



Morphine inhibits cell viability and growth via suppression of vascular endothelial growth factor in human oral cancer HSC-3 cells

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Abstract

Purpose Although many oral cancer patients require opioids, the effects of morphine and related drugs on oral cancer progression have not been well established. Thus, we examined the effects of morphine exposure on the viability of human oral squamous carcinoma HSC-3 cells and aimed to identify the underlying mechanism.

Methods We exposed HSC-3 cells to the various concentrations of morphine (0, 0.1, 1, 10, 100, or 1000 $\mu\text{mol/L}$) for 48 h and, subsequently, evaluated cell viability using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay and cytotoxicity using the lactate dehydrogenase (LDH) assay. To explore the effects of morphine on cell proliferation further, colony formation assay and cell cycle analysis were performed. Additionally, the intracellular expression of nuclear factor kappa B (NF- κB) was analyzed using flow cytometry, and vascular endothelial growth factor (VEGF)-A was evaluated using human VEGF assay.

Results Morphine exposure reduced cell viability and enhanced cytotoxicity in HSC-3 cells in a concentration-dependent manner. The number of colonies in the morphine-treated groups was significantly lower than that in the control group. Consistent with these results, morphine exposure significantly reduced the concentration of VEGF in the cell culture medium in a concentration-dependent manner. However, our data show that morphine at clinical concentrations (0.1–10 $\mu\text{mol/L}$) does not affect cell cycle and apoptosis.

Conclusions Our results suggest that in human oral cancer HSC-3 cells, morphine exposure inhibits cell viability and growth via suppression of VEGF in clinical conditions.

Keywords Morphine · HSC-3 · Proliferation · Nuclear factor kappa B · Vascular endothelial growth factor

Introduction

Cancer is a major global health issue and the leading cause of death worldwide. Surprisingly, since several retrospective clinical studies have shown lower incidence of cancer recurrence after the use of regional anesthesia and reduced doses of opioids after surgery [1–3], there has been an increased interest in understanding the effects of anesthesia-related drugs on cancer progression. While multiple laboratory studies have demonstrated that morphine induces tumor growth for glioblastoma (T98G) and non-small cell lung cancer (H2009) [4, 5] and promotes angiogenesis for human dermal microvascular endothelial cells and breast

cancer (MCF-7) [6, 7], other studies have reported that morphine induces apoptosis and inhibits tumor angiogenesis for leukemia (HL-60), lung cancer (A549 and LLCs), breast cancer (MCF-7), pancreatic cancer (MIA PaCa-2), colon cancer (HT-29), oral cancer (CAL-27), and gastric cancer (MGC-803) [8–11]. Thus, whether morphine promotes or inhibits cancer cell viability remains controversial.

Among all types of cancer, oral cancer is the most common neoplasm in the head and neck region. Although chemotherapy and radiotherapy have improved in recent years, the first line of treatment continues to be surgical removal of cancer lesions. After resection of the primary tumor, free autologous tissue transfer with vascular anastomosis is often required for oral reconstruction, which can lead to prolonged procedure time and postoperative immobility of vascular anastomoses during intensive care unit stay. Thus, such patients are often exposed to opioids for several days during the perioperative period. Additionally, since patients

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with terminal oral cancer often suffer from severe pain, opioids are used in palliative care to control pain and improve the quality of life. Thus, many oral cancer patients are prescribed opioids; however, the effects of opioids on the progression of oral cancer and the underlying mechanisms are far from being understood fully.

The nuclear factor kappa B (NF- κ B), which binds to consensus DNA sequences at the promoter regions of responsive genes to regulate cellular processes, is one of the key players in cancer cell biology. The role of NF- κ B in human cancer initiation, development, metastasis, and resistance to treatment has received widespread attention and there are some reports that NF- κ B promotes cancer cell proliferation, suppresses apoptosis, and regulates tumor angiogenesis [12, 13]. Additionally, vascular endothelial growth factor (VEGF) is recognized as an important regulator of tumor angiogenesis that binds to cell-surface tyrosine kinase receptor-VEGFR [14] to induce and positively regulate a physiological and pathological angiogenic cascade [15].

Therefore, in this *in vitro* study, we aimed to investigate the effects of morphine on the viability of human oral cancer HSC-3 cells and its underlying mechanisms, including changes in the expression of NF- κ B and production of VEGF after treatment with morphine.

Materials and methods

Cell culture

Human oral squamous carcinoma cell line, HSC-3, kindly provided by Dr. Nakagawa (Nara Medical University, Nara), was cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka Japan) supplemented with 10% fetal bovine serum (FBS; ATCC, Manassas, VA, USA) and 1% penicillin–streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cells were incubated under humidified conditions at 37 °C and in atmospheric air supplemented with 5% CO₂. The medium was changed every 3 days, and the cells were passaged every 5–7 days; these passaged cells were used for experiments.

Cell viability and cytotoxicity assay

Cell viability was determined by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates at a density of 2×10^3 cells/well. After 24-h incubation, the used medium was replaced with a fresh medium containing different concentrations (0, 0.1, 1, 10, 100, or 1000 μ mol/L) of morphine (determined

based on previous literature [6, 11, 16]) in the experimental groups, while the normal medium was used in the control group. After 48 h of morphine exposure, all the cells were incubated at 37 °C for 4 h with medium containing 0.5 mg/mL MTT, the medium was then removed, and the formazan product was lysed using 0.1 mL dimethyl sulfoxide. The relative number of viable cells was determined by measuring absorbance at a wavelength of 570 nm with absorbance at 650 nm as reference. Each experiment was performed in triplicate and repeated six times.

Cytotoxicity was determined using the Cytotoxicity lactate dehydrogenase (LDH) assay (Dojindo Molecular Technologies, Inc. Japan) according to the manufacturer's instructions. After sample preparation identical to that described for the MTT assay, the used medium was replaced with a 1% FBS medium containing different concentrations of morphine same as those in the MTT assay in the experimental groups and with 1% FBS medium in the control group. After 48 h of exposure, 10 μ L of lysis buffer was added to each well, was incubated at 37 °C for 30 min, 100 μ L of working solution added to each well and was incubated at room temperature for 30 min. Next, 50 μ L of stop solution was added to each well and absorbance (A) at 490 nm was determined. Absorbance in wells without cells but containing the medium alone was set as the baseline and was used as control. The degree of cytotoxicity (percentage) was calculated by subtracting the baseline or control value from average absorbance of each triplicate set of wells, described by the equation:

$$\% \text{cytotoxicity rate} = (A - \text{control}) / (\text{high control}) \times 100.$$

Each experiment was performed in triplicate and was repeated six times.

Colony formation assay

The standard colony formation assay was used to measure cell proliferation. Cells were plated in 60-mm dishes at a density of 500 cells/dish. After 24 h incubation, the used medium was replaced with a fresh medium containing different concentrations of morphine same as those in the MTT assay in the experimental groups, while the normal medium was used in the control group. Colonies obtained after 10 days of growth were fixed in methanol and were stained with 2% Giemsa solution. The dishes were photographed and macroscopic colonies consisting of approximately 50 cells or more were scored as having grown from single cells using Image J software.

Cell cycle analysis by flow cytometry

For the cell proliferation analysis, cells were plated in 75cm² tissue culture flasks at a density of 36×10^4 cells/flask. After incubation for 24 h, the medium was replaced with

a medium containing 10, 100, or 1000 $\mu\text{mol/L}$ morphine in the experiment groups, and with the normal medium in the control group. After 48 h of exposure, Bromodeoxyuridine (BrdU) was added to the medium to a final concentration of 10 $\mu\text{mol/L}$ at 45 min prior to cell recovery, and cell proliferation was quantified based on BrdU incorporation into newly synthesized DNA using the BD Pharmingen™ BrdU Flow Kit (BD-Pharmingen, San Diego, CA, USA). The cells were stained with anti-BrdU antibody/FITC and 7-AAD, and FACS was performed according to the manufacturer's instructions. Each experiment was repeated six times. Dual parameter fluorescent dot plots showed cells in the S phase that actively synthesize DNA at the top, cells in the G0/G1 phases were represented in the lower left quadrant and cells in the G2/M phases were in the lower right quadrant.

Apoptosis assay by flow cytometry

After sample preparation identical to that used for cell cycle analysis, the cells were exposed to different concentrations of morphine same as those in the cell cycle analysis, and the apoptotic cells were identified using a combination of the FITC Annexin V Apoptosis Detection Kit I (BD-Pharmingen, San Diego, CA, USA) and FACS on a BD FACSCalibur HG™ flow cytometer (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, after 1×10^5 cells were extracted, 5 μL Annexin V-fluorescein-isothiocyanate solution and 5 μL propidium iodide solution were added to the samples. After incubation in the dark for 15 min at room temperature, 400 μL of binding buffer was added to the samples, and subjected to FACS. Dual parameter fluorescent dot plots showed viable cells in the lower left sector, cells in early apoptosis in the lower right sector, and cells in late apoptosis/necrosis in the upper right sector. Each experiment was repeated six times.

Intracellular caspase-3 expression and NF- κB analysis by flow cytometry

After sample preparation identical to that used for cell cycle analysis, the cells were treated with different concentrations of morphine same as those in the cell cycle analysis for 48 h, were fixed and were permeabilized using BD Cytotfix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA, USA), stained with PE-anti-active caspase-3 (BD-Pharmingen, San Diego, CA, USA) or Alexa Flour 647 anti-NF- κB p65 (BD-Pharmingen, San Diego, CA, USA), and FACS performed according to manufacturer's instructions. Briefly, after 1×10^6 cells were extracted, the cells were fixed and permeabilized using 250 μL of fixation/permeabilization solution for 20 min at 4 °C. Then, the cells were washed twice with $1 \times \text{BD Perm/Wash}^{\text{TM}}$ buffer and incubated with 10 μL of PE-anti-active

caspase-3 or Alexa Flour® 647 anti-NF- κB p65 in 40 μL of $1 \times \text{BD Perm/Wash}^{\text{TM}}$ buffer for 30 min at 4 °C. Cells were washed twice, Stain buffer (FBS) (BD-Pharmingen, San Diego, CA, USA) was added to the samples, and subjected to FACS.

Fluorescence intensity was expressed on a logarithmic scale. Caspase-3 activation was defined as positive expression of PE-anti-active caspase-3, and samples from the control group were used as negative controls. Activation of NF- κB was defined as positive expression of Alexa Flour 647 anti-NF- κB p65 with samples stained with Alexa Flour 647 isotype control acting as negative controls.

Human VEGF assay

VEGF-A concentration in cell culture medium was measured using the human VEGF assay kit, IBL (Immuno-Biological Laboratory Co., Gunma, Japan) according to the manufacturer's instructions. Briefly, the cells were plated in 6-well plates at a density of 3.4×10^4 cells/well. After incubation for 24 h, the used medium was replaced with 2 mL of fresh medium containing different concentrations of morphine same as those in the MTT assay in the experimental groups, while the normal medium was used in the control group. After 48 h of exposure, the VEGF-A concentration of each cell culture medium was measured. The kit was a solid-phase sandwich enzyme linked immunosorbent assay (ELISA) using specific polyclonal and monoclonal antibodies, while the coloring agent was tetra methyl benzidine (TMB). The sample (100 μL) was put in a precoated plate [Anti-Human VEGF (16F1)] mouse IgG M0Ab affinity purify (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), then washed after incubating for 60 min at 37 °C. Afterwards, the labeled antibody (HRP conjugated anti-human VEGF rabbit IgG fab' affinity purify (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) was added, incubated, and washed again. Finally, coloring agent was added, followed by stop solution, and the absorbance was determined at 450 nm using a plate reader. VEGF concentration was determined using standard curve. Each experiment was performed in duplicate and repeated six times.

Statistical analysis

GraphPad Prism ver. 6 software (GraphPad Software, San Diego, CA, USA) was used for all the statistical analyses. Comparisons were made between control and each group of morphine using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The data are expressed as the mean \pm SD (standard deviation), and statistical significance was defined as $P < 0.05$.

Results

Cell viability and cytotoxicity

Morphine exposure in HSC-3 cells led to lower cell viability compared to controls (Fig. 1a), and as shown in Fig. 1b, morphine exhibited enhanced cytotoxicity in a concentration-dependent manner.

Colony formation assay and cell cycle analysis

Figure 2 shows the effects of morphine on cell proliferation. The number of colonies formed by HSC-3 cells treated with 0, 0.1, 1, 10, 100, or 1000 $\mu\text{mol/L}$ of morphine after

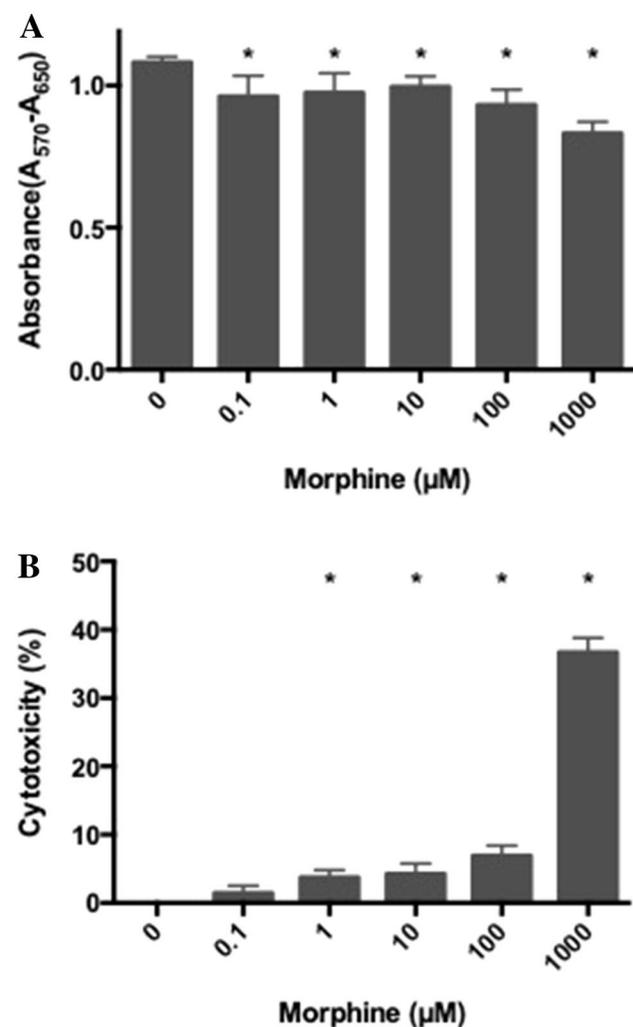


Fig. 1 Effects of morphine on HSC-3 cell viability (a) and cytotoxicity (b). Morphine significantly reduced cell viability and enhanced cytotoxicity in a concentration-dependent manner. All the data are expressed as mean \pm SD of six independent experiments. * $P < 0.05$ compared to control

10 days of culture were 73.5 ± 13.3 , 62.2 ± 15.5 , 48.5 ± 13.0 , 47.7 ± 8.6 , 2.8 ± 2.5 , or 0.5 ± 0.5 , respectively (Fig. 2a). The numbers of colonies formed were significantly lower in the morphine-treated groups than the control group in a concentration-dependent manner.

As shown in Fig. 2b–f, incubation with 1000 $\mu\text{mol/L}$ morphine led to significantly lower percentage of the HSC-3 cells in the S phase (R1) compared to the untreated control cells (8.3% vs 30.6%, $P < 0.05$, respectively), whereas percentage of HSC-3 cells in the G0/G1 phase (R2) was greater than that observed in the control cells (54.9% vs 81.4%, $P < 0.05$, respectively). It was revealed that morphine at clinical concentrations does not affect cell cycle.

Apoptosis and intracellular caspase-3 expression assays

Figure 3 shows the results of apoptosis analysis by flow cytometry. Incubation with 1000 $\mu\text{mol/L}$ of morphine resulted in significantly greater percentage of HSC-3 cells in the early apoptotic stage (lower right sector) compared to the untreated control cells (6.3% vs 2.1%, $P < 0.05$). Additionally, as Fig. 3f shows, the percentage of caspase-3-positive cells in the morphine (1000 $\mu\text{mol/L}$) was significantly greater than that in the control groups (3.7% vs 2.3%, $P < 0.05$, respectively). Taken together, these data suggest that although 1000 $\mu\text{mol/L}$ of morphine can induce apoptosis in HSC-3 cells, apoptosis is not attributed to the lower viability induced by clinical concentrations of morphine.

Intracellular NF- κB expression and production of VEGF

As Fig. 4 shows, incubation with 100 or 1000 $\mu\text{mol/L}$ of morphine led to a significant decrease in the percentage of NF- κB p65-positive cells compared to the control cells (52.0, 38.7% vs 65.2%, $P < 0.05$, respectively), whereas 10 $\mu\text{mol/L}$ of morphine did not show a significant difference. Interestingly, as shown in Fig. 5, morphine exposure to HSC-3 cells led to the reduction of VEGF-A production compared to controls even at lower concentrations of morphine.

Discussion

In this report, we show that morphine can suppress cell viability and growth in HSC-3 cells, and that this is probably due to reduced production of VEGF. Cell cycle arrest or apoptosis induction does not seem to contribute to the effect reported here.

Morphine and chemically related opioids are clinically potent analgesics that are used to relieve surgical pain during

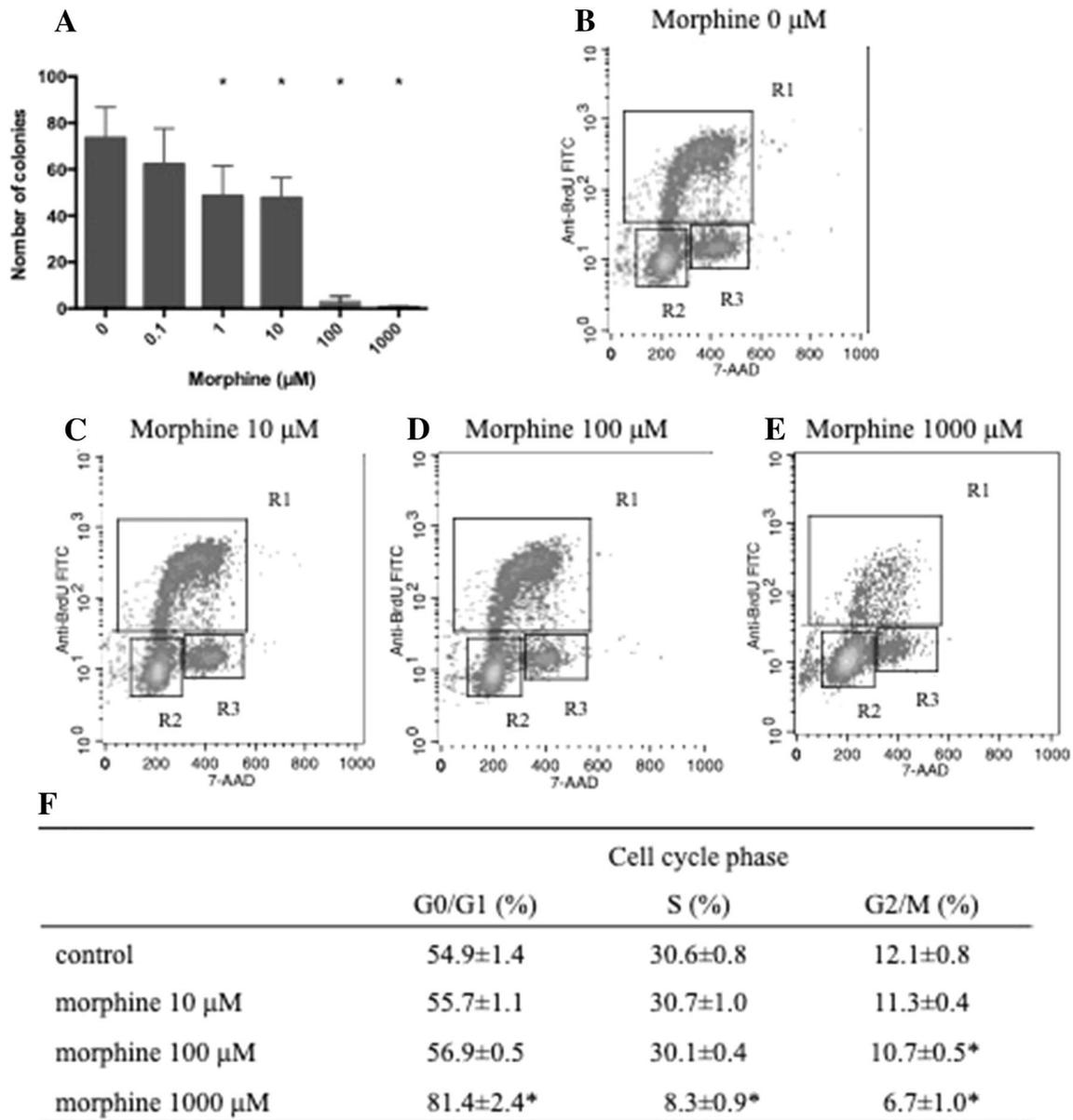


Fig. 2 Effects of morphine on HSC-3 cell proliferation. **a** The HSC-3 cells that were incubated with morphine exhibited fewer colonies than the controls. * $P < 0.05$ compared to control. **b–e** Representative charts of cell cycle analysis using flow cytometry. **f** The percentage of cells in each cell cycle phase. The percentage of HSC-3 cells in the S phase after treatment with 1000 $\mu\text{mol/L}$ of morphine was signifi-

cantly lower compared to the controls while the percentage of cells in the G0/G1 phase after incubation with 1000 $\mu\text{mol/L}$ morphine was greater than that of control cells. All the data are expressed as mean \pm SD of six independent experiments. * $P < 0.05$ compared to control

the perioperative period and to tackle cancer pain in palliative care settings. Previous clinical and laboratory studies have suggested that opioids are important for regulating the growth of normal and neoplastic tissue. Specifically, Wang et al. [17] have reported that patients with non-small cell lung cancer receiving intravenous opioid receptor agonists for postoperative pain might have shorter overall survival and disease-free survival than patients not receiving these agonists. Additionally, Zylla et al. [18] reported that a

greater opioid requirement is associated with shorter progression-free survival and overall survival in patients with metastatic prostate cancer. Conversely, although the situation in previous laboratory studies was complex because the concentrations of morphine and the type of cancer cells used were varied, combining those findings, the low concentrations of morphine seem to promote cancer cell viability [4–6, 19], whereas the high concentrations seem to inhibit the viability [6, 11, 16]. Among these studies, Gupta et al.

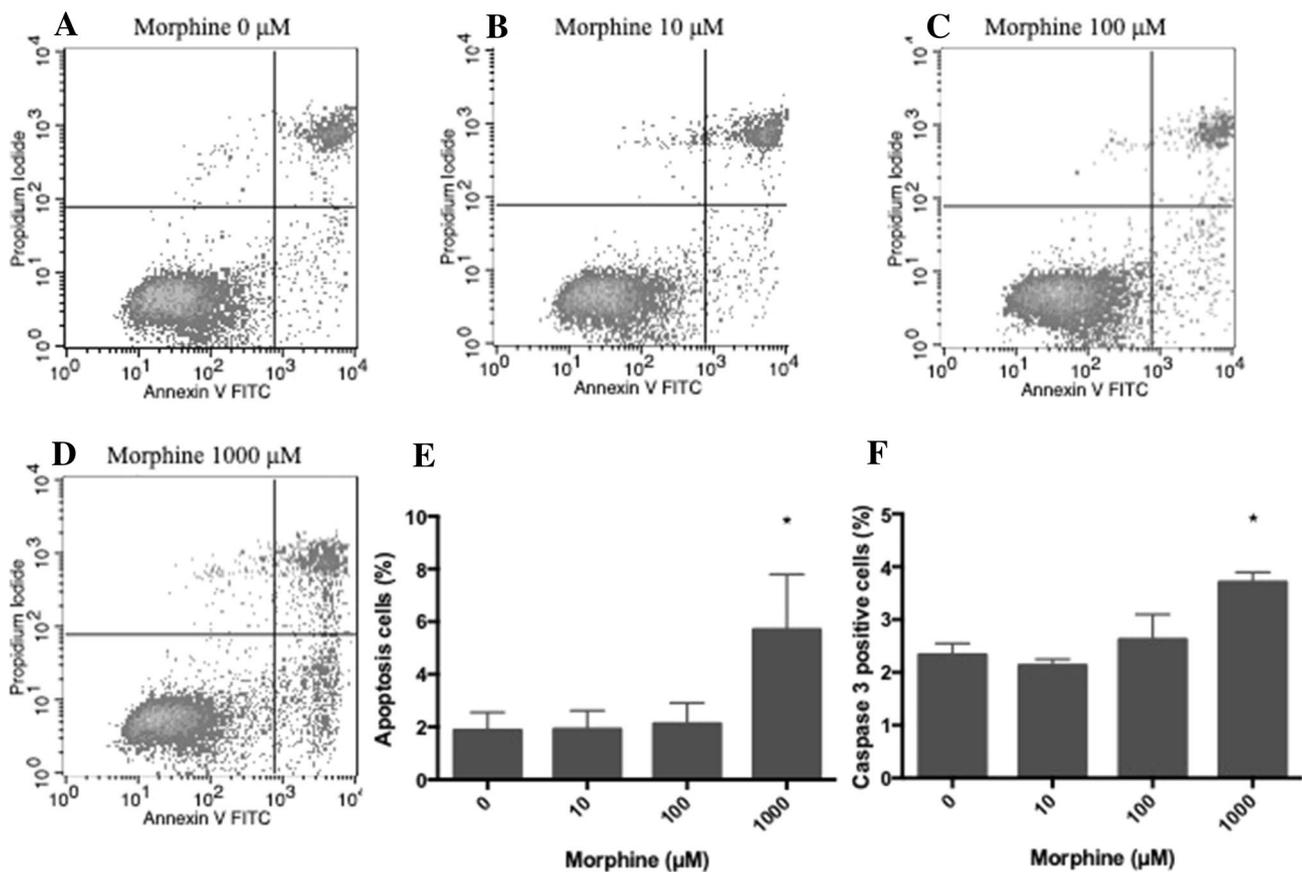


Fig. 3 Effects of morphine on apoptotic cell death in HSC-3 cells. **a–d** Representative charts of apoptosis assayed by flow cytometry. **e** Early apoptotic rates in HSC-3 cells incubated with 1000 μmol/L of morphine were significantly higher than that of control cells. **f**

Percentage of caspase-3-positive cells that had been treated with 1000 μmol/L morphine was higher than that of control. All the data are expressed as mean ± SD of six independent experiments. * $P < 0.05$ compared to control

reported biphasic effects, that is, the low concentrations of morphine (0.01–100 μmol/L) enhanced cell proliferation and higher concentrations (> 1000 μmol/L) inhibited cell viability. Bimonte et al. also mentioned that the low concentrations of morphine may promote cancer cell proliferation and high concentrations may inhibit cancer cells [20]. In the present study, morphine inhibited HSC-3 cell proliferation and reduced colony formation capability in a concentration-dependent manner from low to high concentration.

Qin et al. [11] showed that morphine inhibits gastric cancer cells by cell cycle arrest. In addition, Hatsukari et al. [8] showed that morphine induces apoptosis in leukemic, lung cancer, and breast cancer cells in vitro. However, since cell cycle arrest and apoptosis induction were observed only in the group treated with 1000 μmol/L of morphine in our study, it seems that the reduction in cell viability and growth by clinically relevant concentrations of morphine is not attributable to cell cycle arrest or apoptosis induction.

VEGF is a signal protein produced by cells that stimulate the formation of blood vessels. It is a sub-family of

growth factors and important signaling proteins involved in angiogenesis. Several studies have assessed the relationship between morphine exposure and VEGF expression in cardiac myocytes in vitro and in rodent models [21, 22]; the results from these studies revealed that morphine may inhibit VEGF expression. Consistent with these results, we demonstrated that production of VEGF decreased after morphine exposure at clinical concentrations.

NF-κB is a ubiquitous nuclear transcription factor that plays an important role in the regulation of numerous genes associated with both cell proliferation and apoptosis. Several studies have assessed the relationship between morphine exposure and NF-κB activation in neuronal cells, microglia in vitro, in rodent models [23–26], as well as cancer cells [11, 27, 28], revealing that morphine may inhibit NF-κB activation [23]. In addition, it has been reported that inhibition of NF-κB activity decreases the VEGF mRNA expression in breast cancer cells [29]. Since our data showed that NF-κB-positive HSC-3 cells decreased only after high concentrations (100 or

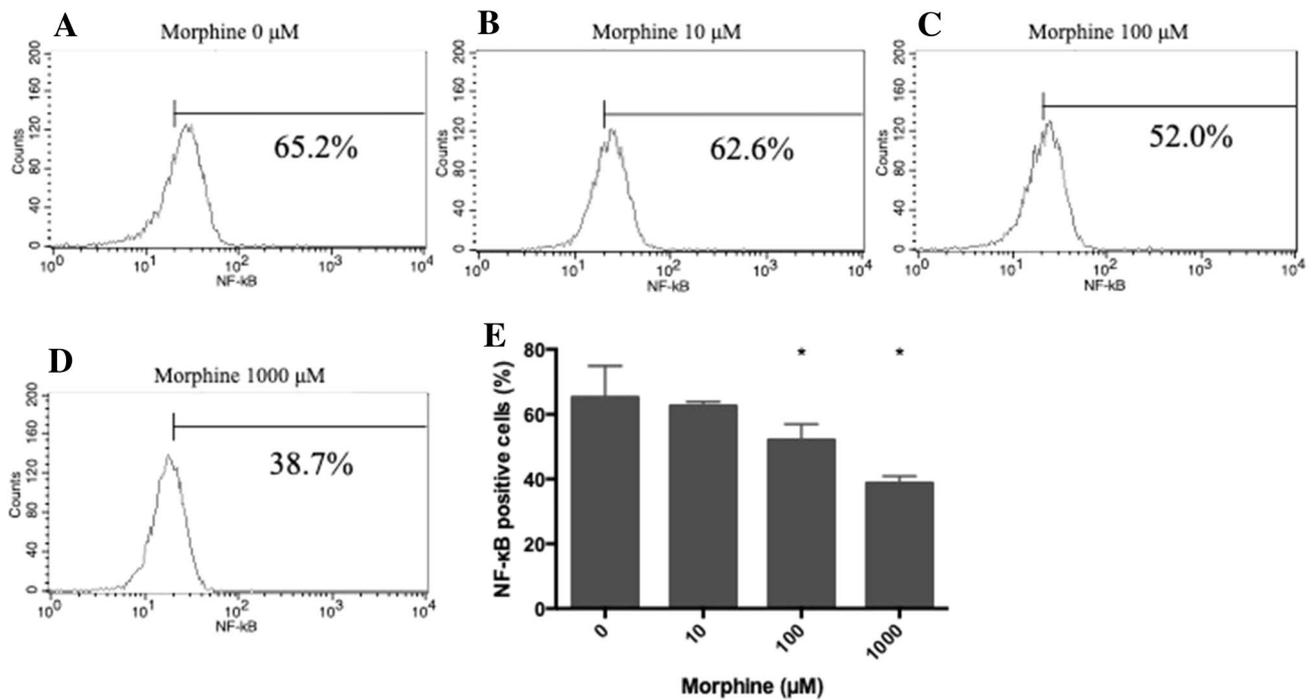


Fig. 4 Intracellular expression of NF-κB after morphine exposure in HSC-3 cells. **a–d** Representative charts of NF-κB p65 positive cells assayed by flow cytometry. **e** The percentage of NF-κB p65-positive

cells incubated with 100 or 1000 μmol/L morphine was lower than that of the control cells. All the data are expressed as mean ± SD of six independent experiments. * $P < 0.05$ compared to control

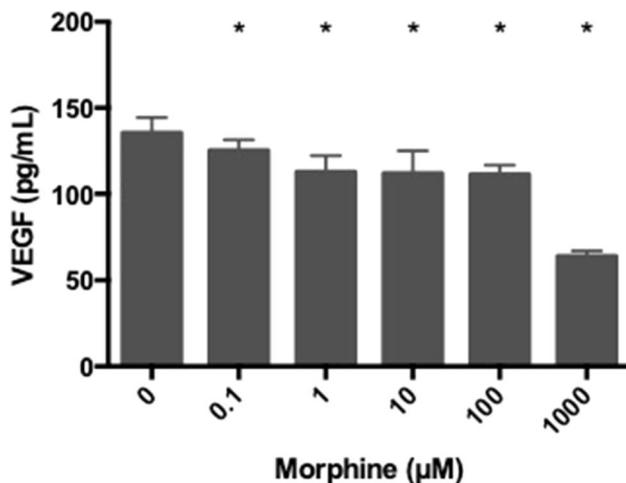


Fig. 5 Concentration of VEGF-A in the cell culture medium after morphine exposure in HSC-3 cells. Morphine significantly reduced the VEGF-A concentration in a concentration-dependent manner. All the data are expressed as mean ± SD of six independent experiments. * $P < 0.05$ compared to control

Our findings should be interpreted within the constraints of the study's limitations. Although clinical concentrations of morphine in the blood were used in the present in vitro study, the exact concentrations of morphine around cancer cells in the in vivo setting may be different. Some scientists have proposed that opioids promote tumor growth due to their negative effects on the immune system, specifically on natural killer cells and monocytes [30–32]. However, in the present in vitro study, such interactions could not be tested; therefore, further in vivo studies are necessary to investigate whether morphine can affect oral cancer growth.

In summary, clinically relevant concentrations of morphine inhibit cell viability and growth in HSC-3 cells probably due to reduced production of VEGF, and not due to cell cycle arrest or apoptosis induction.

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1000 μmol/L) of morphine exposure while production of VEGF decreased at even lower concentrations, the reduction in intracellular NF-κB expression may be related to the anti-proliferative effect of morphine on HSC-3 cells.

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