Morphine Promotes the Angiogenesis of Postoperative Recurrent Tumors and Metastasis of Dormant Breast Cancer Cells

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Abstract

Background: Surgery plays a significant role in the comprehensive treatment of breast cancer, and opioids are often the first-choice analgesics in the perioperative period. However, recent studies showed that opioids may enhance the angiogenesis of breast cancer and the recurrence and metastasis of tumor cells. Objectives: We aim to investigate the influence of opioids on recurrence and metastasis of breast cancer in nude mice. Methods: Forty female nude mice with breast tumor were randomly divided into 4 groups (n = 10). They were treated with (i) normal saline (10 mL/kg), (ii) morphine (10 mg/kg), (iii) morphine plus naloxone (10 + 4 mg/kg), and (iv) naloxone (4 mg/kg) for 2 weeks. Four groups of MDA-MB-231 cells were administered (i) Dulbecco’s Modified Eagle’s Medium, (ii) morphine (10 μmol/mL), (iii) morphine plus naloxone (10 + 10 μmol/mL), and (iv) naloxone (10 μmol/mL). The influence of morphine in each treated group was evaluated by immunocytochemistry and Western blotting. Results: Mice in the morphine group had higher rates of Ki67-positive cells, lower rates of apoptotic index, and a significant increase in the microvessels density of the tumor as evidenced by CD31 staining (p < 0.05). Furthermore, the MDA-MB-231 cells in the morphine group showed an increase in p-Akt, c-Myc, and thrombospondin-1 expression. Conclusion: In the current study, we found that morphine promotes the angiogenesis of the recurrent postoperative tumors of nude mice with breast cancer and the proliferation of tumor cells and such promotion may be related to the PI3K-c-Myc signaling pathway.

Introduction

Breast cancer is a serious threat to women’s health and quality of life, and bone marrow micrometastasis is detected in approximately 30.6% of patients with newly diagnosed breast cancer, with possibility of hematogenous spread in the case of early-stage cancer; high-resolution imaging technology is incapable of detecting these micrometastases. Nearly 30% of patients with axillary lymph node-negative breast cancer die within 5 years after surgery [1–3].

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It was generally accepted that the effects of perioperative anesthetics on the body were transient, according to the state of the metabolism of drugs used. Therefore, the body should return to the preanesthetic state after the anesthetics were fully metabolized. However, an increasing number of studies show that anesthetic drugs have long-term effects on the body, which may increase the postoperative metastasis of malignant tumor cells [4, 5]. In a study of 129 breast cancer patients undergoing mastectomy and axillary dissection, a 32 ± 5-month follow-up revealed that the recurrence and metastasis-free survival of paravertebral block and opioid analgesia in general anesthesia patients were 94 and 82% (at 24 months) and 94 and 77% (at 36 months), respectively [6]. This suggests that opioids may be involved in cancer recurrence.

Tumor growth depends on angiogenesis and requires new vessels when the diameter of the tumor exceeds 1 mm. At this point, if angiogenesis fails to occur, the tumor may then behave as an isolated cell or small cell mass and remain quiescent for long periods or known as tumor dormancy. In vitro morphine was found to enhance endothelial cell migration and proliferation in a concentration-dependent manner [1]. Compared to the control (phosphate-buffered saline [PBS]) group, administration of morphine promoted the growth of breast tumors in mice, and the tumor vessels were higher in density and more dilated than mice in the control groups [7, 8]. The opioids may lead to the “switching on” of the tumor angiogenic switch and generating new blood vessels. These new blood vessels may activate “dormant” breast cancer cells, impair the cancer cell proliferation/apoptosis balance, and cause tumor cell proliferation, leading to tumor recurrence and metastasis in breast cancer patients [9]. Therefore, we hypothesize that some breast cancer patients may have micrometastasis during treatment and that these cancer cells are dormant, leading to the inability of general imaging modalities to detect them. Opioids may induce the growth of dormant tumors by promoting vascularization.

Methods

Cell Culture and Animals

Human breast adenocarcinoma cells (MDA-MB-231) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (Grand Island, NE, USA) containing 10% fetal bovine serum (Grand Island, NE, USA) at 37°C, in 5% CO2 atmosphere. Five- to six-week-old BALB/c-nu specific-pathogen-free, SPF nude mice were purchased at the Experimental Animal Center of Sun Yat-sen University (University Town). Nude mice were reared in the SPF barrier environment of the Experimental Animal Center of Sun Yat-sen University and were approved by the ethics committee of Sun Yat-sen University.

Establishment of a Lung Metastasis Model in Nude Mice with Breast Cancer

Pulmonary Metastasis

MDA-MB-231 cells (5 x 10^6/0.2 mL PBS) were inoculated into the left second nipple mammary gland fat pad (n = 24). In order to observe when the lung shown metastasis, a group of nude mice (n = 6) were killed weekly, and paraffin sections were then taken from the lung tissue to perform hematoxylin and eosin staining (H&E) staining. We found that 3 weeks after inoculation, the lung tissue began to show metastasis.

Drug Exposure

MDA-MB-231 cells (5 x 10^6/0.2 mL PBS) were inoculated into the mammary fat pad of nude mice (n = 40). Three weeks after inoculation (the lung tissue has shown metastasis), the mice were anesthetized with inhalation of sevoflurane, and the tumors were removed and discarded. After surgery, the mice were randomly divided into 4 groups (n = 10), each group was treated with an in intraperitoneal injection (i.p.) of (i) normal saline (10 mL/kg), (ii) morphine (10 mg/kg), (iii) naloxone (4 mg/kg), or (iv) morphine + naloxone (10 + 4 mg/kg) for 2 weeks. One week later, the recurrent tumor tissue and lung tissue were removed. A portion of the tumor tissue was frozen in liquid nitrogen and then transferred to a −80°C refrigerator for Western blotting analysis; the other tumor tissues and all the lung tissues were preserved in 10% neutral formalin, and paraffin sections were prepared.

H&E Staining

Representative 4-μm lung cross-sections were cut from the paraffin-embedded tissue, and 3 sections were chosen from each paraffin block. The lung sections were stained with H&E, and 3 different visual fields were selected for each lung tissue to count the number of metastases.

Histology and Immunohistochemistry

The tissue section obtained above was placed in boiling Citric acid buffer (pH 6.0) for 5 min. The section was then further heated for 15 min in low heat. Section 1 was stained with a 1:200 dilution of anti-Ki67 antibody (abcam ab15580, Cambridge, MA, USA), and section 2 was stained with a 1:50 dilution of anti-CD31 antibody (abcam ab28364, Cambridge, MA, USA). To assess the fragmented DNA, the sections were labeled using the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling technique (Roche, Basel, Switzerland). The number of positive cells was counted by random reading in 5 or more high-power fields, and the apoptotic index (AI) was calculated according to the formula:

\[ AI = \frac{\text{positive cell number}}{\text{total cell number}} \times 100\% \]

Western Blot

Protein expression related to tumor recurrence was detected via immunoblot analysis. The recurrent tumor tissue was adjusted to a uniformed concentration of 1.5 μg/μL and denatured at 98°C for 10 min. A total of 30 μg of protein was resolved on 8% sodium
dodecyl sulfate polyacrylamide gel electrophoresis and was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk and incubated with primary antibodies to thrombospondin-1 (TSP-1; 1:1,000 Cell Signaling #14778, Danvers, MA, USA), p-Akt (Ser 473, 1:2,000 Cell Signaling #4060, Danvers, MA, USA), c-Myc (1:1,000 Cell Signaling #13987, Danvers, MA, USA), Akt (1:1,000 Cell Signaling #9272, Danvers, MA, USA), and β-actin (1:1,000 Cell Signaling #4970, Danvers, MA, USA). Antibody binding was detected by incubating blots with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using an enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA).

**Study on the Mechanism of Angiogenesis**

**In vitro Analysis**

MDA-MB-231 cells were grown in large petri dishes. The cells were divided into 4 groups and treated with (i) Dulbecco’s Modified Eagle’s Medium, negative control; (ii) morphine (10 μmol/mL); (iii) naloxone (10 μmol/mL); or (iv) morphine + naloxone (10 + 10 μmol/mL) for 2 h. Cells were then collected, and the total protein was extracted. A total of 50 μg protein was resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and was transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk and incubated with primary antibodies to thrombospondin-1 (TSP-1; 1:1,000 Cell Signaling #14778, Danvers, MA, USA), p-Akt (Ser 473, 1:2,000 Cell Signaling #4060, Danvers, MA, USA), c-Myc (1:1,000 Cell Signaling #13987, Danvers, MA, USA), Akt (1:1,000 Cell Signaling #9272, Danvers, MA, USA), and β-actin (1:1,000 Cell Signaling #4970, Danvers, MA, USA).

**In vivo Analysis**

Forty BALB/c-nu female nude mice were inoculated with MDA-MB-231 cells according to the methods mentioned above. They were then randomly divided into 5 groups (n = 8). After tumor removal, the 5 groups of mice were given (i) normal saline (10 mL/kg; i.p.), (ii) morphine (10 mg/kg; i.p.), (iii) morphine + Akt inhibitor (AZD5363; Beyotime, Shanghai, China; morphine 10 mg/kg; i.p. + AZD5363 200 mg/kg oral), (iv) morphine + c-Myc inhibitor(10058-F4; Beyotime, Shanghai, China; morphine 10 mg/kg; i.p. + 10058-F4 20 mg/kg intravenous injection; i.v.), or (v) morphine + TSP-1 inhibitor (LSKL peptide; a peptide with a sequence of Leu-Ser-Lys-Leu; AnaSpec, Freemont, CA, USA; morphine 10 mg/kg; i.p. + LSKL 30 mg/kg; i.p.) for 2 weeks. Inhibitors dosage was given in accordance with instructions from previous studies [10]. After administration, nude mice were kept under the same conditions for 1 week. Recurrent tumor tissues were removed and stored in 10% neutral formalin. Paraffin sections were prepared for immunohistochemical study of angiogenesis (CD31 marker).

**Statistical Analysis**

All the data are expressed as mean ± SD. Data processed by SPSS 13.0 software (IBM, Armonk, NY, USA), and the statistics icons were plotted with Graphpad Prism 5.0 (GraphPad Prism Inc., San Diego, CA, USA). All data were analyzed by one-way analysis of variance. Differences were considered statistically significant at p < 0.05.

**Results**

**Morphine Promotes Lung Metastasis after Breast Cancer Surgery in Nude Mice**

To mimic the presence of micrometastases in breast cancer patients during surgery, we observed the lung condition of mice. After 3 weeks of inoculation, the lungs began to exhibit signs of metastasis (Fig. 1).

For quantitative analysis of the lung metastases, the lung tissue section was observed under low-power mi-
croscopy, and number of lung metastasis was counted. As seen in Figure 2, observed lung metastases in saline group, morphine group, morphine + naloxone group, and naloxone group were 2.37 ± 1.69, 8.60 ± 0.80, 3.3 ± 1.36, and 2 ± 1.35 (number/field), respectively.

**Morphine Promotes Postoperative Recurrence, Tumor Proliferation, and Angiogenesis and Reduces the Rate of Tumor Cell Apoptosis**

The Ki67 staining of the tumor cells in morphine group was deeper, the increment index was higher, and the number of CD31-positive cells and the microvessel density (MVD) were higher than that in control group (p < 0.01; Fig. 3a, b, e, f). The AI of the tumor cells in morphine group was lower than that of those in control group (p < 0.01; Fig. 3c–e).

**The Effects of Morphine on the PI3K-c-Myc Signaling Pathway in Recurrent Tumors**

To investigate whether morphine promotes tumor growth and if angiogenesis is related to the PI3K-c-Myc signaling pathway, we examined the expression of the related proteins in the recurrent tumor tissues. We found that the expression of proteins p-Akt, c-Myc, and PI3K was higher in the morphine group compared to the control group (p < 0.01; c–e). Compared to the control group, *p < 0.05; compared to the morphine group, # p < 0.05 (e, f). TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling; MVD, microvessel density.

(Figure continued on see next page.)
TSP-1 in morphine group was increased compared to that in control group ($p < 0.05$; Fig. 4). We used morphine and naloxone to treat breast cancer MADMB-231 cells and detected the expression of these proteins. The results were consistent with those observed in animal experiments (Fig. 5). In the nude mice model, we found that when Akt, c-Myc, and TSP-1 inhibitors were added, the angiogenesis of recurrent tumors decreased significantly compared with morphine group ($p < 0.05$; Fig. 6).
Fig. 4. The recurrent tumor tissue was used to detect protein expression. Membranes were blocked with 5% nonfat milk and incubated with primary antibodies to TSP-1 (1:1,000), p-Akt (Ser 473, 1:2,000), c-Myc (1:1,000), Akt (1:1,000), or β-actin (1:1,000). The expressions of p-Akt (a), c-Myc (b), and TSP-1 (c) in Morphine group were increased compared with the control group, and the difference was statistically significant (* p < 0.05). Western blot analysis of p-Akt, Akt, c-Myc, TSP-1, and β-actin in the recurrent tumor tissue (d). Compared to the control group, * p < 0.05; compared to the morphine group, # p < 0.05. TSP-1, thrombosponin-1.

Fig. 5. MDA-MB-231 cells were inoculated in large petri dishes. When the cell density reached about 80%, the cells were starved for 24 h in a serum-free medium. The cells were divided into 4 groups and treated with DMEM, morphine (10 μmol/mL), naloxone (10 μmol/mL), or the same concentration of morphine plus naloxone for 2 h. The expressions of p-Akt (a), c-Myc (b), and TSP-1 (c) were detected. The results were consistent with those observed in animal experiments. The expressions of p-Akt, c-Myc, and TSP-1 in the morphine group were increased compared with the control group, and the difference was statistically significant (* p < 0.05). Western blot analysis of p-Akt, Akt, c-Myc, TSP-1, and β-actin in MDA-MB-231 cells (d). Compared to the control group, * p < 0.05; compared to the morphine group, # p < 0.05. TSP-1, thrombosponin-1.
Discussion

Tumor dormancy is commonly observed in glandular epithelial tumors, such as breast cancer, prostate cancer, neuroblastoma, and retinoblastoma [11, 12]. The development of tumors is closely related to the ability of the tumor to induce angiogenesis. Many factors, such as hypoxia, metabolic stress, immune and inflammatory reaction, can promote the turning on of the angiogenic switch [13–17]. In fact, the short period of angiogenesis can allow for the tumor to escape dormancy [18]. New blood vessels induce tumor cells to escape dormancy and continue proliferation, leading to breast cancer metastasis and recurrence [19]. Opioids play an irreplaceable role in perioperative analgesia and cancer pain in breast cancer cases [20]. In C3TAG mouse breast cancer model, compared with PBS treatment, morphine treatment of 3-month-old mice resulted in a significant increase in tumor burden, higher density of tumor vascular system, more vasodilation, and significantly shorter survival. Morphine-induced tumor growth was antagonized by naloxone, an opioid receptor antagonist. This suggests that morphine may promote the growth of existing tumors through opioid receptor mechanism, thus shortening the survival time of mice bearing tumors [7]. Opioids can promote the proliferation of malignant cells in many ways. By activating cyclooxygenase-2, increasing the production of prostaglandin E-2 promotes angiogenesis and tumor progression. In order to control the survival and migration of cancer cells, opioids can also directly affect the proliferation or apoptosis of cancer cells, affect their ability
to adhere and invade, regulate immune response or cell pathway [4, 21]. However, reports are inconsistent depending on the type of cell studied and the dosage of opioids. The direct effect of morphine on cancer cells and whether it can inhibit or promote the proliferation and survival of malignant cells are still controversial. Long-term morphine therapy has been reported to reduce leukocyte migration and recruitment, thereby reducing angiogenesis and tumor growth. High-dose morphine (1–10 mmol/L) has been proved to have anticancer effect by inhibiting the growth of several cancer cell lines and the expression of tumor necrosis factor-alpha in vitro. At the same time, morphine has antitumor effect by stimulating the release of nitric oxide or reactive oxygen species [21–25]. These contradictory results may be related to in vivo or in vitro experiments. The differences of animal models, endothelial cells, tumor cells, drug concentration, and action time may affect the experimental results. The present findings are controversial in terms of the effect of morphine on angiogenesis. In vitro morphine-enhanced endothelial cell migration and proliferation in a concentration-dependent manner. When low concentration of morphine was administered (1 μmol/L), the extent of its proliferation was equivalent to that of 100 ng/mL of VEGF165-treated cells [1]. In vivo, morphine promoted the growth of breast tumors in mice, and the tumor vessels were denser and more dilated than in the controls [7, 8]. However, high concentrations of morphine presented toxic side effects on endothelial cells in vitro, and this may prevent the VEGF formation induced by hypoxia and affect the expression of leukocytes and the endothelial cell adhesion molecule. This may lead to delays in its penetration to the tumor tissue, thereby preventing angiogenesis [21, 24, 26, 27]. In experiments showing that opioids have antiangiogenesis effects, the dose of morphine is always high (100 μmol/L or 1 mmol/L, or 30 mg/kg in vivo), which may be toxic to endothelial cells; this inhibits the proliferation of endothelial cells [9, 21]. It is worth noting that in real clinical setting, it is difficult to reach an opioid dose of 30 mg/kg for perioperative analgesia; therefore, analgesic morphine administered was more prevalent to lower dose, which we assume that the effect of opioids on angiogenesis is more common and significant.

We removed the tumor under anesthesia and the metastasis model of breast cancer was established. Continuous drug treatment that is started immediately after surgery is more closely related to the use of opioids in the case of clinical breast cancer surgery and the perioperative period. The presence of minimal residual tumors is inevitable when tumor resection is performed, and some small cell masses may also flow through the bloodstream due to surgical stimulation or bleeding. Sustained exposure to morphine may stimulate these residuals and micrometastases and promotes angiogenesis in these sites, creating conditions for tumor growth and thereby promoting tumor dormancy free. In animal experiments, according to the FDA guidelines, when the drug dose was calculated according to body surface area, the dose used in mice was 12.3 times larger than that used in humans. Therefore, 10 mg/kg/day morphine in mice is equivalent to 48 mg/day morphine for a 60 kg adult [28]. In vitro experiments, a large number of literatures have studied the effects of different doses of morphine on breast cancer cells and endothelial cells. The results show that morphine concentration at 0–100 μmol/L can promote cell proliferation, while higher concentration of morphine is unfavorable for cell proliferation [8, 9, 29]. Therefore, we directly treated breast cancer cells with morphine concentration of 10 μmol/L. Results of H&E staining of lung tissues and recurrent tumor immunohistochemistry (Ki67 and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling) show that morphine at 10 μmol/L can promote the proliferation and metastasis of breast cancer cells after surgery and reduce the apoptosis of cancer cells; these effects can be antagonized by naloxone. These results confirmed our previous hypothesis that in nude mice, the surgical resection of tumors of the lungs and other organs that have dormancy of micrometastases. It is difficult to avoid the existence of micro residual tumor cells, and these dormant cells may enter blood circulation due to surgery. Under morphine stimulation, the positive and negative regulation balance of angiogenesis is broken in the micrometastases, micro-residual foci, and their surrounding tissues. Tumor angiogenesis is necessary for tumor growth, and morphine breaks the balance between the proliferation and apoptosis of dormant tumors; the tumor cells are thereby isolated from the dormant state and eventually relapse and metastasize [14].

Naumov et al. [14] found that when breast cancer cells transformed from the nonvascular proliferative phenotype into the vascular proliferative phenotype, the expression of the p-Myc and Myc proteins increased but the TSP-1 expression decreased. Decreased expression of c-Myc is predominantly caused by the activation of the PI3K-Akt pathway. When PI3K is inhibited, the tumor may enter a dormant state [30–32]. The above studies show that angiogenesis, tumor dormancy, and recurrence are closely related to the PI3K-Akt-c-Myc signaling pathway. It was also show that in leukemic cells, morphine activates the PI3K-Akt pathway [33]. Therefore, we put forward a hypothesis: when morphine activates the PI3K-Akt pathway, the activated Akt may further increase the ex-
expression of protein c-Myc, that activates the PI3K-Akt-c-Myc signaling pathway, inhibits the expression of TSP-1, and promotes the turning on of the angiogenic switch, leading to tumor neovascularization. Western blotting showed that morphine can activate the PI3K-c-Myc pathway and promotes Akt phosphorylation; therefore, the activated Akt can further activate c-Myc and promotes tumor growth. When 3 pathway inhibitors were added, the MVD of recurrent tumors was lower than that in morphine-treated mice, which further confirmed that morphine promoted tumor angiogenesis through PI3K-Akt-c-Myc pathway (Fig. 7). Previously, TSP-1 was regarded as an inhibitor of tumor angiogenesis [34]. When the PI3K-c-Myc signaling pathway is activated, the activated Myc reduces the expression of TSP-1 [35]. However, this study found that when PI3K-Akt-c-Myc was activated by morphine, the expression of TSP-1 in the cancer tissues did not decrease with the increased expression of c-Myc. Similar results have been obtained in other studies, suggesting that TSP-1 is associated with increased tumor invasiveness, vascular invasion, high Ki67 expression, high MVD, and low survival rates, and that its upregulation plays an important role in tumor metastasis, and the more migratory tumor cells express more TSP-1 [36–39]. These conflicting results suggest that the TSP-1 function does not simply inhibit or promote vascular growth. Morphine administration increased the expression of TSP-1, TSP-1 promoted the migration of vascular endothelial cells, and promoted tumor cell binding to platelets, providing optimal conditions for tumor metastasis.

Fig. 7. Morphine can activate the PI3K-Akt pathway and promotes Akt phosphorylation; the activated Akt further activates c-Myc/TSP-1 and promotes angiogenesis. These new blood vessels impair the cancer cell proliferation/apoptosis balance and cause tumor cell proliferation, leading to tumor recurrence and metastasis. TSP-1, thrombosponin-1.
A limitation of our study is that it does not describe how morphine promotes the increase in TSP-1 expression. We plan to focus on this aspect in our future studies.

Conclusions

Morphine promotes the angiogenesis of the recurrent postoperative tumors of nude mice with breast cancer and the proliferation of tumor cells. It also promotes the metastasis of dormant breast cancer cells, and such promotion may be related to the PI3K-c-Myc signaling pathway.

Declarations

We declare that the manuscript has not been published previously and is not currently submitted for review to any other journal.

References

Effects of Morphine on Recurrence and Metastasis in Breast Cancer


