hours at 37 ℃. After being washed, the sections underwent incubation with horseradish peroxidase-goat anti-rabbit antibody at 37 ℃ for 30 minutes. The peroxidase reaction was performed using 3, 3-diaminobenzidine (DAB) solution in DAB buffer substrate. Sections were visualized with DAB, counterstained with hematoxylin, mounted in neutral gum,

and analyzed with a bright-field microscope.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA (2.5 μg) was reverse transcribed with the Prime Script RT Master Mix Kit (Takara, Tokyo, Japan) following the manufacturer's protocol. The genes were amplified from cDNA by PCR. The cDNA was amplified with PCR using the following specific primers: Bcl-2 forward 5'- GACTTCGCCGAGATGTCCAG -3', reverse 5'-CATCCCAGCCTCCGTTATCC-3'; Bax forward 5'-AAGAAGCTGAGCGAGTGT -3', reverse 5'-CCGGAGGAAGTCCAATGTC-3'; cyclind1 forward 5'- CCCTCGGTGTCCTACTTCAA -3', reverse 5'-GGGGATGGTCTCCTTCATCT -3'; VEGF forward 5'- TCGAACTTTGACAGCGACAAGAA -3', reverse 5'-TCAGGGCGAGGACCATAGAGG -3', and GAPDH forward 5'- CGGGAAGCTTGTCATCAATGG-3', reverse 5'- GGCAGTGATGGCATGGACTG-3'. PCR was performed in the following steps: denaturation (5 min at 94 ℃), amplification (95 ℃ for 30 s, 54–58 ℃ for 1 min, and 72 ℃ for 1 min), and extension (72 ℃ for 5 min). RT-qPCR was conducted with the One Step SYBR® Prime Script® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China). The GAPDH mRNA level was used as an internal control. The relative expression of mRNA was assessed using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

The proteins from tumor tissues were washed with 1× PBS and lysed with RIPA buffer consisting of protease inhibitors (Beyotime Biotechnology, Shanghai, China). Afterwards, the proteins were collected and extracted, and their concentrations were evaluated (BCA Protein Assay Kit, Beyotime Biotechnology Co., Ltd., Haimen, China). The samples (20 μg/lane) were electrophoresed by 12% SDS-PAGE and electrically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) in transfer buffer. Following being blocked with 5% bovine serum albumin in Tris-buffered saline/Tween 20 (TBST) for 2 h at room temperature, the membranes underwent incubation at 4 ℃ overnight using the following primary antibodies: anti-NF-κB (#4764, CST, USA), anti-Bcl-2 (#3498, CST, USA), anti-Bax (#5023, CST, USA), anti-cyclind1 (#2978,

CST, USA), anti-VEGF (#2479, CST, USA), and GAPDH (ab181602, Abcam). Following incubation with the suitable primary antibodies, the membranes were washed 3 times and incubated for 1.5 h at room temperature using a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (ab205718, Abcam). The quantification of immunoreactive intensity was visualized with ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA). The relative amounts of protein in each lane were obtained after normalization with the GAPDH values in the same lane.

Statistical analysis

Date are presented as the mean ± SEM and were calculated with one-way analysis of variance (ANOVA) and t test with Dunnett's comparison using Prism (version 6.0; GraphPad Software Inc., San Diego, CA, USA). A P value <0.05 was taken as indicating a statistically significant difference.

Results

Morphine inhibits growth of human gastric cancer in nude mice.

The growth of the tumor was evaluated by the growth curves of the tumor. According to the results, the tumor growth in group M (M1, M2, M3, M4) were obviously inhibited in comparison to those in groups C, NS, N, and MN (P<0.05) (Figure 1).

Morphine reduced the expression levels of bcl-2, cyclind1, and VEGF, while raising Bax expression.

Cyclind1, bcl-2, Bax, and VEGF were selected to estimate the expression differences between the tumors in different treatment groups by RT-qPCR. According to the results, cyclind1, Bcl-2, and VEGF mRNA of tumors in group M (M1, M2, M3, M4) were obviously inhibited in comparison to those in groups C, NS, N, and MN, while the expression of Bax was increased (P<0.05) (Figure 2).

Morphine decreased the expression of NF-κB, Bcl-2, cyclind1, and VEGF and increased the expression of Bax.

The expression of protein in tumor tissue was detected by immunochemistry staining.

As shown in Figure 3, the positive cells of NF-κB, Bcl-2, cyclind1, and VEGF in group M were more than those in group C, NS, N, and MN, while the number of positive cells of Bax in group M was less than those in groups C, NS, N, and MN. (P<0.05). The related protein changes of tumor tissue were detected by a western blot
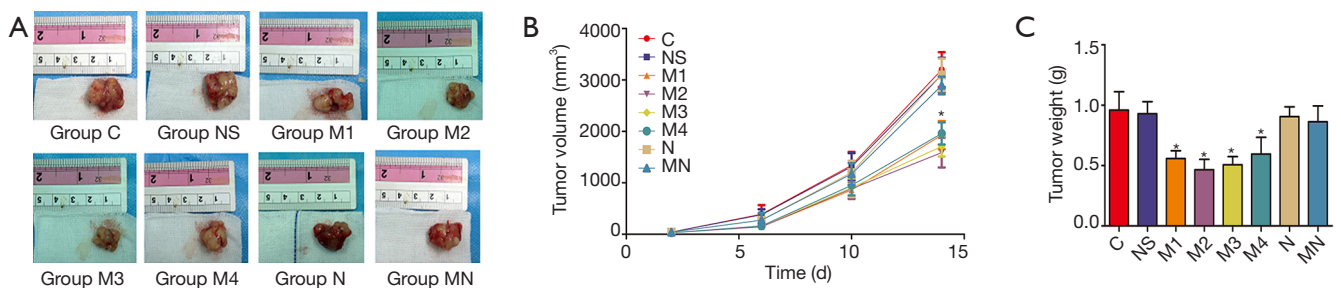
Figure 1 Growth curves of tumor xenografts in nude mice. (A) The growth of the tumor was observed on days 2, 6, 10, and 14. (B) The mean tumor size of mice in each group. (C) The tumor weight of mice in each group on day 14. Data are expressed as the mean ± SEM. *, P<0.05 *vs.* group C, NS, N and MN. Group C, control; Group NS, 1.5 mL/kg NS; Group M1, 5 mg/kg morphine; Group M2, 10 mg/kg morphine; Group M3, 20 mg/kg morphine; Group M4, 40 mg/kg morphine; Group N, 1 mg/kg naloxone; Group MN, 20 mg/kg morphine and 1 mg/kg naloxone.
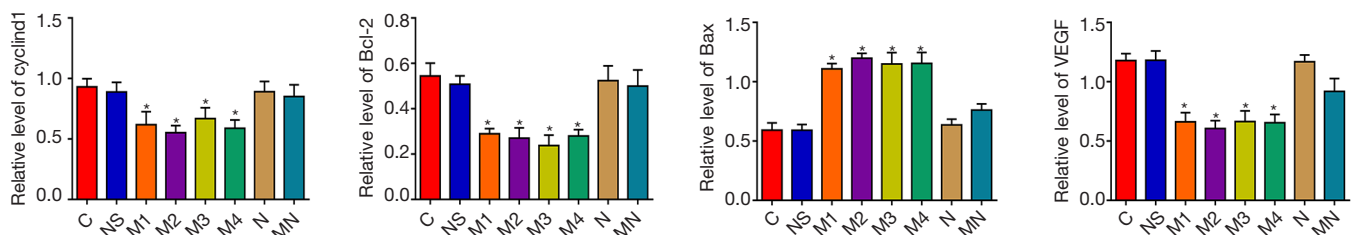
Figure 2 Morphine induces Bcl-2, cyclind1, and VEGF mRNA expression downregulation and upregulation of Bax in tumors of nude mice. Data are expressed as the mean ± SEM. *, P<0.05 *vs.* group C, NS, N and MN. Group C, control; Group NS, 1.5 mL/kg NS; Group M1, 5 mg/kg morphine; Group M2, 10 mg/kg morphine; Group M3, 20 mg/kg morphine; Group M4, 40 mg/kg morphine; Group N, 1 mg/kg naloxone; Group MN, 20 mg/kg morphine and 1 mg/kg naloxone.

assay. According to the results, NF-κB, Bcl-2, cyclind1, and VEGF protein of tumors in group M were lower, while that of Bax was higher compared with groups C, NS, N, and MN, (P<0.05) (*Figure 4*).

Discussion

The incidence and death rate of cancer are increasing, and cancer pain is one of the most common clinical symptoms in the middle and late stages of cancer. Morphine, a representative of potent opioids, is frequently used for moderate to severe cancer pain. Previously, morphine and other opiates were found to have an anti-cancer effect, including the promotion of cell death in tumor cells. Kawase *et al.* (11) reported that opiates could induce cell death in human oral tumor cell lines. While relieving pain, morphine significantly inhibited melanoma growth and lung metastasis in a mouse model of cancer pain (12). Koodie *et al.* (13) also demonstrated that morphine could inhibit migration and suppress angiogenesis associated

with tumor growth in mice. On the other hand, morphine was also found to increase the proliferation of tumor cells and promote angiogenesis, leading to the acceleration of tumor progression. Additionally, morphine was also shown to induce cancer recurrence by disturbing the behavior of the regulatory T cells (14). In another study, morphine promotes the angiogenesis of postoperative recurrent tumors and metastasis by PI3K-c-Myc pathway (15). Therefore, the effects of morphine on cancer development have been a controversial topic affecting its clinical use, and the way that morphine influences cancer growth is not clear.

Our previous study showed that morphine could inhibit human gastric tumor cells *in vitro*, and the effects might be related to the activation of caspases and the inhibition of NF-κB and survivin. In the current study, we observed how morphine affected the growth of human gastric tumor in a murine MGC-803 cell xenografting model and the expression of NF-κB and some of its downstream target genes. Our data showed that morphine effectively inhibited the tumor growth in the nude mice. Morphine
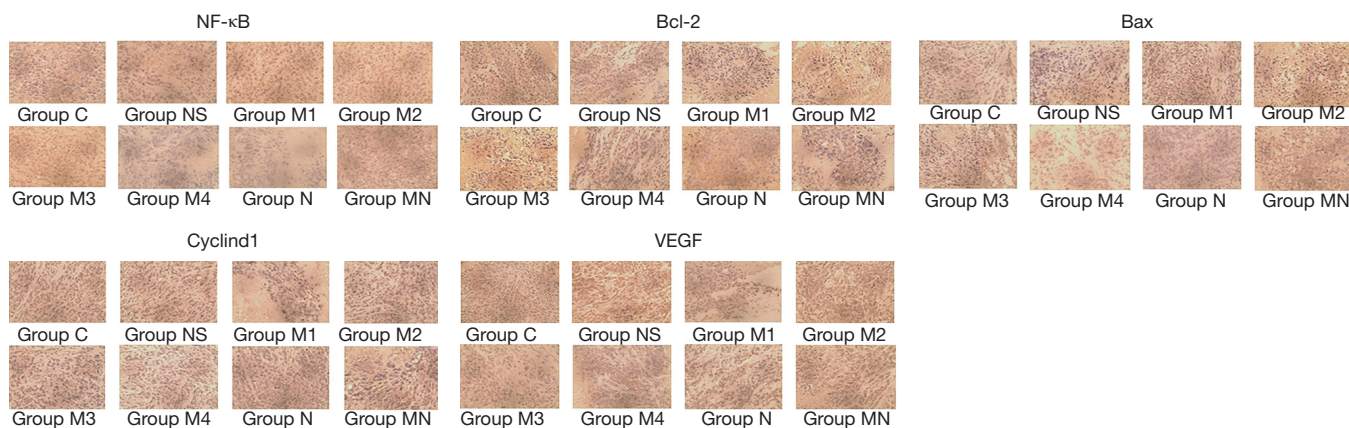
Figure 3 Morphine increases Bax protein expression while NF-κB, Bcl-2, cyclind1, and VEGF are decreased in tumors of the nude mice using immunochemistry staining. Group C, control; Group NS, 1.5 mL/kg NS; Group M1, 5 mg/kg morphine; Group M2, 10 mg/kg morphine; Group M3, 20 mg/kg morphine; Group M4, 40 mg/kg morphine; Group N, 1 mg/kg naloxone; Group MN, 20 mg/kg morphine and 1 mg/kg naloxone.
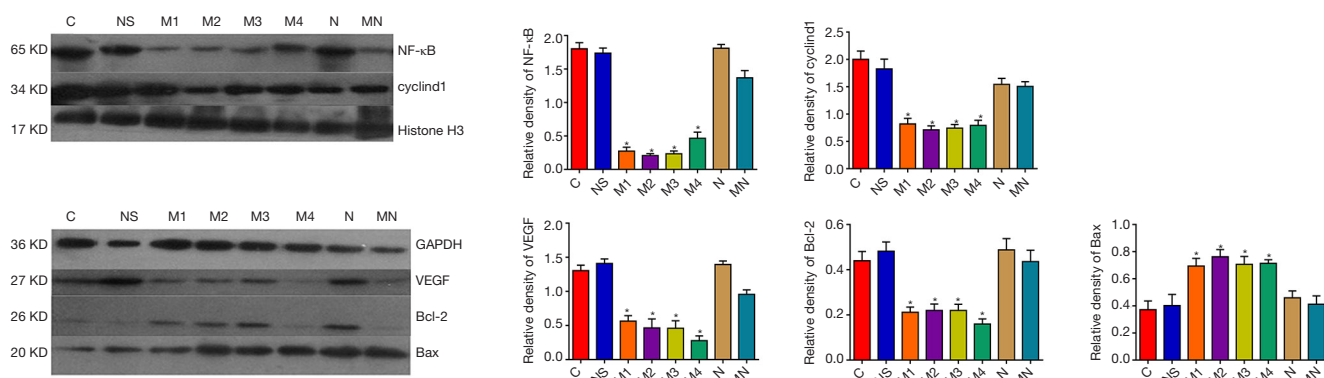

Figure 4 Morphine increases Bax protein expression while NF-κB, Bcl-2, cyclind1. and VEGF are decreased in tumors of the nude mice through western blot. Data are expressed as the mean ± SEM. *, P<0.05 *vs.* group C, NS, N and MN. Group C, control; Group NS, 1.5 mL/kg NS; Group M1, 5 mg/kg morphine; Group M2, 10 mg/kg morphine; Group M3, 20 mg/kg morphine; Group M4, 40 mg/kg morphine; Group N, 1 mg/kg naloxone; Group MN, 20 mg/kg morphine and 1 mg/kg naloxone.

downregulated the expression of NF-κB, Bcl-2, cyclind1, and VEGF, while upregulating Bax in the tumors.

NF-κB activation may promote tumor growth, and the inhibition of NF-κB could suppress the development of cancer. A few other studies have also speculated that the mechanism of the anti-cancer effects of morphine might involve NF-κB inhibition (16). Bcl-2 and Bax belong to the Bcl family which is critically involved in the regulation of apoptosis, with Bcl-2 inhibiting apoptosis and Bax promoting apoptosis. The decrease of Bcl-2/Bax ratio could induce the apoptosis (17). In our study, morphine downregulated the expression of Bcl-2 while Bax expression

was upregulated, which might induce apoptosis of the cancer cells in the tumor and result in the inhibition of tumor growth. Cyclind1 is one of the regulators of the cell cycle, the overexpression of which will result in malignant cell proliferation (18,19). In this study, morphine inhibited the expression of cyclind1, and reduced cell proliferation. Meanwhile, VEGF, which regulates vascular development, plays an important role in tumor angiogenesis, bolsters the forming of new blood vessels to maintain tumor growth, and triggers endothelial cell proliferation and metastasis (20-22). Balasubramanian *et al.* (23) found that morphine could inhibit hypoxia-induced VEGF expression in

Page 6 of 7

Li et al. Morphine inhibits the growth of gastric cancer

cardiac myocytes and endothelial cells, thus inhibiting the neovascularization caused by myocardial ischemia. Koodie *et al.* (4) observed that administration of morphine in clinically relevant analgesic doses significantly reduce tumor cell-induced angiogenesis in a murine Lewis lung carcinoma tumor model. As Bcl-2/Bax, clind1and, VEGF are the downstream target genes of NF-κB, the potent regulation of these genes by morphine may inhibit NF-κB.

Recently, many researchers have found that the μ-opioid receptor (MOR) might regulate cancer progression (24-26). Both MOR knockout and silent MOR expression can markedly attenuated tumor growth and metastasis (27). In the case where tumors that express MOR and have no loss of p53 mutations, opioid receptor antagonists increase the inhibitory effect of morphine on tumor growth (5). These experimental data imply that the MOR is involved in tumor growth and metastasis. Herein, our study data showed that morphine could inhibit tumor progression, while the opioid receptor antagonist, naloxone, could reverse the effects of morphine on tumor progression. This finding suggests that opioid receptors might be involved in morphine's inhibition of tumor development in the MGC-803 cell xenograft model.

Conclusions

In summary, morphine could inhibit human gastric tumor growth *in vivo*: the morphine signals could be transduced by opioid receptors and then downregulate the expression of NF-κB, leading to the regulation of the downstream target genes (Bcl-2/Bax, clind1, and VEGF) and the eventual inhibition of tumor growth.

Acknowledgments

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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