RESEARCH COMMUNICATION

Exogenous Morphine Inhibits Human Gastric Cancer MGC-803 Cell Growth by Cell Cycle Arrest and Apoptosis Induction

Yi Qin[&], Jing Chen[&], Li Li[&], Chun-Jie Liao, Yu-Bing Liang, En-Jian Guan, Yu-Bo Xie*

Abstract

Morphine is not only an analgesic treating pain for patients with cancer but also a potential anticancer drug inhibiting tumor growth and proliferation. To gain better insight into the involvement of morphine in the biological characteristics of gastric cancer, we investigated effects on progression of gastric carcinoma cells and the expression of some apoptosis-related genes including caspase-9, caspase-3, survivin and NF-κB using the MGC-803 human gastric cancer cell line. The viability of cells was assessed by MTT assay, proliferation by colony formation assay, cell cycle progression and apoptosis by flow cytometry and ultrastructural alteration by transmission electron microscopy. The influences of morphine on caspase-9, caspase-3, survivin and NF-κB were evaluated by semi-quantitative RT-PCR and Western blot. Our data showed that morphine could significantly inhibit cell growth and proliferation and cause cell cycle arrest in the G2/M phase. MGC-803 cells which were incubated with morphine also had a higher apoptotic rate than control cells. Morphine also led to morphological changes of gastric cancer cells. The mechanism of morphine inhibiting gastric cancer progression in vitro might be associated with activation of caspase-9 and caspase-3 and inhibition of survivin and NF-κB.

Keywords: Morphine - gastric cancer - cell cycle arrest - apoptosis

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Introduction

Morphine is a first-line opioid analgesic which can relieve pain in a variety of patients, including those with cancer. For those patients with severe pain associated with metastasis of cancer, morphine may be one of the most effective drugs (Stefano et al., 2005; Hara, 2008). However, there is evidence that morphine has extra-analgesic actions that lie outside the realm of anti-nociception and appear to be mediated by indirect mechanisms. One of the most important extra-analgesic actions is that morphine significantly alters tumor progression.

Complementary pre-clinical and clinical studies have monitored inhibitory effects of pharmacologically administered morphine on tumor growth in selected animal models and in human patient populations. For example, a pre-clinical murine animal model of tumor progression has demonstrated that repeated administration of morphine results in significantly diminished tumor cell-induced tissue destruction (El Mouedden and Meert, 2007). Pre-clinical observations are complemented by a series of clinical studies demonstrating that both pre- and post-operative administration of analgesic dosages of morphine as an adjuvant to cancer surgery operationally reduces systemic cell dissemination of tumor cells (Page et al., 1994; 1998). However, morphine is also suggested to promote the development of cancer because several researches demonstrated that morphine enhanced the growth of several different tumor cell lines in vivo (Ishikawa et al., 1993; Fujioka et al., 2011).

The morphine-tumor interaction is very complex and the specific mechanisms by which morphine influences tumor progression are far from being fully understood. Researchers have proposed a protective role for morphine against tumor progression through induction of apoptosis in tumor cells. Maneckjee and Minna (1994) demonstrated that treatment of human lung cancer cells with morphine resulted in morphological changes and cleavage of DNA into nucleosome-sized fragments characteristic of apoptosis. The process of apoptosis is regulated by several proteins that either inhibit or promote apoptotic cell death. Caspase-9 and caspase-3, members of the cysteine protease family, are considered to be the central proteins in the execution of apoptosis (Bhardwaj et al., 2003; Du et al., 2012) while survivin is a identified member of the inhibitor of apoptosis protein family (Idenoue et al., 2005). Suzuki and Shiraki (2001) reported that survivin plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting the activity of caspases. Moreover, survivin is one of the downstream targets of

Departments of Anesthesiology, The First affiliated Hospital, Guangxi Medical University, Nanning, China [&]Equal contributors
**For correspondence: xieyubo715001@yahoo.com.cn*

NF-κB which is a key transcription factor regulating various genes and may be a cause of cancer initiation and promotion (Pacifico et al., 2004; Athar et al., 2009).

Since there was evidence that serum morphine concentrations of cancer patients who received from 80 mg to 160 mg of morphine daily reached 0.8 to 2.2 mmol/L (Sueoka et al., 1998), the concentrations of morphine in our study (0.1 μmol/L, 10 μmol/L or 1 mmol/L) were relatively suitable.

In this study, we aimed to investigate the effects of morphine on biological characteristics of gastric cancer and on expression of caspase-9, caspase-3, survivin and NF-κB in an in vitro cell model. The longterm goals of our project are to understand the safety of morphine in cancer pain therapy and the possible mechanisms of morphine-tumor interaction.

Materials and Methods

Cell Culture

The poorly differentiated human gastric adenocarcinoma cell line, MGC-803, was purchased from Cell Bank, Chinese Academy of Science, Shanghai, China, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured in an incubator at 5% CO_2 in air at 37 ˚C, with medium changes every 3 days.

Cell Proliferation and Survival Assay

The viability and proliferation of cells were determined by an MTS assay using a CellTiter 96 AQueous assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, the cells were plated at 1×10^3 cells/well in 96-well plates. The cells in experiment groups were incubated with 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine for 72 h. MTS mixed with medium without serum was added to the cell cultures after removal of the old medium. The plates were incubated at 37 ˚C for 2 h, and the absorbance (A) at 490 nm was determined using a 96-well Opsys MRTM microplate reader (ThermoLabsystems, Chantilly, VA, USA) and RevelationTM QuickLink Software. The blank control wells with medium only were set as zero absorbance controls. The percentage of cell survival was calculated using the background-corrected absorbance: % proliferation rate =100 × A of experimental well/A of control well. All experiments were performed at least three times.

Colony Formation Assay

Cell suspensions from each group were diluted in DMEM supplemented with 10% FBS, and immediately replated in 6-well plates at 20 cells/cm^2. The plates were incubated until the cells in the control wells had formed sufficiently large colonies at 7 day. The colonies were fixed with 6% glutaraldehyde and stained with 0.5% crystal violet. The plates were photographed and their digital images were manually analyzed to determine the colony number.

Cell Cycle Analysis by Flow Cytometry

For cell cycle analysis, MGC-803 cells (1×10^6) were incubated for 24 h and then washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4 ˚C for 30 min. The cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACscan (Becton Dickinson, Mountain View, CA, USA). The data were analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, USA).

Apoptosis Assay by Flow Cytometry

Apoptotic cells were identified by the Annexin V/FITC Apoptosis Detection Kit (Jingmei Biotech Co., Shenzhen, China) and an EPICS XL-MCL flow cytometer (Becton Dickinson) according to the manufacturer's instructions after MGC-803 cells were incubated for 24 h. Briefly, 1×10^6 cells were stained with Annexin V/FITC for 30 min at 4 ˚C in the dark and propidium iodide for 10 min before flow cytometric analyses.

Morphological Observation under Scanning Electron Microscopy (SEM)

After cells were incubated for 24 h, they were fixed in 4% buffered glutaraldehyde and then postfixed in 1% osmium tetroxide at 4 ˚C. Dehydration was conducted sequentially in 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol for 15 min at each concentration twice, first at 4 ˚C and then at room temperature. The samples were dried in 50%, 70%, 80%, 90%, 95% and finally 100% acetonitrile for 15 min at room temperature and then by vacuum drying. Vacuum plating was used to coat the samples with carbon and then gold in a specimen supporter. Observation was performed under a H-500 scanning electron microscopy (Hitachi Ltd., Japan).

Semi-Quantitative RT-PCR Analysis

Total RNAs were extracted from positive cell clone using TRIzol Reagent (Invitrogen). Neo gene segments were amplified and verified by semi-quantitative RT-PCR. Complementary deoxyribonucleic acids (cDNAs) were reverse-transcribed from 2 μg of total RNA. Primers used in this study were as follows: caspase-9 forward 5'-GGCTGTCTACGGCACAGATGGA-3', reverse 5'-CTGGCTCGGGGTTACTGCCAG-3', 200 bp; caspase-3 forward 5'-AAGCGAATCAATGGACTC-3', reverse 5'-TTCCTGACTTCATATTTCAA-3', 192 bp; survivin forward 5'-AAATGCACTCCAGCCTCTGT-3', reverse 5'-TGTCGAGGAAGCTTTCAGGT-3', 311 bp; NF-κB forward 5'- TGCTGTGCGGCTCTGCTTCC -3', reverse 5'- AGGCTGGGGTCTGCGTAGGG -3', 321 bp; GAPDH(1) forward 5'- ACAGCAACAGGGTGGTGGAC -3', reverse 5'- TTTGAGGGTGCAGCGAACTT -3', 252 bp; GAPDH(2) forward 5'- CGGGAAGCTTGTCATCAATGG-3', reverse 5'- GGCAGTGATGGCATGGACTG-3', 358 bp; GAPDH(3) forward 5'- ACCACAGTCCATGCCATCAC-3',

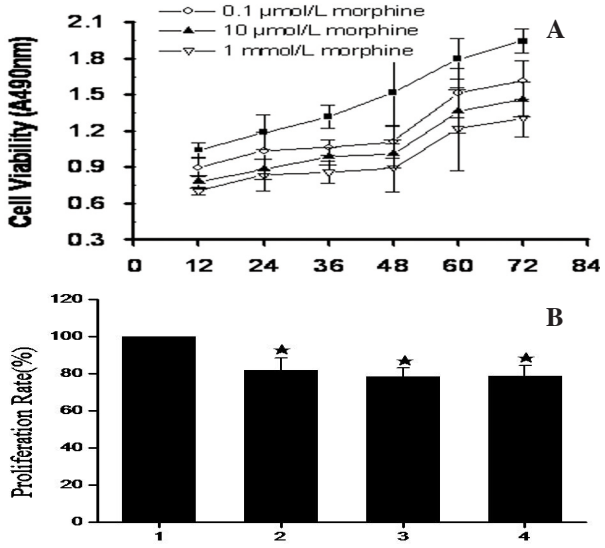

Figure 1. Cell Survival was Assessed by MTS Assay. The results represent the means of at least three independent experiments. (A) The cell viability was observed after MTS treatment in MGC-803 cells for 72 h. (B) The mean cell proliferation rates of MGC-803 cells which were incubated with morphine for 72 h after MTS treatment were significantly lower compared with that of the control cells (*P<0.05). Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine

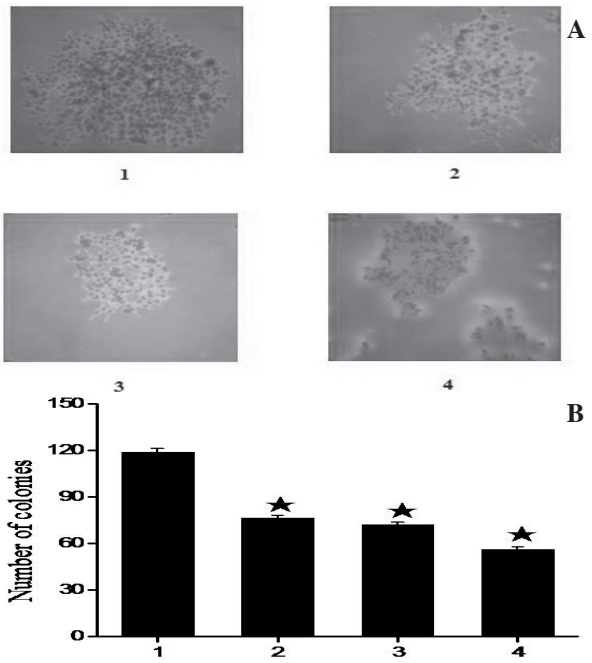

Figure 2. Results of Colony Formation Assay. (A) MGC-803 cells were plated in 6-well plates. The surviving fraction of cells (visible colonies) was stained with gentian violet, photographed, and counted manually. (B) The MGC-803 cells which were incubated with morphine exhibited fewer colonies than the control cells (*P<0.05). Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine

reverse 5'- TCACCACCCTGTTGCTGTA -3', 450 bp. The products of PCR were checked by agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of GAPDH mRNA.

Western Blot Analysis

Cell lysates were prepared in a buffer containing 100 mM NaCl, 10 mM Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml PMSF, and 1%(v/v) NP40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three buffer system. The membrane was blocked with 5% BSA in PBST (PBS, pH 7.5, containing 0.1% Tween-20) and incubated with a 1:1000 dilution of primary antibody (anti-caspase-9, anti-caspase-3, anti-survivin or anti-NF-κB) (CST, USA) overnight at 4 ˚C. The membrane was then washed with PBST and incubated with a peroxidase-conjugated secondary antibody (1:5000) (Santa Cruz Biotechnology Inc., USA) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, USA), according to the manufacturer's recommendations. Western blot film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, USA). After development, the membrane was stripped and reprobed with antibody against GAPDH (1:1000) (Santa Cruz Biotechnology) to confirm equal sample loading.

Statistical Analysis

Data are expressed as mean±standard error of the mean (SEM) analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and Origin 7.5 software programs (OriginLab Co., Northampton, MA, USA). Student's t-test was used to measure statistical significance between two treatment groups. Multiple comparisons were performed with a one-way analysis of variance (ANOVA). Data were considered significant if $P < 0.05$.

Results

Morphine Inhibits Cell Growth and Proliferation in Gastric Cancer MGC-803 Cells

We determined the in vitro survival rates of MGC-803 cells. MGC-803 cells which were incubated with morphine exhibited significantly reduced cell survival, as assessed by the MTS assay (Figure1). 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine made the mean proliferation rates of MGC-803 cells lower than the control cells (P<0.05). We also observed that the cells in morphine groups grew slower than the control cells, indicative of a suppressive effect of morphine on MGC-803 cell growth and survival. To confirm the inhibitory effect of morphine on the growth of MGC-803 cells, colony formation was assayed to check the capability of the cell lines to grow in an anchorage-independent environment (soft agarose). The cell lines were able to form colonies in soft agarose, but the number and size of the colonies were not consistent across the cell lines (Figure 2A). The numbers of colonies formed by MGC-803 cells which were incubated with 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine after 7 days of culture were 76.3 ± 1.8, 72.1 ± 1.7 or 56.0± 1.8, which represented a 35.9%, 39.5% and 53.0% decrease, respectively, when compared with the control cells (P<0.05) (Figure 2B). Together, the data suggest that morphine inhibits cell growth and proliferation in the gastric cancer cell system.

Table 1. Cell Cycle Analysis by Flow Cytometry in Gastric Cancer MGC-803 Cells

Group	Cell cycle phase		
	G0/G1 (%)	S(%)	G2/M(%)
control	51.3±6.0	47.4±3.5	1.3±0.2
0.1 μmol/L morphine	42.5±3.1*	39.9±3.9*	17.6±2.2*
10 μmol/L morphine	43.5±4.9*	39.6±3.5*	16.9±1.7*
1 mmol/L morphine	42.7±5.2*	39.2±2.8*	18.1±3.4*

*P<0.05, MGC-803 cells in morphine groups vs. MGC-803 cells in control group

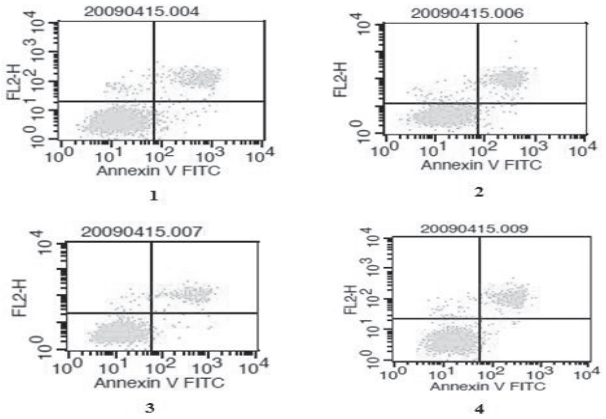

Figure 3. Percentages of Apoptotic Cells Analyzed by Flow Cytometry. Numbers in the quadrants reflect the percentage of MGC-803 cells. The apoptotic rates in MGC-803 cells which were incubated with morphine were significantly higher than that in the control cells. Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine

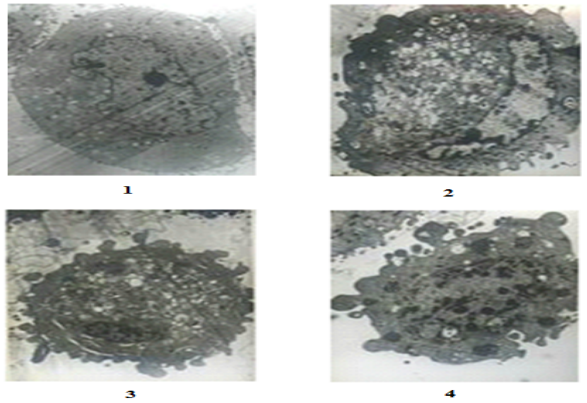

Figure 4. Results of Scanning Electron Microscopy of Gastric Cancer MGC-803 Cells. Morphine resulted in morphological changes of MGC-803 cells. Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine

Effect of Morphine on Cell Cycle Control in Gastric Cancer MGC-803 Cells

Flow cytometry was used to determine whether the inhibitory effect of morphine on MGC-803 cell proliferation was mediated, at least in part, through affecting cell cycle progression. The following cell cycle profile for the cells which were incubated with 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine was seen: 42.5%, 43.5% or 42.7% were in G0/G1 phase and 17.6%, 16.9% or 18.1% were in G2/M phase, with about 13-fold higher in G2/M phase cell population, and with a 17.2%, 15.2%

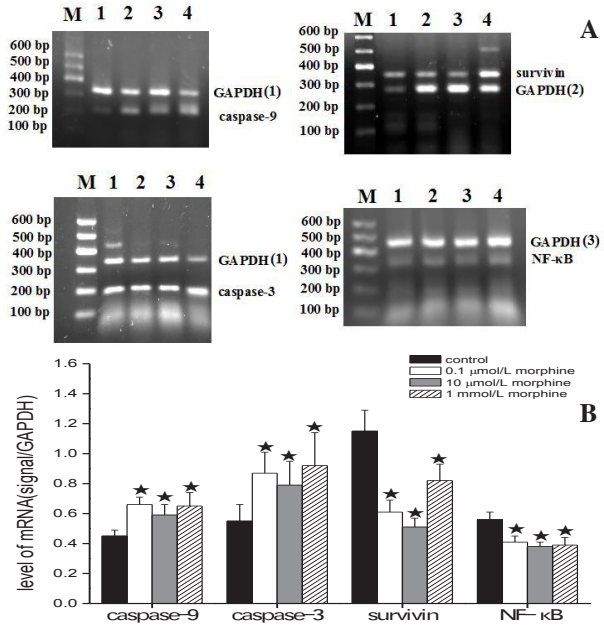

Figure 5. Morphine Induces Caspase-9 and Caspase-3 mRNA Expression Upregulation and Downregulation of Survivin and NF-κB. (A) Semi-quantitative RT-PCR analysis of caspase-9, caspase-3, survivin, NF-κB and GAPDH in MGC-803 cells from four groups respectively. Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine. (B) caspase-9, caspase-3, survivin and NF-κB mRNA levels were measured at four groups, normalized to those of GAPDH and presented as means ± SEM. *P<0.05 compared with control group, using ANOVA and Student-Newman-Keuls analyses

and 16.8% decrease in G0/G1 phase cell population compared to the control cells, respectively (P<0.05) (Table 1). The data indicate that cell growth inhibition by morphine is associated with significant cell cycle arrest at the G2/M phase and suggest that morphine suppresses cell proliferation by controlling the G2/M checkpoint and inducing a specific block in cell cycle progression.

Morphine Induces Cellular Apoptosis

To further study the effect of morphine on MGC-803 cell apoptosis, cells were stained with Annexin V/FITC and PI, and then subsequently analyzed by flow cytometry. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, the cells at apoptosis were in the right quadrant (including the upper right quadrant and the lower right quadrant). As indicated in Figure 3, the percentages of MGC-803 cells which were incubated with 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine in the right quadrant were 19.1%, 20.9% or 24.4%, which were significantly higher than that of the control cells (11.4%) (P<0.05). It implies that morphine could induce apoptosis in gastric cancer MGC-803 cells.

Morphological Study of Morphine Affecting Gastric Cancer Cells

As shown in Figure 4, 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine resulted in morphological changes of MGC-803 cells, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear

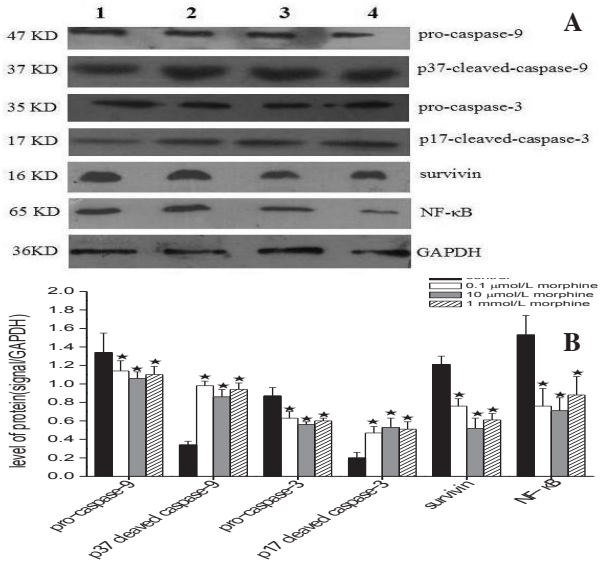

Figure 6. Morphine Could Increases p37 Cleaved Caspase-9 and p17 Cleaved Caspase-3 Protein Concentrations while Pro-caspase-9, Pro-caspase-3, Survivin and NF-κB are Decreased in MGC-803 Cells. (A) Western blot analysis of pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3, p17 cleaved caspase-3, survivin, NF-κB and GAPDH in MGC-803 cells from four groups respectively. Lane 1, control; lane 2, 0.1 μmol/L morphine; lane 3, 10 μmol/L morphine; lane 4, 1 mmol/L morphine; GAPDH: internal control protein. (B) pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3, p17 cleaved caspase-3, survivin, NF-κB protein levels were measured at four groups, normalized to those of GAPDH and presented as means ± SEM. *P<0.05 compared with control group, using ANOVA and Student-Newman-Keuls analyses

fragmentation, chromatin condensation, chromosomal DNA fragmentation and apoptotic bodies, as observed by scanning electron microscopy (SEM).

The Expression of Genes Using Semi-Quantitative RT-PCR and Western Blot

Caspase-9, caspase-3, survivin and NF-κB were selected to estimate expression differences between the cells which were incubated with 0.1 μmol/L, 10 μmol/L or 1 mmol/L morphine and the control MGC-803 cells by semi-quantitative RT-PCR and Western blot. As shown in Figure 5, densitometric analysis showed that caspase-9 and caspase-3 mRNA of MGC-803 cells in morphine groups were higher than that in control group while survivin and NF-κB mRNA in morphine groups was lower than that in control group (P<0.05), and no differences were found among morphine groups (P>0.05). As shown in Figure 6, morphine could lead to the cleavage of pro-caspase-9 (47 kDa) and pro-caspase-3 (35 kDa) into other multiple, cleaved, maturation products (data not shown), but only the 37-kDa form of cleaved caspase-9 and 17-kDa form of cleaved caspase-3 were observed. Densitometric analysis showed that p37 cleaved caspase-9 and p17 cleaved caspase-3 protein of MGC-803 cells in morphine groups were higher while pro-caspase-9, pro-caspase-3, survivin and NF-κB were lower than that of control group (P<0.05), and no differences were found among morphine groups (P>0.05).

Discussion

Morphine and chemically related opiate alkaloids have been implicated in a wide variety of pharmacological and physiological functions (Chida, 2011). In addition to their use in the treatment of pain, they appear to be important in the growth regulation of normal and neoplastic tissue. Many studies have demonstrated that morphine may decrease the incidence, development or spread of certain cancers, because it has been associated with downregulation of developmental processes and cell activity, including tumor growth retardation. Payabvash et al (Payabvash et al., 2006) found that morphine inhibited mice liver cancer growth. Also, morphine has been demonstrated to decrease growth of human breast cancer cells in vitro (Hatzoglou et al., 1996). However, we see the double-edged sword in morphine physiology and regulation. Morphine also potentially induces angiogenesis in cancer, thereby possibly facilitating tumor growth potential (Singleton et al., 2006). The complexity of morphine-tumor interaction can be observed in many experiments and how morphine influences tumor growth are far from being fully understood. In the present study, human gastric carcinoma MGC-803 cells were incubated with morphine and our results clearly showed that morphine could inhibit growth and proliferation of MGC-803 cells, reduce the capability of colony formation and make cell cycle arrest in G2/M phase. Moreover, morphine led to morphological changes of MGC-803 cells and induced cell apoptosis.

Apoptosis is an activated cellular death process that is induced by physiological or pathological factors to eliminate redundant and damaged cells. During initiation of apoptosis, the key event is the triggering of caspase cascade (Cardone et al., 1998; Yang and Yu, 2003). In this study, caspase-9 was activated by morphine which subsequently results in the downstream activation of caspase-3 (Green and Reed, 1998). Caspase-3 is the main executor of apoptosis and its higher activity results in morphological changes of cells, such as nuclear fragmentation, chromosomal DNA fragmentation and apoptotic bodies (Kumar, 1997; D'Amelio et al., 2010). This leads eventually to cell death (Martin and Green, 1995).

In the other hand, as a member of inhibitors of apoptosis proteins (IAP) family, survivin plays an important role in the regulation of cell death and cell survival. Survivin expression is an unfavorable prognostic factor in several malignancies, such as gastric carcinoma, breast carcinoma and acute myeloid leukemia. The higher the survivin expression, the worse the prognosis (Ibrahim et al., 2012). Clearly, inhibition of survivin activity would facilitate propagation of caspase cascade and enhance apoptosis (Chen et al., 2012; Dai et al., 2012). NF-κB is a ubiquitous nuclear transcription factor that plays a key role in the regulation of a large number of genes which related to apoptosis, tumorigenesis, inflammation, and immune diseases. Many investigations suggested that the activation of NF-κB might be a tumor promoter and the suppression of NF-κB could inhibit the growth of cancer

cells (Carbone and Melisi, 2012; Khan et al., 2012). Some researches reported that the anticancer effect of morphine might be mediated through inhibition of NF-κB (Sueoka et al., 1998; Yin et al., 2006).

In this study, as survivin is one of the downstream target genes of NF-κB, the potent inhibition of survivin expression by morphine was thought to bind to the inhibition of NF-κB, and then augment the activity of caspase-9 and caspase-3 and lead to apoptosis in gastric carcinoma MGC-803 cells eventually. Consequently, morphine may induce apoptosis in MGC-803 cells as a result of downregulation of NF-κB and survivin and upregulation of caspase-9 and caspase-3. It will be further identified in our future research.

Acknowledgements

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