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Induction of Apoptosis by Morphine in Human Tumor Cell Lines *In Vitro*

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Abstract. *Most previous studies of the induction of tumor cell apoptosis by morphine have been conducted with concentrations very much higher than those used clinically. An investigation of the ability of morphine to induce apoptosis at its clinical concentration (10^{-8} M) was therefore undertaken. Cytotoxicity was tested by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, induction of early apoptosis and necrosis by fluorescence-activated cell sorter (FACS) analysis with Annexin V and propidium iodide (PI), activation of caspase -2, -3, -8 and -9 by cleavage of specific substrates, DNA fragmentation by agarose gel electrophoresis, radical intensity and O_2^- scavenging activity by ESR spectroscopy. Millimolar concentrations of morphine showed higher cytotoxicity against human tumor cell lines (HL-60, A549, MCF7) than against normal human cells (HGF, HPC, HPLF). The clinical concentration of morphine produced early apoptotic markers in HL-60 and A549 cells whereas it induced higher numbers of necrotic cells in MCF7 cells, both in a naloxone-sensitive manner. The clinical concentration of morphine failed to activate any caspase species and induced only trace amounts of internucleosomal DNA fragmentation, in contrast to cytotoxic concentrations of morphine. Morphine, with a C-3 hydroxyl group, showed higher cytotoxicity and O_2^- scavenging activity than codeine, in which the hydroxyl group at C-3 was replaced with a methoxy group, suggesting the involvement of a radical-mediated reaction. The present report may offer new strategies for treatment and prevention of cancer using a clinical concentration of morphine not only as an anti-nociceptive, but also as an apoptosis or necrosis inducer.*

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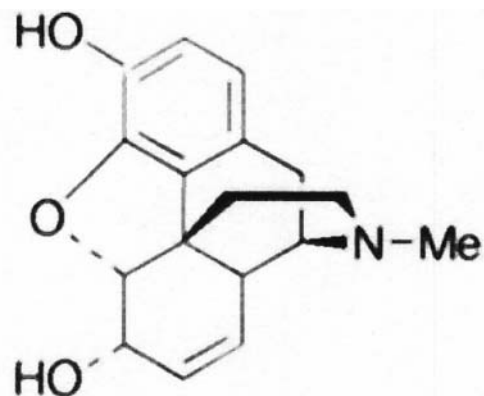
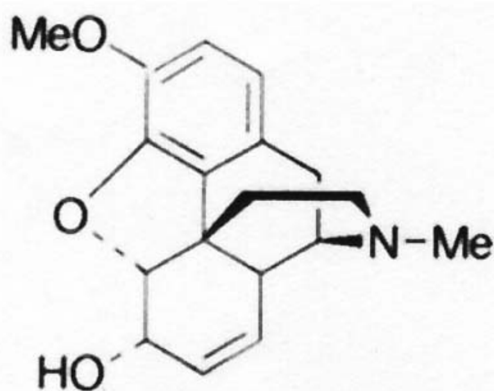
The control of cancer pain is one of the most important therapies for patients with cancer. Pain occurs in more than 80% of cancer patients before death (1-3). Morphine (Figure 1) is the most commonly used cancer pain relief agent in the world. The pain relieving effect of morphine may extend both length and quality of life. In contrast, morphine has shown side-effects that reduce the host immune response. Previous investigations have studied the effects of morphine on the mononuclear phagocyte system (4-7). This effect of morphine is partially attributable to morphine-induced macrophage apoptosis, which may result in the shortening of a patient's life (8, 9).

Although there are many extensive studies on morphine, it is not well understood whether morphine itself directly modifies the growth of tumor cells. Some reports have indicated that higher concentrations of morphine reduce the growth of tumor cells (8, 10-12). Morphine has been demonstrated to induce apoptotic cell death in human tumor cell lines, by a yet unknown mechanism (13, 14). However, these investigations, including ours, have all used extremely high concentrations of morphine (10^{-3}-10^{-6} M) and thus are not suitable for clinical use. Tegeder *et al.* have reported that the plasma concentration of morphine in cancer patients receiving chronic morphine treatment for pain relief is from $0.9 \sim 3.4 \times 10^{-6}$ M (15), while Smith *et al.* have reported that the baseline concentration of morphine in cerebrospinal fluid is approximately 0.4×10^{-6} M (16). The plasma concentration of morphine would be expected to be higher than the baseline concentration. No clear-cut



Morphine



Codeine

Figure 1. *Chemical stucture of morphine and codeine.*

relationship has been reported between morphine plasma concentration and cancer pain relief, but its lower limit for optimal pain control was found to be $40x10^{-8}$ M (17).

The tumor-specific cytotoxic activity of morphine has therefore been investigated here, using both several normal cells (periodontal ligament fibroblast HPLF, gingival fibroblast HGF, pulp cell HPC) and human tumor cell lines (squamous epithelial lung cancer A549, mammary gland tumor MCF7, promyelocytic leukemia HL-60). Also the ability of morphine at a clinical concentration to induce apoptosis, judged by the stainability with Annexin V as a probe of aminophospholipid exposure (18, 19), the induction of internucleosomal DNA fragmentation (20) and the effect of naloxone, an opioid receptor antagonist (21), has been assessed. The signaling pathway of morphine-induced apoptosis, using fluorescence-based caspase activation assays (22), has been determined.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT), hypoxanthine (HX), xanthine oxidase (XOD), dimethylenetriaminepentaacetic acid (DETAPAC), propidium iodide (PI) (Sigma Chem. Ind., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan) and Annexin V-FITC (Beckman Coulter Company, Marseille, France). Morphine hydrochloride, codeine phosphate and naloxone hydrochloride were obtained from Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan.

Cell culture. Human tumor cell lines (A549, MCF7) and human normal cells (HGF, HPLF and HPC) were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS under a 5% CO_2 humidified atmosphere. HGF, HPLF and HPC were prepared from the explants of periodontal gingival, ligament and pulp cells from first premolars extracted for orthodontic purposes, after obtaining approval from the Institutional Review Board of the Ethics Committee, Meikai University School of Dentistry, Japan. In this study, these cells were used between the fifth and tenth passages. MCF7 cells were obtained from the RIKEN Cell Bank, Tukuba, Japan. A549 cells were obtained from Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% FBS.

Assay for cytotoxic activity. Near confluent cells were incubated for 24 hours in DMEM or RPMI-1640 medium supplemented with 10%FBS containing various concentrations of the opioid (morphine and codeine) (96-microwell plate, Falcon). Each compound was dissolved in DMSO at 50 mg/mL and diluted with culture medium. DMSO at lower than 1% was found to be non-cytotoxic. After washing once with phosphate-buffered saline (PBS), the cells were incubated at 37˚C for four hours with 0.1 mL of fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, the cells were lysed with 0.1 mL DMSO. The relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate. Viability of HL-60 cells was determined by trypan blue exclusion. The 50% cytotoxic concentration (CC_{50}) of each test sample was determined from the dose–response curve.

Morphological evaluation of apoptotic and necrotic cells. The appearance of apoptotic and necrotic cells was monitored at the individual cell level by staining with Hoechst (H)-33342 (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI). Cells ($1x10^6$ /ml) were incubated with or without morphine for 24 hours. At the end of the incubation period, (H)-33342 (final concentration 1 μg/ml) was added and incubation continued for 10 min at 37˚C. The cells were placed on ice and PI (final concentration 1 μg/ml) was added to each well. The cells were incubated with dyes for 10 min on ice, protected from light, and then examined under ultraviolet light with the use of a Hoechest filter (Nikon, Melville, NY, USA).

Table I. *Cytotoxic activity of opioids against various human cultured cells.*

	CC_{50} (mM)					
	Tumor cell lines			Normal cells		
	HL-60	A549	MCF7	HGF	HPC	HPLF
Morphine HCl	0.51±0.05	2.03±1.09	4.05±1.59	2.83±1.22	4.72±0.75	2.79±1.33
Codeine phosphate	0.62±0.15	>10	>10	>10	>10	>10
Naloxone HCl	0.89±0.02	>10	>10	>10	>10	>10

Each value represents mean±SEM from 6 determinations. Near confluent cells were incubated for 24 hours with the indicated concentrations of morphine or codeine. The relative viable cell number was then determined by the MTT method and the CC_{50} value was determined from the dose-response curve.

Assay for apoptosis induction. To determine the number of necrotic and apoptotic cell populations, a fluorescence-activated cell sorter (FACS) (Becton Dickinson and Co., Franklin Lakes, NJ, USA) was used. The human tumor cell lines were incubated for four hours with or without the test compounds (morphine and naloxone) under control and experimental conditions. Cells were then collected by centrifugation at 1000 xg for five minutes at room temperature. Cells were washed twice in 500 μL cold (4˚C) PBS. One μL Annexin V-FITC kit solution and 10 μL PI kit solution were added to the samples, according to the manufacturer's instructions. After incubation in the dark for 15 minutes at room temperature, 400 μL binding buffer was added to the stained samples which were then subjected to FACS (18, 19).

The cell populations were separated into four groups: The cells that were stained with Annexin V, but not with PI were classified as early apoptotic cells (lower right quadrant, Figure 3). The cells that were positive for Annexin V and PI were classified as late apoptotic cells (upper right quadrant). The cells that were negative for Annexin V, but positive for PI were classified as necrotic cells (upper left quadrant). The cells that were negative for both Annexin V and PI were classified as normal viable cells (lower left quadrant).

Fluorescence-based caspase activation assays. Caspase-2, -3, -8 and -9-like activities were measured by a fluorometric microplate assay (MBL Medical & Biological Laborateries Co., LTD., Nagoya, Japan). The activity was measured as fluorescence generated by the addition of an appropriate fluorogenic peptide substrate (7-amino-4-trifluoromethyl coumarin (AFC) derivative) to the cell lysates. The fluorescence produced in the presence or absence of a specific tetrapeptide aldehyde inhibitor was measured and the difference was calculated to assess the level of caspase-mediated substrate cleavage. The substrates used to measure caspase-2, -3, -8 and -9 activity were Ac-VDVAD-AFC, Ac-DEVE-AFC, Ac-IETD-AFC and Ac-LEHD-AFC, respectively. Briefly, HL-60 cells ($5x10^5$/mL) were incubated for 0~4 hours with various concentrations of morphine. The cells were pelleted by centrifugation at 1000 xg for five minutes, and 50 μL lysis buffer, 50 μL 2x reaction buffer and 5 μL of each substrate were added and they were then incubated for one hour at 37˚C. Caspase activity was determined by the optimal intensity of liberated fluorescence of AFC (Ex 400 nm, Em 505 nm), using a Biolumin 960 fluorescence plate reader (Molecular Dynamics, Sunnyvale, CA) (23).

Assay for radical intensity. The radical intensity of the test samples was determined in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.0), or in 0.1 M KOH (pH 12.5) at 25˚C using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). The instrument settings were: center field 335.6±5.0 mT; microwave power 8 mW; modulation amplitude 0.1 mT; gain 630; time constant 0.03 seconds; scanning time 2 minutes. Radical intensity was determined 40 seconds after mixing and defined as the ratio of the peak height of the radical intensity produced by test samples to that of MnO (24).

For determination of the superoxide anion radical (O_2^-) produced by the HX and XOD reaction (total volume: 200 μL) [2 mM HX in the mixture of 50 μL of 0.1 M phosphate buffer (pH 7.4) (PB), 20 μL of 0.5 mM DETAPAC, 30 μL of 8% DMPO, 40 μL of sample (in DMSO), 30 μL of H_2O or SOD, and 30 μL XOD (0.5 U/mL in 0.1 M PB)], the gain was changed to 500. The radical intensity was determined by ESR spectroscopy 1 minute after mixing. The O_2^- scavenging activity was expressed as SOD unit/mg sample by calibration with a standard curve of SOD.

Statistical analysis. For comparison of mean values between two groups, the unpaired *t*-test was used. To compare values between multiple groups, analysis of variance (ANOVA) was applied and Newman-Keuls multiple range test was used to calculate the *p*-value. All values are represented as mean ± SEM unless otherwise stated. Statistical significance was defined as $p<0.05$, $p<0.01$ or $p<0.001$.

Results

Cytotoxic activity. A total of six target cells were incubated for 24 hours with various concentrations (0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 or 10 mM) of morphine hydrochloride, codeine phosphate or naloxone hydrochloride, and the 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve. Morphine showed higher cytotoxicity to all cell lines tested, than codeine (Table I). The tumor cell lines (HL-60, A549, MCF7) showed variable sensitivity to morphine with the HL-60 cells (CC_{50}=0.51 mM) being the most sensitive. The normal cells (HGF, HPLF, HPC) were relatively resistant to morphine (CC_{50}=2.83~4.72 mM). On the other hand, codeine and naloxone showed much

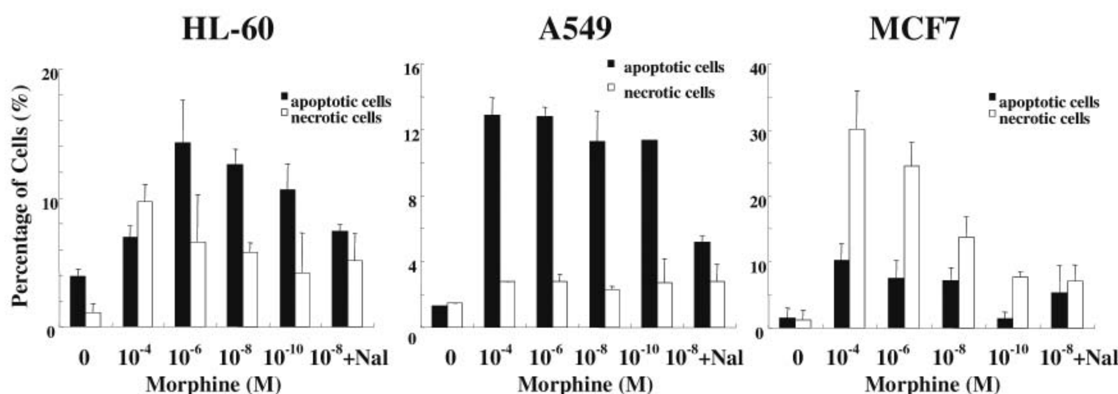


Figure 2. *Induction of apoptosis and necrosis by morphine in three human cancer cell lines. Cells were incubated for 24 hours with the indicated concentrations of morphine in the presence or absence of naloxone (10^{-6} M) and applied to FACS. Each value represents the mean from 4 determinations.*

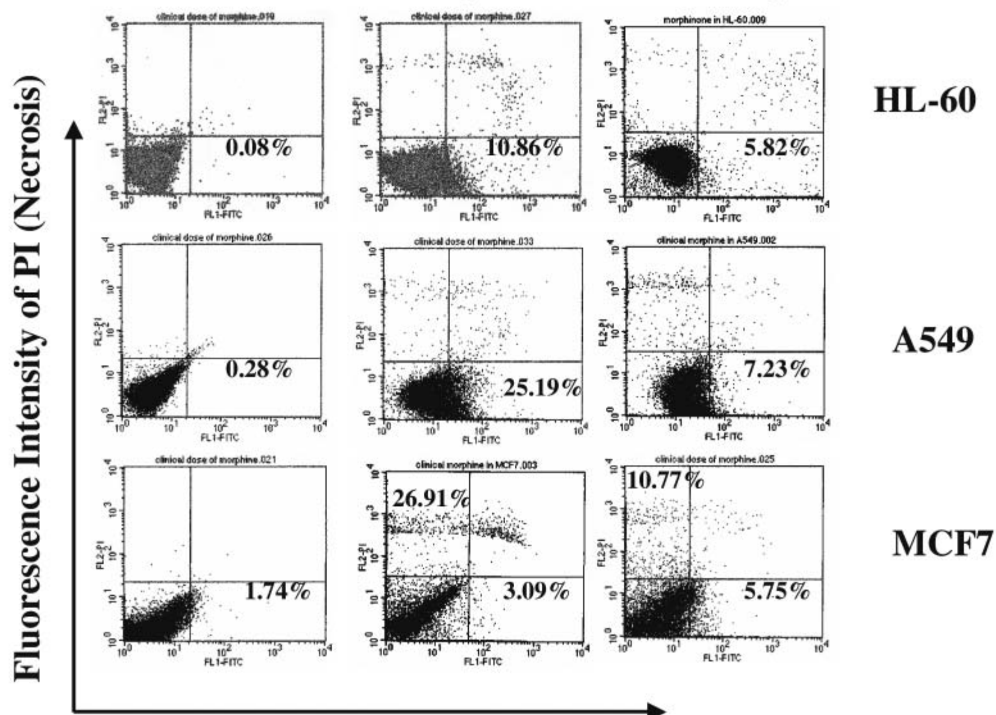


Figure 3. *FACS analysis of the presence of apoptotic and necrotic cell populations after treatment with a clinical concentration (10^{-8} M) of morphine in the presence or absence of 10^{-6} M naloxone in tumor cell lines. The number in each quadrant represents the percentages of cells. Similar results were reproducibly obtained in another 3 independent experiments.*

less cytotoxicity to all of the cells (CC_{50}>10 mM), except the HL-60 cells (CC_{50}=0.62 and 0.89 mM, respectively) (Table I).

FACS analysis. To evaluate the direct effect of morphine on human cell lines, they were incubated with various concentrations of morphine for 4 hours (Figure 2). Flow cytometry demonstrated that morphine dose-dependently stimulated apoptosis (Annexin-positive cells) or necrosis (PI-positive cells). The optimal concentration of morphine for induction of these effects was 10^{-6}-10^{-4} M.

The clinically effective concentration of morphine in the plasma (0.4 ~ 3.4 μM) is considerably lower than its cytotoxic dose described above (Table I). Accordingly, we set the concentration of morphine in this study at 10^{-8} M,

Table II. *Effect of morphine with or without naloxone on the induction of apoptosis and necrosis.*

	% of total cells		
Cell type	Control	Morphine	Naloxone+ Morphine
HL-60			
Necrotic	2.88±0.39	2.25±0.46	1.43±0.10
Late apoptotic	1.49±0.29	2.34±0.82	1.99±0.01
Early apoptotic	3.11±0.10	11.12±3.20**	5.81±0.53
Viable	92.7±1.3	84.3±4.3	90.8±0.6
A549			
Necrotic	1.87±0.81	2.29±0.24	1.57±0.42
Late apoptotic	0.56±0.22	1.19±0.28	0.61±0.09
Early apoptotic	1.31±0.51	17.56±5.20***	3.91±1.10##
Viable	96.3±1.2	79.0±5.3	93.9±1.5
MCF7			
Necrotic	2.41±1.16	17.77±2.26***	9.02±0.06*,#
Late apoptotic	0.12±0.05	1.17±0.20***	0.40±0.06##
Early apoptotic	1.18±0.41	5.47±2.06*	3.83±0.31
Viable	96.3±1.6	75.63±2.7	86.8±0.4

Cells were incubated for 4 hours without (control), or with 10^{-8} M of morphine or 10^{-8} M morphine + 10^{-6} M naloxone. Each value (% cells) represents mean±SEM from 4 independent FACS analyses. Figure 2 shows one representative example of such experiments.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with control; #$p<0.05$; ##$p<0.01$ compared with morphine.

considering that the concentration of morphine in the center of a tumor may be lower than its plasma concentration. The representative data of the induction of apoptotic and necrotic cell populations by 10^{-8} M morphine are shown in Figure 3. The experiments were independently repeated four times and these data are summarized in Table II. In the HL-60 cells, morphine (10^{-8} M) increased the proportion of early apoptotic cells from 3.11±0.10% (control) to 11.12±3.20% (mean±SEM(%); $p<0.01$). In the A549 cells, morphine also increased the proportion of early apoptotic cells from 1.31±0.51% (control) to 17.56±5.20% ($p<0.001$). However, in the MCF7 cells, morphine produced a larger population of necrotic cells (from 2.41±1.16% to 17.77±2.26%; $p<0.001$) than early apoptotic cells (from 1.18±0.41% to 5.47±2.06%; $p<0.05$). In addition, morphine also significantly increased the proportion of late apoptotic cells in MCF7 cells (from 0.12±0.05% to 1.17±0.20%; $p<0.001$).

To confirm the mechanism of action of the morphine-induced apoptosis and necrosis, the tumor cells were incubated with morphine (10^{-8} M) and naloxone (10^{-6} M) for four hours. The simultaneous addition of naloxone significantly reduced the number of both apoptotic and necrotic cells induced by morphine alone in all the tumor cell lines (Figure 3, Table II).

Caspase pathway. Fluorometric protease assays showed that millimolar concentrations ($3x10^{-3}$ M) of morphine activated caspase-2, -3 and -9, but not caspase-8 in HL-60 cells (Figure 4). Lower and clinical concentrations (10^{-8}-10^{-6} M) of morphine did not induce such activation of any caspase species.

O_2^- scavenging activity. ESR spectroscopy showed that morphine dose-dependently reduced the radical intensity of DMPO-OOH, a DMPO adduct of O_2^-, produced by the HX-XOD reaction (Figure 5). The O_2^- scavenging activity of morphine was calculated to be 1.66 SOD unit/mg, whereas codeine did not show O_2^- scavenging activity. Neither morphine or codeine produced detectable radical signals (radical intensity was below 0.03 under alkaline conditions (pH 10.0 and 12.5)).

Discussion

The present study has demonstrated that a clinical concentration of morphine induced apoptosis and necrosis in human tumor cell lines. In HL-60 and A549 cells, clinical concentrations of morphine produced Annexin-positive cells and the induction of a trace of internucleosomal DNA fragmentation (data not shown), possibly due to the lack of caspase activation at 10^{-8} M. However, morphine produced a higher number of necrotic cells in the MCF7 cells. These results indicate that the clinical dose of morphine acted differentially against lung and breast tumor cells. In addition, all of these cytotoxic actions were mediated by the opioid receptor, as judged from their efficient blockade by naloxone, a specific opioid antagonist.

Opioid effects on tumor growth have been a controversial topic of discussion. Opioids, acting *via* specific receptors, have been reported to inhibit the growth of human lung cancer cells, while nicotine, acting through nicotinic acetylcholine receptors, reverses the growth inhibition (11, 12). Morphine inhibited the proliferation of MCF7 cells at a concentration of over 10^{-5} M while it caused cell death, *via* a p53-dependent mechanism in adenocarcinoma cells at concentrations over $5x10^{-4}$ M, a very much higher concentration of morphine than normal clinical plasma concentration (15). A clinical concentration of buprenorphine induced apoptosis in A549 and MCF7 cells by activation of caspase-3 like protease (25), but these anti-cancer effects were not mediated by the opioid receptor. Therefore it is recognized that morphine and other opioids can induce apoptosis or necrosis and inhibit the growth of different cancer cells by activating different signal pathways. Apoptosis of MCF7 cells has also been shown to be triggered by a novel p53- and caspase-independent

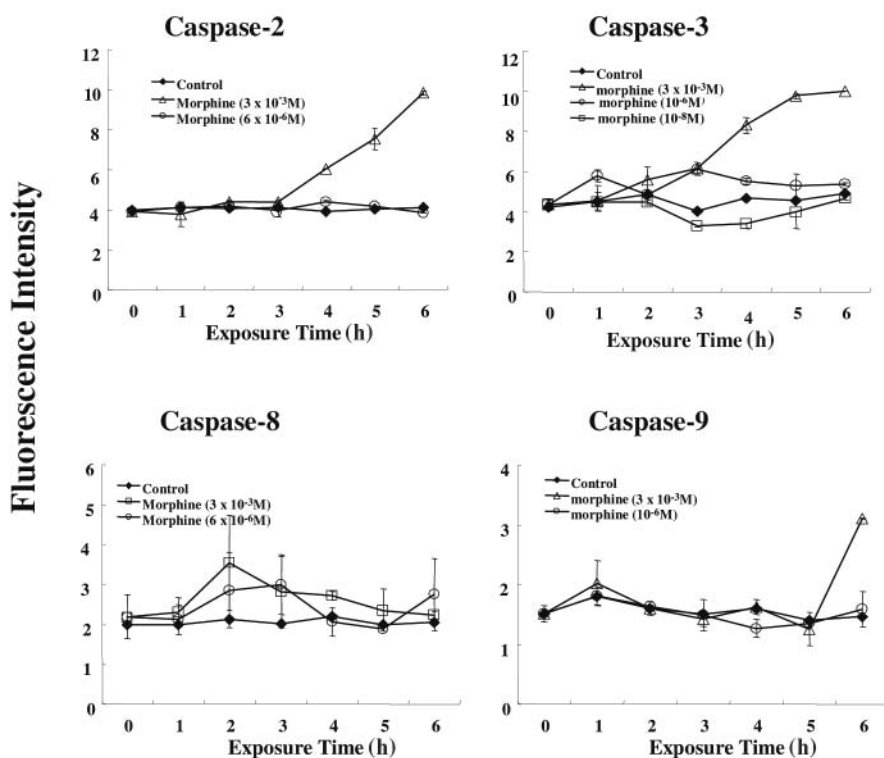


Figure 4. *Activation of caspase-2, -3, -8 and -9 by cytotoxic concentrations of morphine. HL-60 cells were incubated for the indicated times with 0 (●) (control), 10^{-8} M (□), 10^{-6} M (○) or $3x10^{-3}$ (△) morphine, and 4 different caspase activator were assayed by measuring the cleavage of specific substrate.*

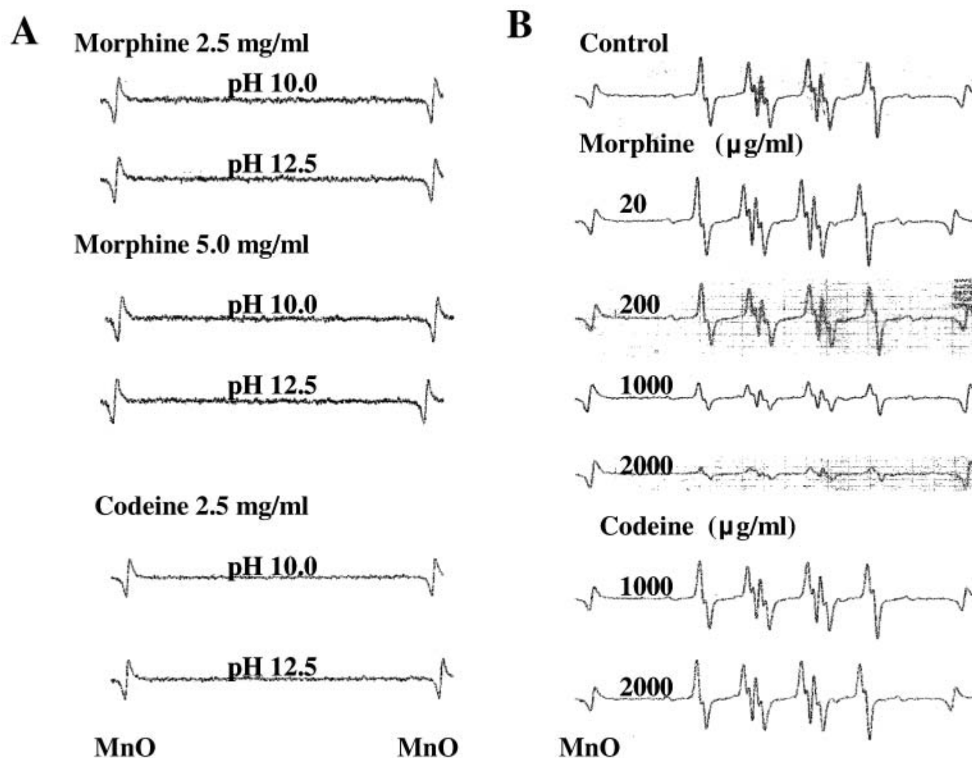


Figure 5. *ESR analysis of O_2^- scavenging activity and radical intensity of opioids. (A) Radical generation by morphine and codeine at alkaline pHs. (B) Effect of morphine and codeine on the intensity of DMPO-OOH, DMPO addict of O_2^-, generated by HX-XOD reaction.*

apoptotic pathway using a sigma-2 receptor (26-31). CNE2 human epithelial tumor cell lines have been shown to undergo apoptosis by the activation of a kappa-opioid receptor *via* a phospholipase C pathway (32). Delta opioid peptide inhibited the apoptosis of pheochromo-cytoma cells (PC12) by a delta2 opioid receptor- mediated increase of Fas-ligand (33). These investigations suggest the importance of more specific pain and cancer treatments for each cancer patient. However, an effect of a much lower, clinical concentration of morphine on the target cancer cell lines has not been investigated, making the direct comparison between their results and ours difficult.

Unexpectedly, a difference in the physiological activity of morphine and codeine was found. Supraclinical concentrations of morphine showed higher cytotoxicity and O_2^- scavenging activity than codeine. The only structural difference between these opioids is found at the C-3 position where morphine has a hydroxyl group, whereas codeine has a methoxy group (Figure 1). The presence of the hydroxyl group at C-3 seems to be essential for conferring the radical-mediated cytotoxic activity on morphine. Further study is required to elucidate the mechanism by which morphine induces apoptosis.

Opioids alleviate pain for cancer patients, improve their appetite and allow them sound sleep. The present study may suggest new strategies for the treatment and prevention of cancer, using opioids as anti-nociceptive and apoptosis or necrosis inducer.

Acknowledgements

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