The effect of morphine on the proliferation of human ovarian epithelial cancer A2780cp cell line

Mohammad Nabiuni1*, Hanieh Jalali2, Maryam Rezaeigazik2

(1) Department of Cell and Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.
(2) Department of Animal Sciences Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.

Abstract
Background: Morphine is used widely to treat severe pain in some medical conditions including cancer. Previous studies have shown conflicting reports about the effect of this analgesic drug on the proliferation and growth of various cancer cells. The aim of the present study was determining the effect of this drug (20-600 µg/ml) on the proliferation of ovarian cancer A2780cp cell line. To investigate the effect of morphine on A2780cp cell line, morphological changes were studied with inverted microscope, the viability of cells was determined with trypan blue staining and MTT assay, and the type of cell death was determined using flowcytometry analysis.

Results: Morphological and molecular analysis have revealed traces of apoptosis. Trypan blue staining and MTT assay have shown deceasing of viability in does and time dependent manners with IC50 from 385-510 µg/ml for 24-72 hours. Flowcytometry analyzes revealed 20.97% apoptosis after treatment with 300 µg/ml morphine for 48 hours.

Discussion: Our findings suggest that morphine can reduce the proliferation and growth of A2780cp cell line with inducing apoptosis more than necrosis in this type of cells.

Keywords: Morphine, A2780cp, ovarian cancer, proliferation.

1 Background

Morphine is the main and the most efficient component of opium which is contained 7-14% of it [1]. Morphine is used widely by cancerous patients to relieve pain especially in terminal phases of cancer [2]. Although several studies have shown anti proliferation effects of morphine, there are conflicts about its effects. Has showed injection of morphine in mouse models of breast cancer significantly reduced tumor growth [3], [4], also some in vitro studies have shown reduction of MCF-7 cancer cells growth treated with morphine [5]. However a study on A549 lung cancer cells demonstrated that clinical concentrations of
morphine can induce the expression of apoptotic markers [6]. It has been demonstrated that morphine may inhibit growth of PC-9, HL-60 and KATO III cancer cell or cancer lines mediated through inhibition of TNF-α release and TNF-α mRNA expression [7]. The effect of morphine on non-cancerous cells has been reported too and morphine has induced apoptosis in endothelial cells [8], T lymphocytes, macrophages [9], microglia and neurons derived from rat brain [10].

In contrast, there are studies demonstrating that clinical concentrations of morphine can increase tumor growth associated with increase of angiogenesis and decrease of apoptosis [11]. Effect of morphine on human glioblastoma T98G cell proliferation was studied and provided evidence that morphine can increase cell proliferation [12]. In addition MAD.MB231 breast cancer cells treated with morphine have been shown similar results. In a leukemia cancer mouse model this drug caused enhancement of tumor growth and immunosuppressive effects [13].

Epithelial ovarian cancer is the most lethal gynecological cancer worldwide [14] and it is the sixth most common cancer among women [15]. More than 90% of ovarian cancers have been thought to arise from epithelial cells that cover the ovarian surface. It is associated with severe pain in last therapeutic phases [15] which can originated directly from the tumor or as a result of therapy aimed at reducing the tumor, including surgery, chemotherapy, radiation therapy, and hormonal therapy [16]. Morphine is the best choice for a variety of common pain syndromes in gynecologic malignancies [17].

According to inevitable need to use analgesic drugs in patients under chemotherapy and their probable drug interferences or other side effects, and also on the basis of different effects of morphine on various cancer cells, it is important to investigate the effect of this drug on various cell lines separately and its effects must be under study to avoid unexpected and undesired results in cancer treatment. The aim of this study was determining the effect of morphine on the growth and proliferation of cisplatin resistant epithelial ovarian cancer A2780cp cell line.

2 Methods and materials

2.1. Cell culture

Human ovarian cancer A2780cp cells were purchased from NCBI (National Cell Bank of Iran). Cells were cultured in RPMI-1640 (Gibco-Invitrogen) with 10% (v/v) fetal bovine serum (FBS, Gibco-Invitrogen) and 1% of antibiotics (100U/ml penicillin and 100mg/ml streptomycin) at 37°C in a 5% (v/v) CO₂ and 95% humidified incubator. Medium was changed every 48 hours; cells were seeded in the number of 5×10⁴ in 24 well plate and were prepared for treatment.

2.2. Chemicals

Morphine sulfate was purchased from Daroupakhsh Co., DMSO from MERK Co., MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) from Sigma and Annexin V-FITC Apoptosis detection kit from Abcam company.

2.3. Morphological observations

Morphological changes of the cells in response to treatment with the morphine were monitored and periodically captured and analyzed. To observe the morphological changes the 5×10⁴ cells were cultured in 24-well plates and at 60-65% confluence, the cells were treated for 24-72 h with desired doses of morphine (200–600 µg/ml), Morphological changes were observed under an invert microscope and were compared to the untreated cells. A phase contrast microscope was used to determine the cell morphologies of untreated and morphine-treated cells.
2.4. Cell viability assessment using trypan blue
A total number of $5 \times 10^4$ A2780cp cells were cultured in each well of a 24-well culture dish and the dishes were kept in a CO$_2$ incubator for 24 hours. Then the supernatants were removed and 1 mL of RPMI 1640 medium containing 0 (control), 200, 400 and 600 µg/mL concentrations of morphine was added to each well. After 24, 48 or 72 hours, the cells were detached, stained with trypan blue, and the number of alive cells counted using a Neubauer chamber.

2.5. MTT Assay
To determine the viability percentage of cultured cells and also to measure the toxicity of morphine on A2780cp cells, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed as previously described [18]. Briefly, A2780cp cells were cultured in 24-well plates and treated with different concentrations of morphine for 24, 48 or 72 hours. Upon completion of treatment, 100µl of MTT solution (5mg/ml in PBS) was added per well. Cells were incubated at 37°C for 4 hours in darkness. Subsequently, the medium was removed and 1ml of DMSO was added to each well, and the plate was kept at room temperature for 20 minutes. The absorbance was measured at 570nm. The percentage of viable cells was calculated by fractionation of optical density of each experimental group on optical density of control group x100. Also, the 50% inhibitory concentration (IC50) values were determined from MTT obtained results.

2.6. Flowcytometry analyses
To find the mechanism by which morphine can induce cell death, flowcytometric analyze was performed to detect the phosphatidylserine, an early marker of apoptosis (4). Briefly, 4x $10^5$ of A2780cp cells were cultured in 25 cm$^2$ culture flasks and treated with 300 µg/ml of morphine as a concentration with 30% toxicity on the A2780cp cells for 48 hours. Cells were trypsinized and stained using the ANNEXIN V-FITC apoptosis detection kit (Abcam) according to the manufacturer's instructions. Stained cells were analyzed using a Flow cytometer BD FACS calibur instrument and the data obtained was analyzed using FCS Express 4 flowcytometry software.

2.7. Statistical analysis
All experiments were performed at least three times and data were analyzed using InStat.3 and Microsoft Excel Worksheet software. $p$ values less than 0.05 were considered significant.

3 Results

3.1. Morphine treatment modified the morphology of ovarian cancer cells
Morphological changes were observed after treatment with 200-400 µg/ml of morphine and compared with untreated cells. Cells treated with 200 µg/ml did not undergo significant morphological changes related to cell death, but there were few cellular shrinkage. The cells treated with higher concentrations showed significant morphological changes related to cell death and the cells gradually became flat and wrinkled with the appearance of small vesicle bodies which were related to apoptotic bodies. Arrows are showing shrunken cells and apoptotic bodies in figure 1. Upon the incubation of cells with increasing concentrations of morphine or the time of incubation, apoptotic cells were more frequently seen. This results demonstrated that morphine affected A2780cp cells in dose and time dependent manners.
3.2. Morphine inhibited cell proliferation in dose and time dependent manners

Cell counting showed morphine caused significant decrease of proliferation in dose and usually time dependent manners except in 200 µg/ml of morphine which had not shown dependence on time. Morphine treatment led to decrease of the cell number and as the figure 2 shows, The averages number of cells reached from $196 \times 10^4$ in control to $52.5 \times 10^4$ in 600 µg/ml of morphine treated group throughout 72 hr treatment ($p<0.001$).

![Figure 1: Morphological comparison of normal and treated cells. Non treated (control) cells (A), and treated with cell death inducing concentrations of morphine (B). Arrows are showing shrinkage of the cells and apoptotic bodies (Magnification 10×, observation under invert microscope).](image)

![Figure 2: The number of viable cells after staining with trypan blue and counting using a Neubauer chamber. The number of viable cells decreased in dose and time dependent manners after treatment with various concentrations of morphine for 24, 48 and 72 hours are shown. The degree of signification of decrease of the number of viable cells is shown with stars:* $p<0.05$, ** $p<0.01$, *** $p<0.001$.](image)

The comparison of the average cell viabilities obtained from MTT assay after 24, 48, and 72 hours revealed a significant difference between groups. The cell viability was decreased in treated groups in comparison with the control group (figure 3). Morphine treatment led to strong cell death and decreased cell viability ranging from 39.45-86.40 ($p<0.001$), 29.00-78.8 ($p<0.01$) and 74.00-25.00 ($p<0.05$) in A2780cp cells at 24, 48 and 72 hours, respectively.
Figure 3: Viability of cells arrived from MTT assay. Dose and time dependent effects of morphine on ovarian cancer cells (A2058cp) were shown. Viability of cells was decreased with increase of time or morphine concentration. The degree of signification of decrease of the percentage of viability after treatment with morphine is shown with stars:*P<0.05, **P<0.01, ***P<0.001.

Based on the IC50 values, the morphine exhibited anti proliferative activity on A2780cp cells at 510, 420 and 385 µg/ml at 24, 48 and 72 hours, respectively (Table 1).

Table 1: IC50 values. IC50 value of various times of treatment earned form dose response curves.

<table>
<thead>
<tr>
<th>time of treatment</th>
<th>IC50 µg/ml</th>
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<tbody>
<tr>
<td>24hr</td>
<td>510</td>
</tr>
<tr>
<td>48hr</td>
<td>420</td>
</tr>
<tr>
<td>72hr</td>
<td>385</td>
</tr>
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3.3. Morphine induced apoptosis in ovarian cancer cells

Detection of apoptotic and necrotic cells by flowcytometry is shown in Figure 4. The results of this assay showed that 300 µg/ml morphine which had 70% viability after 48 hours of treatment induced cell death in A2780pc cells mainly through apoptosis pathway. Because of the absence of significant apoptotic cells in 200 µg/ml and presence of many apoptotic cells in 400 µg/ml of morphine, we chose middle does of this drug. Statistical analysis of results indicated 11.75% of early apoptotic death in 300 µg/ml of morphine treated cells which was significant in compared to 7.40% early apoptotic death in control cells without treatment with morphine (Figure 4). The percentage of late apoptotic cells increased 6.39% and reached from 2.83% in control group to 9.22% in 300 µg/ml morphine treated group.
Figure 4: FACS analysis of A270cp treatment cells with morphine. The left 2D dot plot is control and the right is treated with 300 µg/ml of morphine. The cell population was separated into four groups: The cells stained with Annexin V, but not with PI classified as early apoptotic cells (low-right). The cells that were positive for Annexin V and PI classified as late apoptotic cells (up-right). The cells that were negative for Annexin V, but positive for PI were classified as necrotic cells (up-left) and those were negative for both Annexin V and PI were classified as normal viable cells (low-left). The number in each quadrant represents the percentages of cells. Similar results were obtained in another independent experiment.

4 Discussion

Cancerous patients use morphine to relieve severe pain to improve the quality of life [2]. Morphine has many physiological and pharmacological functions mediated through its µ, δ and κ receptors. The probably effects of this drug on the proliferation of cancerous cells have to be considered. In this study we investigate the effect of morphine on the proliferation and growth of epithelial ovarian cancer A2780cp cell line. We found that morphine significantly reduced cell viability in dose and time dependent manners in compared with the untreated cells. The percentage of viable cells decreased with increasing the dose of morphine and also extending the time of cell exposure to morphine. Morphine inhibited the proliferation and induced cell death through apoptosis pathway in A270cp cell line. These results were in accordance with results from previous studies suggesting that morphine induce apoptosis cell death in various cell lines or cancer induced mouse models.

The mechanism by which morphine can induce apoptosis is not well known but various studies propose that it can induce apoptosis through opioid receptors or independent of receptor mediated pathways. Some studies demonstrated that morphine treatment was able to induce apoptosis in SH-SY5Y cells in an opioid receptor-independent manner and c-Jun N-terminal kinase (JNK) has an important role in this process [19]. Activation of JNK with morphine led to generation of reactive oxygen species and caused release of cytochrome c and caspase-9/3 activation by increasing the expression of Bim, a pro-apoptotic protein, and reduction of expression of the anti-apoptotic protein Bcl-2. In addition decreased levels of JNK in cells transfected with specific small interfering RNA resulted in resistance to the pro-apoptotic effect of morphine [20]. Also morphine could induce apoptosis in human endothelial cells through both of the nitric oxide and ROS pathways [8]. Morphine inhibited growth of PC-9, HL-60 and KATO III cancer cells lines mediated through inhibition of TNF-α release and TNF-α mRNA expression [7]. It has been revealed that the µ-opioid receptor, by which morphine exerts its main action, directly regulates tumor growth. Binding of morphine to the µ-opioid receptor, regulates cell cycle progression in endothelial cells [21]. It can also mediate apoptosis by activating phosphatidylinositol 3-kinase /protein kinase B (Akt) pathway [22]. Morphine can promote the apoptosis of MCF-7 cells by a novel sigma-2 receptor pathway [23], [24] and CNE2 human epithelial tumor cell line by the activation of the κ-opioid receptor via the phospholipase pathway [25]. Therefore morphine can induce apoptosis or necrosis and inhibit the growth of different cancer cells by activating different signal pathways.
In conclusion, this study demonstrates that morphine impairs the viability and promotes the apoptosis of A2780cp cells and its effect is in dose and time dependent manners. This suggest that morphine effects as an anti pain drug must be under more attention to use in medical purposes.

Acknowledgment

The authors wish to thank Drug control Headquarters and Animal House Unit of Kharazmi University of Tehran for supporting this study.

References


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