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Comparative Analysis of Apoptosis-inducing Activity of Codeine and Codeinone

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Background: **There are relatively few studies about the antiproliferative effects of codeine-related compounds on human cancer cell lines, compared with those of morphine-related compounds. The authors previously found that codeinone, an oxidation metabolite of codeine, among 10 opioids, showed the highest cytotoxic activity (DNA fragmentation-inducing activity) against human promyelocytic leukemic cell lines (HL-60). This was counteracted by an antioxidant, *N*-acetyl-L-cysteine (NAC). These findings prompted us to perform a more detailed study of apoptosis induction after codeinone treatment.**

Methods: **Apoptosis was induced by treating HL-60 cells for 1–6 h with codeine or codeinone. DNA fragmentation was assessed by both agarose gel electrophoresis and fluorometric determination of the fragmented DNA after staining with diamidinophenylindole (DAPI). The appearance of apoptotic cells was monitored by microscopic observation after staining with Hoechst (H)-33342, and fluorescence activated cell sorter (FACS) after staining with Annexin. The release of cytochrome c and cytochrome oxidase from mitochondria and activation of caspase 3 were monitored by Western blot analysis. Intracellular caspase 3–like activity was confirmed by FACS, using cell permeable substrate. Mitochondrial manganese-containing superoxide dismutase (MnSOD) activity and mRNA expression were assayed by activity staining after separation on the polyacrylamide gel electrophoresis, and reverse transcriptase-polymerase chain reaction (RT-PCR), respectively.**

Results: **Codeinone induced internucleosomal DNA fragmentation and production of Annexin-positive apoptotic cells more potently than codeine in HL-60 cells. Codeinone stimulated the release of both cytochrome c and cytochrome oxidase, and cleavage of procaspase 3 without significant changes in both the activity and expression of MnSOD.**

Conclusions: **Codeinone was found to possess both apoptosis and necrosis-inducing activity, in addition to the reported antinociceptive activity, further substantiating its antitumor potential.**

MOST previous studies of opioids have dealt with their analgesic effects, addiction, adverse drug reactions, and a variety of other side effects.[1–9] Although there are many extensive reports about opioids as cancer pain killers, it is not well understood whether opioids themselves directly inhibit the growth of tumor cells. Recently, morphine 6-glucuronide, an active metabolite of morphine and other synthetic derivatives, has been reported to induce apoptosis in human cancer cells.[10] However, the mechanism of apoptosis induction by these compounds is still obscure. Furthermore, tumor-specific cytotoxic action of morphine-related compounds has not extensively been investigated.

Alternately, codeine, which has been used as the second step on the analgesic ladder proposed by the World Health Organization's cancer treatment guidelines, had not yet been investigated for its apoptosis-inducing capability. In both human and animals, the major metabolites of codeine are codeine 6-glucuronide, morphine, and norcodeine.[11] However, in the presence of nicotinamide-adenine dinucleotide (NAD), 9,000*g* supernatant of guinea pig liver homogenate can transform codeine into codeinone.[12] Currently, the exact metabolic pathway of codeine to codeinone in human liver has not yet been reported. However, it has been reported that the antinociceptive activity of systemically administered codeinone, using the acetic acid writhing test, was four times stronger than that of codeine, although its pharmacologic characteristics are not yet fully understood.[13–18]

Based on this background, we have synthesized codeinone by oxidizing codeine, and recently reported that codeinone showed the greatest cytotoxicity, among 10 morphine-related compounds, against human promyelocytic leukemia cells (HL-60), human oral squamous cell carcinoma (HSC-2), and human submandibular gland carcinoma cell (HSG).[18] We also found that codeinone induced internucleosomal DNA fragmentation in HL-60 cells and its activity was significantly reduced by *N*-acetyl-L-cysteine (NAC), but not by other antioxidants, such as vitamin C and catalase.[18] However, the mechanism of the cytotoxic action was still unclear.

In this study we compared the tumor-specific cytotoxic activity of codeine, codeinone, and morphine 6-glucuronide, using several normal cells and tumor cells, and their ability to induce various apoptosis-associated characteristics in HL-60 cells (used as a model

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Table 1. Cytotoxic Activity of Opioids (μM)

	CC_{50} (μM)					
	Tumor Cell Lines			Normal Cell Lines		
	A549	MCF7	HL-60	HPLF	HGF	HPC
Morphine 6-glucuronide	424 ± 55	nd	4610 ± 249	nd	1440 ± 180	437 ± 137
Morphine chloride	2522 ± 357	6552 ± 180	1359 ± 105	4266 ± 285	4374 ± 950	8847 ± 293
Codeinone	16.5 ± 0.5	14.0 ± 4.5	4.7 ± 0.1	34.6 ± 5.0	23.9 ± 6.5	28.6 ± 2.9
Codeine phosphate	42721 ± 539	11770 ± 157	>325	25251 ± 3630	24430 ± 2645	22479 ± 2075

All values are expressed as mean ± SE, (n = 4). The cells were treated for 24 h with various concentrations of each compound, and the viable cell number of HL-60 cells and other cells was determined by trypan blue exclusion or MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method, respectively. The CC_{50} (μM) was then determined from the dose-response curve.

nd = not determined; A549 = human lung carcinoma; MCF7 = human mammary gland tumor; HL-60 = human promyelocytic leukemic cells; HPLF = human periodontal ligament fibroblast; HGF = human gingival fibroblast; HPC = human pulp cells.

system). In addition, morphine has recently been reported to elevate the mitochondrial manganese-containing superoxide dismutase (MnSOD) content during apoptosis.[19] Since the inhibition of SOD by 2-methoxyestradiol significantly enhanced the susceptibility to apoptosis induction, SOD has been suggested to be a promising target for selective killing of cancer cells.[20–21] Therefore, we also investigated whether codeine and codeinone modify the activity or mRNA expression of MnSOD, and Cu/Zn containing SOD (Cu/ZnSOD).

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated companies: RPMI1640 medium, Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY); fetal bovine serum, (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), riboflavin, phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Ind., St. Louis, MO); and dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan). Morphine hydrochloride and codeine phosphate (codeine) were obtained from Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan. Morphine 6-glucuronide was obtained from Shionogi & Co. Ltd., Osaka, Japan. Codeinone was prepared from codeine according to the literature.[22]

Cell Culture

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

Assay for Cytotoxic Activity

Near confluent cells were incubated for 24 h with 0.1 ml of culture medium containing various concentrations of test samples (96-microwell plate, Falcon, Becton Dickinson and Company, Franklin Lakes, NJ). The viable cell number of HL-60 cells in suspension culture was then determined by trypan blue exclusion (table 1). The viable cell number of other adherent cells and HL-60 cells was determined by the MTT method (fig. 1). In brief, the cells were washed once with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 9.6 mM NaH_2PO_4, 1.4 mM K_2HPO_4, pH 7.4) and incubated for 4 h with 0.2 mg/ml MTT in fresh DMEM supplemented with 10% FBS. After removing the medium, cells were lysed with DMSO, and the absorbance at 540 nm (which reflects the relative viable cell number) was measured. The 50% cytotoxic concentration (CC_{50}) of test samples was determined from the dose–response curve.

Assay of DNA Fragmentation

DNA was extracted and applied to 2% agarose gel electrophoresis for detection of DNA fragmentation as described previously.[18] DNA isolated from ultraviolet (UV)-irradiated HL-60 cells was run in parallel as markers of oligonucleosomal DNA fragments, together with the DNA size marker.

Quantitative Analysis of DNA Fragmentation

The extent of DNA fragmentation was quantified by fluorometric determination of DNA recovered from the supernatant of cell lysate. Briefly, cells were lysed in 5 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA)-0.5% Triton X-100 for 20 min on ice. The lysate and its supernatant obtained after centrifugation at 27,000*g* for 20 min were sonicated and their DNA contents were measured by the diamidinophenylindole (DAPI) method, using fluorescence spectrophotometer

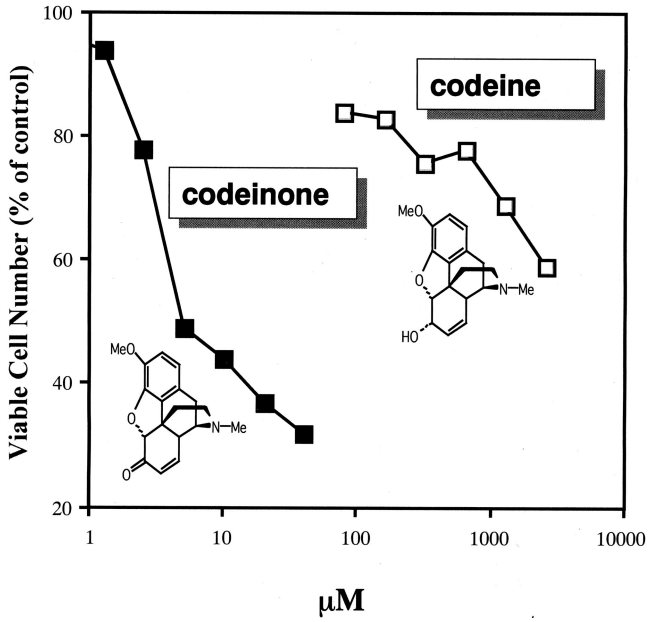

Fig. 1. Structure and cytotoxic activity of codeine and codeinone. HL-60 cells (1 × 10^6/ml) were incubated for 24 h without (control) or with the indicated concentrations of codeine or codeinone. Cells were incubated with MTT reagent, and the relative viable cell number was then determined and expressed as % of control absorbance at 540 nm. Each value represents mean from 4 determinations. HL-60 = human promyelocytic leukemic cells; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

(F-4500 HITACHI, Hitachi, Ltd., Tokyo, Japan). The percentage of DNA fragmented was defined as the ratio of DNA content in the supernatant to that of total DNA in the lysate.[23–24]

Morphological Evaluation of Apoptotic 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), and Propidium Iodide (PI) (Sigma Chemical Co., St. Louis, MO).[19] HL-60 cells (1 × 10^5/ml) were incubated without or with codeinone for 6 h. At the end of the incubation period, (H)-33342 (final concentration, 1 μg/ml) was added and incubated for 10 min at 37°C. Cells were placed on ice and PI (final concentration, 1 μg/ml) was added to each well. Cells were incubated with dyes for 10 min on ice, protected from light, and then examined under ultraviolet light with the use of a Hoechst filter (Nikon, Melville, NY).

Detection of Apoptotic Cells by Fluorescence Activated Cell Sorter (FACS)

Cells were incubated with test samples for 4 h. Cells were collected by centrifugation at 1,000*g* for 5 min at room temperature, and washed twice in cold PBS. A PNIM3546 ANNEXIN V-VITC Kit (Bechman Coulter Company, Marseille, France) was used for FACS (Becton, Dickinson and Company, Franklin Lakes, NJ).[25,26] Cells were added 1 μl Annexin V-FITC and 10 μl PI, and incubated in the dark for 15 min at room temperature. The stained samples were added 400 μl 1× binding buffer and processed by FACS. Cells that have bound Annexin V but do not take up PI (bottom right quadrant, fig. 4) are early apoptotic. Annexin V positive cells that take up PI are late apoptotic (top right quadrant). Annexin negative cells that take up PI are necrotic (top left quadrant). Cells that are negative for both Annexin V and PI (bottom left quadrant) are nonapoptotic and nonnecrotic viable cells.

Assay for Expression of Apoptosis-Related Proteins

The cell pellets were resuspended in 100 μl of homogenizing buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF), and disrupted by passing through a 21.5 gauge syringe 10 times (for the cytochrome c release assay with intact mitochondrial system) or by three cycles of freezing (at −80°C and thawing [on ice] for the caspase 3 degradation assay with disrupted cell system).[27] We did not add Trixton X-100 (1%) in the homogenizing buffer to avoid the leak of these proteins from mitochondria. The cell homogenates were centrifuged at 10,000 rpm, 5 min at 4°C to pellet down the mitochondria, and nuclear fraction, and the supernatant was collected. Cytosol fraction was obtained as supernatant after centrifugation of post-mitochondrial supernatant at 105,000*g* 15 min from the supernatant. The protein concentrations were measured using a Protein Assay Kit (Bio Rad, Hercules, CA). The equal amounts of the protein (20 μg) were mixed with 2× sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptoethanol), boiled for 10 min, and the aliquots equivalent to 20 μg protein were applied to the 15% SDS polyacrylamide gel electrophoresis, and then transferred to nitro cellulose membranes. Then membranes were blocked with 5% skim milk in Tris-HCl buffered saline plus 0.05% Tween 20 for 1.5 h and incubated with antibodies against cytochrome c (BD Biosciences, Pharmingen, San Diego, CA), antibodies against cytochrome oxidase subunit II (Molecular Probes, Inc., Eugene, OR), and caspase-3 (Transduction Laboratories, Lexington, KY) 1:1000 for 1.5 h at room temperature or overnight at 4°C, and then incubated with horseradish peroxidase-conjugated antirabbit immunoglobulin G (IgG) for 30 min at room temperature. Immunoblots were developed with a Lumi-Light^{PLUS} western blotting substrate (Roche, Molecular Biochemicals, Germany), according to the manufacturer's instructions.

Intracellular Caspase 3–like Activity

Intracellular caspase 3–like activity was measured using the PhiPhiLuxG_1D_2 (OncoImmunin, Gaithersburg, MD).[28] HL-60 cells (5 × 10^5/ml) were treated for 30 min or 1 h at 37°C with 34 μM codeinone. Cells were pelleted

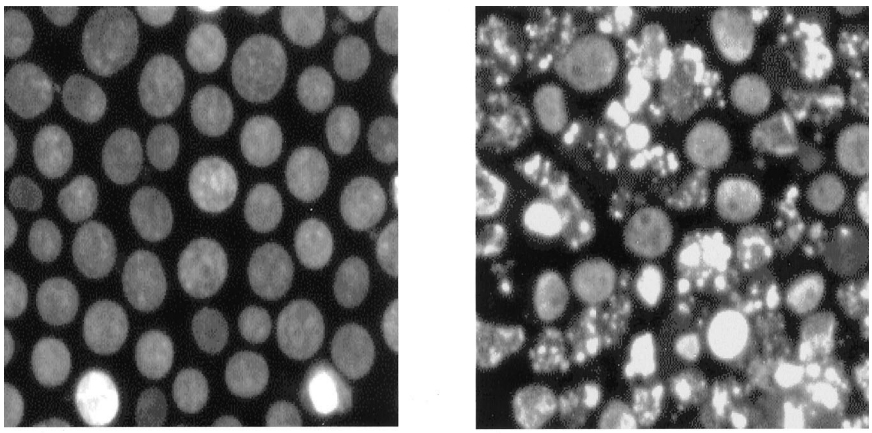

Fig. 2. Morphological changes induced by codeinone in HL-60. HL-60 (1×10^5/ml) cells were incubated without (control) or with codeinone for 6 h. H-33342 (final concentration, 1 μg/ml) were then added and incubated further for 10 min at 37°C. Cells were placed on ice and PI (final concentration, 1 μg/ml) was added to each well. Cells were stood for 10 min on ice, protected from light, and then examined under ultraviolet light with the use of a Hoechst filter. Most control cells were not stained with PI, whereas codeinone-treated cells were H-33342 and PI-stained and rich in apoptotic bodies with fragmented nuclei. HL-60 = human promyelocytic leukemic cells; H-33342 = Hoechst-33342; PI = propidium iodide.

and cultured for 1 h at 37°C in 75 μl of 10 μM PhiPhiLuxG_1D_2 substrate. After washing, caspase 3–like activity was measured as follows. Cells were gated to eliminate the PI-stained cells according to the protocol, and the fluorescence intensity in 1×10^4 cells were measured at the Fluorescence 1 (FL1) channel. Cells that were not stained were used to set the threshold point.

Assay of Superoxide Dismutase Activity

MnSOD and Cu/ZnSOD activities were measured by activity staining with nitro blue tetrazolium (NBT) (Sigma Chemical Co., St. Louis, MO) and riboflavin directly on the gel after separation on polyacrylamide gel electrophoresis.[29] In brief, cells were washed once with cold PBS, dissolved in the sample buffer (1% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and applied to 9% polyacrylamide gel electrophoresis (45 min, 20 mA). The gel was stained with 2 mg/ml NBT in water for 15 min in the dark, replaced with 10 μg/ml riboflavin in the dark for 15 min, and then illuminated overnight. During illumination, the gel became uniformly blue except for the position containing SOD. The gel was destained by washing in water, and subjected to image processing by scanner (Canoscan N656U, Canon Inc., Tokyo, Japan).

Assay for mRNA Expression

Total RNA was isolated using a Purescript RNA Isolation kit (Gentra systems, Minneapolis, MN) protocol. HL-60 cells (1×10^6/ml) were lysed in 300 μl cell lysis solution, then 100 μl Protein-DNA precipitation solution was added. Lysis fluids were centrifuged at 15,000*g* for 3 min. The supernatant was added 300 μl isopropanol. After centrifugation at 15,000*g* for 3 min, the pellet was

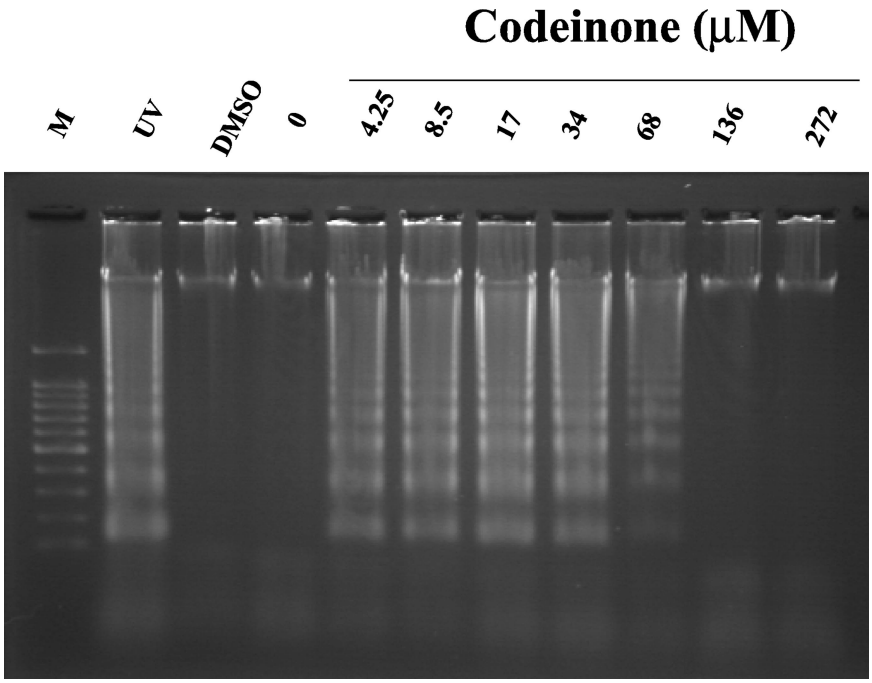

Fig. 3. Induction of internucleosomal DNA fragmentation by codeinone. HL-60 (1×10^6/ml) cells were incubated with the indicated concentration of codeinone for 6 h. DNA was extracted and applied to 2% agarose gel electrophoresis for detection of DNA fragmentation. DNA isolated from UV-irradiated HL-60 cells was run in parallel, as markers of oligonucleosomal DNA fragment, together with DNA size marker (M). HL-60 = human promyelocytic leukemic cells; UV = ultraviolet DMSO = dimethyl sulfoxide.

Table 2. Induction of DNA Fragmentation by Codeinone in HL-60 Cells

Concentration (μM)	DNA Fragmentation (%)
Codeinone	
0 (control)	25 ± 0.3
17.0	24.7 ± 1.6
34.0	48.9 ± 0.7*,†
165.0	50.2 ± 1.4*,†
Codeine	
0 (control)	16.6 ± 3.5
672	17.3 ± 3.6
3360	33.5 ± 0.3
6720	21.2 ± 0.2

HL-60 cells ($1 \cdot 10^5 \cdot ml^{-1}$) were incubated for 6 h with the indicated concentrations of codeinone or codeine and the extent of DNA fragmentation was assessed by the DAPI method. Each value represents mean ± SE from four determinations. To compare values between multiple groups, analysis of variance (ANOVA) was applied and a Least Significant Difference method was used to calculate a *P* value.

* $P < 0.001$ compared with respective controls; † $P < 0.001$ compared with the concentration of 17.0 μM codeinone.

HL-60 = human promyelocytic leukemic cells; DAPI = diamidinophenylindole.

washed in 300 μl 75% ethanol. After centrifugation at 15,000*g* for 1 min, the pellet was air dried for 15 min and dissolved in diethyl pyorcarbonate (DEPC)-treated H_2O. A reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with 1.0 μg of total RNA using the Rever Tra Ace (Toyobo Co., LTD, Osaka, Japan). Single strand cDNA obtained by RT reaction with oligo $(dT)_{20}$ primer was amplified using the KOD plus (Toyobo Co., LTD, Osaka, Japan), using MnSOD specific primers of (5'-TCCCCGACCTGCCCTACGAC-3' and 5'-CATTCTCCCAGTTGATTACAT-3'), and Cu/ZnSOD specific primers (5'-ATGGCCACGAAGGCCGTGTGC-3' and 5'-GGAATGTTTATTGGGCGATCCCA-3'), G3PDH specific primers (5'–TCCACCACCCTGTTGCTGTA-3' and 5'–ACCACAGTCCATGCCATCAC-3'), according to the protocol. The RT-PCR products were applied to 2% agarose gel, and the ethidium bromide-stained gel was then photographed under UV light.

Statistical Analysis

All data in this study were expressed as mean ± SE. To compare values between multiple groups in DAPI method, analysis of variance (ANOVA) was applied and a Least Significant Difference Method was used to calculate a *P* value. Statistical significance was defined as *P* less than 0.01.

The appearance of apoptotic cells and caspase 3–like activity were compared between control and the groups using the Student *t* test. Statistically significant difference was assumed if the *P* value was less than 0.05.

Results

Codeinone dose-dependently reduced the viable cell number of HL-60 cells (fig. 1). The cytotoxic activity of codeinone in HL-60 (CC_{50} = 4.7 ± 0.1 μM) was more than 65 times higher than that of codeine ($CC_{50} > 325$ μM) (table 1). Codeinone induced various apoptosis-associated characteristics in HL-60 cells. Morphological observation with H-33342 staining demonstrated that codeinone produced significant amounts of apoptotic bodies with fragmented nuclei (fig. 2). Agarose gel electrophoresis demonstrated that codeinone induced internucleosomal DNA fragmentation (fig. 3), confirming our previous report. Codeinone, but not codeine, dose-dependently induced DNA fragmentation, as judged by the accumulation of DNA fragments (recovered in the supernatant fraction after centrifugation) (table 2).

FACS analysis demonstrated that codeinone increased the production of apoptotic cells, detected by Annexin staining, from 5.0 ± 2.2% (control, n = 7) to 41.5 ± 5.7% (codeinone-treated; $P < 0.001$, n = 4) (One of the representative data are shown in fig. 4). It is also notable that codeinone produced small numbers of late apoptotic (top right quadrant, fig 4) and necrotic cells (top left quadrant, fig. 4) with PI. We found that codeine (6450 μM) did not significantly increase the number of

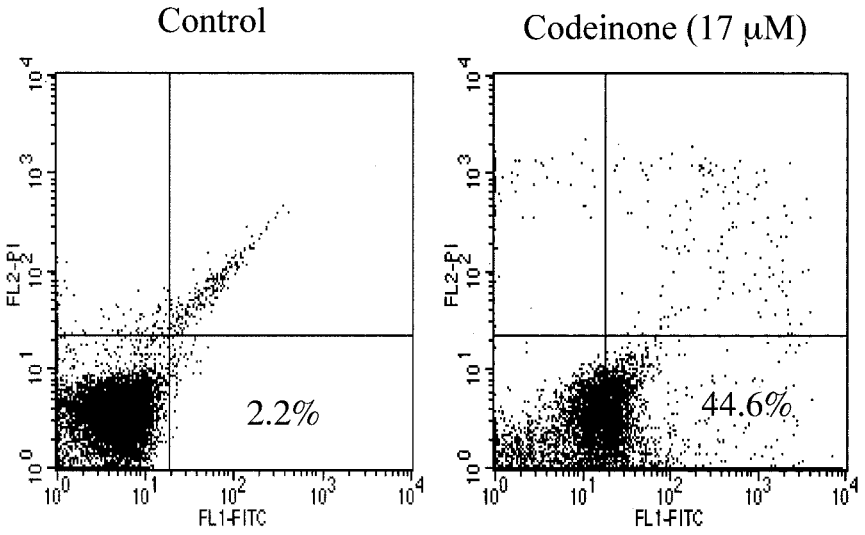

Fig. 4. Induction of apoptosis and necrosis by codeinone. HL-60 cells were incubated for 6 h without (control) or with 17 μM codeinone. The percent of apoptotic cells (*bottom right*) was determined by FACS after Annexin staining. Cells that have bound Annexin V, but do not take up PI (bottom right quadrant of dot plot) are early apoptotic. Annexin V positive cells that also take up PI are late apoptotic (*top right*). Cells that take up PI but not stained by Annexin V are necrotic cells (*top left*). Cells that are negative for both Annexin V and PI (*bottom left*) are nonapoptotic and nonnecrotic viable cells. Similar results were reproducibly obtained in another 3–6 independent experiments. The percent of the apoptotic cells was increased from 5.0 ± 2.2% (control, n = 7) to 41.5 ± 5.7% (codeinone-treated; $P < 0.001$, n = 4). HL-60 = human promyelocytic leukemic cells; FACS = fluorescence activated cell sorter; PI = propidium iodide.

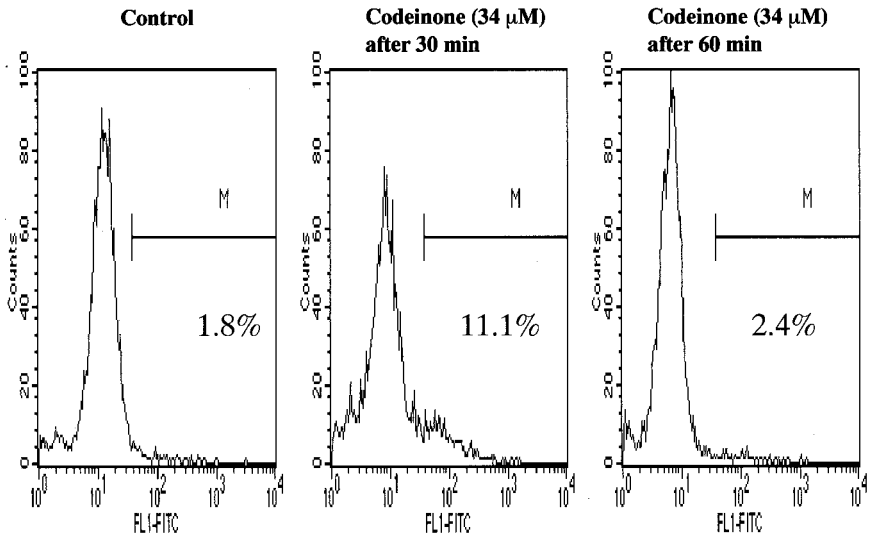

Fig. 5. Elevation of caspase 3–like activity at early stage of codeinone-induced cell death. Data from a representative flow cytometric analysis of intracellular caspase 3–like activity in HL-60 cells are shown. Cells were incubated for 30 min or 1 h without (control) or with 34 μM codeinone. The relative fluorescence intensity of cleaved PhiPhiLuxG$_1$D$_2$ substrate (FL1/x-axis) is given in the abscissa as log scale. The percentage of intracellular caspase 3–like activity appears at the range indicated by M. Repeated experiment demonstrated that the increase of caspase 3–like activity was transient, peaked at 30 min after codeinone treatment (9.8 ± 1.1%; $P < 0.05$, n = 4), and declined at 1 h, returning to control level [2.6 ± 0.5% *versus* 2.5 ± 0.4% (control), not significant, n = 4]. HL-60 = human promyelocytic leukemic cells; FL1 = fluorescence 1; FITC = fluorescein isothiocyanate.

apoptotic cells (data not shown). It is well known that DNA fragmentation is executed by caspase-activated DNase (CAD), which has to be first activated by cleavage of inhibitor of CAD by caspase 3.[30,31] We confirmed that this actually occurred. Codeinone (34 μM) activated the caspase 3–like activity shortly after treatment (fig. 5). Our repeated experiments further confirmed that the increase of caspase 3–like activity was transient, peaked at 30 min after codeinone treatment (9.8 ± 1.1%; $P < 0.05$, n = 4), and declined at 1 h, returning to control level (2.6 ± 0.5% *versus* 2.5 ± 0.4% [control], not significant, n = 4). Caspase activation (fig. 5) was followed by the cleavage of caspase 3 (bottom panel, figure 6). It remains to investigate whether caspase 3 was activated *via* mitochondrial pathway or *via* caspase 8 independently of mitochondria.[32–34] We next examined whether codeinone can induce the cytochrome c release from mitochondria. We found that codeinone actually induced the cytochrome c release, at 34 μM, but it also induced the release of cytochrome oxidase, a mitochondrial enzyme, at a slightly lower concentration (fig. 6). This indicates some mitochondrial damage.

We next investigated whether codeinone modified the activity of mitochondrial MnSOD. When HL-60 cells were incubated for 30 min with lower concentrations of codeinone, the MnSOD activity was slightly reduced. The MnSOD activity returned to the control level at higher concentrations of codeinone. We reproducibly found these concentration-dependent changes of MnSOD activity in a total of four independent experiments (data not shown). On the other hand, codeine did not significantly change the MnSOD activity. However, in contrast to the minor change in MnSOD activity, codeinone did not significantly affect the MnSOD mRNA expression (data not shown).

Discussion

The current study demonstrates that codeinone, but not codeine, induces apoptosis in HL-60 cells, as judged by several biochemical criteria of apoptosis. The Annexin staining experiment confirmed that codeinone actually induces apoptosis at the individual cell level. However, we unexpectedly found that codeinone pro-

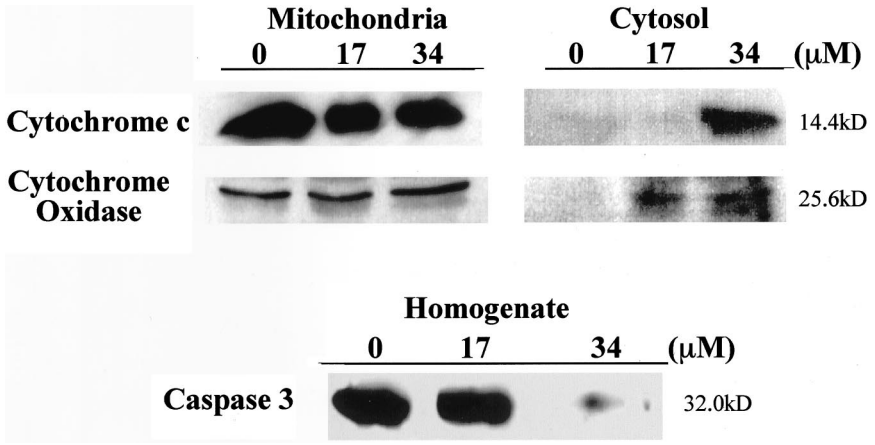

Fig. 6. Codeinone induces the release of both cytochrome c, cytochrome oxidase, and the cleavage of caspase 3. HL-60 cells were incubated for 1 h with the indicated concentrations of codeinone. Both the mitochondrial and cytosolic fractions or homogenates (which is a mixture of both mitochondria and cytosol) (*bottom*) were subjected to SDS PAGE and analyzed by Western blot, using specific antibodies. HL-60 = Human promyelocytic leukemic cells; SDS = sodium dodecyl sulfate; PAGE = polyacrylamide gel electrophoresis.

duces small proportions of necrotic cells as well as apoptotic cell populations [H-33342 staining (fig. 2) and FACS analysis (fig. 4)], and released cytochrome oxidase into cytosol. These data suggest that codeinone may induce the mitochondrial damage, possibly caused by the induction of necrosis. There remains to investigate whether both apoptotic and necrotic characteristics are expressed in the same cell, or codeinone produces a mixture of apoptotic and necrotic cell populations. Similarly, vitamin K analog induced both apoptosis and necrosis in HL-60 cells.[35] Compared with morphine (CC_{50} = 1,359–6,552 μM), morphine 6-glucuronide (CC_{50} = 424–4,610 μM) (table 1), morphine 3-glucuronide (CC_{50} = 3,900–5,200 μM),[10] codeinone showed much higher cytotoxicity (CC_{50} = 4.7–16.5 μM). Furthermore, codeinone showed some tumor-specific cytotoxic action (table 1). It has been reported that when humans were orally administered 30 mg codeine, the plasma concentration of codeine reached 46 ng/ml after 30–120 min.[36] When codeine (60 mg) was orally administered, the maximal plasma concentrations after 1.2 h was 88.1 ng/ml.[37] The plasma concentration of codeine is considered to be much lower than that required for apoptosis-induction. This suggests that it is important to establish an efficient delivery system of codeinone for the clinical application. We found that codeinone failed to stimulate both activity and mRNA expression of MnSOD in contrast to morphine.[19] This suggests that the apoptosis induction by codeinone is not coupled with the changes in the activity and expression of MnSOD, which is a rescue enzyme for oxidative stress. This is supported by our observation that the cytotoxic activity of codeinone was not significantly affected by various antioxidants other than NAC.[18] The reason why the cytotoxic activity was significantly reduced by NAC may be explained by the covalent binding of NAC to α,β-unsaturated ketone moiety in codeinone *via* the Michael reaction. Similarly, the inactivation of codeinone by glutathione, a popular cysteine-containing antioxidant, has been reported.[16–17] The log *P* values of morphine (log *P* = 0.24), codeine (log *P* = 0.82), and codeinone (log *P* = 0.78) were calculated by CLOGP (Pomana College Medical Chemistry Project, Clermont, CA). This demonstrates that higher cytotoxic activity of codeinone, compared with codeine, was not simply caused by the difference in hydrophobicity. Further studies are required to elucidate the mechanism by which codeinone induces the apoptosis/necrosis, cytochrome c release, caspase activation, and finally leads to the internucleosomal DNA fragmentation by the activated DNase, as well as to clarify which pathway, either extrinsic or intrinsic,[38] codeinone triggers by investigating the expression of more critical apoptosis markers such as caspase 8, 9, Bax, and Bcl.

Codeinone has been reported to display comparable antinociceptive activity with codeine in mice.[13] It has also been evidenced by the tail flick test in rats that codeinone has antinociceptive activity, consistent with a previous report that methoclocinnamox, a novel codeinone was fully effective in warm-water tail-withdrawal assay in rhesus monkeys.[39] We have demonstrated for the first time that codeinone shows tumor-specific cytotoxic activity by induction of apoptosis and/or necrosis, and its activity exceeds those of morphine, morphine 6-glucuronide, and codeine. This property of codeinone further adds a possible chemotherapeutic efficacy to be used in combination treatments with other anticancer drugs for patients being treated for cancer pain.[40–41]

References

1. Fagerlund TH, Braaten O: No pain relief from codeine. . .? An introduction to pharmacogenomics. Acta Anaesthesiol Scand 2001; 45:140–9
2. Mystakidou K, Befon S, Kouskouni E, Gerolymatos K, Georgaki S, Tsilika E, Vlahos L: From codeine to transdermal fentanyl for cancer pain control: A safety and efficacy clinical trial. Anticancer Res 2001; 21:2225–30
3. Caraceni A, Portenoy RK: An international survey of cancer pain characteristics and syndromes. IASP Task Force on Cancer Pain. International Association for the Study of Pain. Pain 1999; 82:263–74
4. Sear JW: Recent advances and developments in the clinical use of i.v. opioids during the peroperative period. Br J Anaesth 1998; 81:38–50
5. Eriksen J, Sjogren P: Opioids in pain management. Acta Anaesthesiol Scand 1997; 41:1–3
6. Alexander-Williams JM, Rowbotham DJ: Novel routes of opioid administration. Br J Anaesth 1998; 81:3–7
7. Daeninck PJ, Bruera E: Opioid use in cancer pain. Is a more liberal approach enhancing toxicity? Acta Anaesthesiol Scand 1999; 43: 924–38
8. Bruera E, Lawlor P: Cancer pain management. Acta Anaesthesiol Scand 1997; 41:146–53
9. Collett B-J: Opioid tolerance: the clinical perspective. Br J Anaesth 1998; 81:58–68
10. Sueoka E, Sueoka N, Kai Y, Okabe S, Suganuma M, Kanematsu K, Yamamoto T, Fujiki H: Anticancer activity of morphine and its synthetic derivative, KT-90, mediated through apoptosis and inhibition of NF-κB activation. Biochem Biophys Res Commun 1998; 252:566–70
11. William DG, Hatch DJ, Howard RF: Codeine phosphate in paediatric medicine. Br J Anaesth 2001; 86:413–21
12. Nagamatsu K, Terao T, Toki S: In vitro formation of codeinone from codeine by rat or guinea pig liver homogenate and its acute toxicity in mice. Biochem Pharmacol 1985; 34:3143–6
13. Toki S, Yamano S: Production of morphinone as a metabolite of morphine and its physiological role. in Japanese - Yakugaku Zasshi 1999; 119:249–67
14. Unterlinner B, Lenz R, Kutchan TM: Molecular cloning and functional expression of codeinone reductase: The penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. Plant J 1999; 18:465–75
15. Lister DL, Kanungo G, Rathbone DA, Bruce NC: Transformations of codeine to important semisynthetic opiate derivatives by *Pseudomonas putida* m10. FEMS Microbiol Lett 1999; 181:137–44
16. Nagamatsu K, Inoue K, Terao T, Toki S: Effects of glutathione and phenobarbital on the toxicity of codeinone. Biochem Pharmacol 1986; 35:1675–8
17. Ishida T, Yano M, Toki S: In vivo formation of codeinone-glutathione adduct: isolation and identification of a new metabolite in the bile of codeinone-treated guinea pig. J Anal Toxicol 1998; 22:567–72
18. Kawase M, Sakagami H, Furuya K, Kikuchi H, Nishikawa H, Motohashi N, Morimoto Y, Varga A, Molnar J: Cell death-inducing activity of opiates in human oral tumor cell lines. Anticancer Res 2002; 22:211–4
19. Singhal PC, Kapasi AA, Reddy K, Franki N, Gibbons N, Ding G: Morphine promotes apoptosis in Jurkat cells. J Leukoc Biol 1999; 66:650–8
20. Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W: Superoxide dismutase as a target for the selective killing of cancer cells. Nature 2000; 407:390–5
21. Sueoka N, Sueoka E, Okabe S, Fujiki H: Anti-cancer effects of morphine through inhibition of tumour necrosis factor-α release and mRNA expression. Carcinogenesis 1996; 17:2337–41
22. Rapoport H, Reist HN: A method for preparing codeinone. J Am Chem Soc 1955; 77:490–1
23. Kuribayashi N, Sakagami H, Sakagami T, Niimi E, Shiokawa D, Ikekita M, Takeda M, Tanuma S: Induction of DNA fragmentation in human myelogenous leukemic cell lines by sodium 5,6-benzylidene-L-ascorbate and its related compounds. Anticancer Res 1994; 14:969–76
24. Sorger T, Germinario RJ: A direct solubilization procedure for the determination of DNA and protein in cultured fibroblast monolayers. Anal Biochem 1983; 131:254–6

25. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT: Annexin V as a probe of aminophospholipid exposure and platelet membrane, vesiculation: A flow cytometry study showing a role for free sulfhydryl groups. Blood 1993; 81:2554–65

26. Aubry JP, Blaecke A, Lecoanet-Henchoz S, Jeannin P, Herbault N, Caron G, Moine V, Bonnefoy JY: Annexin V used for measuring apoptosis in the early events of cellular cytotoxicity. Cytometry 1999; 37:197–204

27. Aiuchi T, Mihara S, Nakaya M, Masuda Y, Nakajo S, Nakaya K: Zinc ions prevent processing of caspase-3 during apoptosis induced by geranylgeraniol in HL-60 cells. J. Biochem 1998; 124:300–3

28. Matsuoka H, Kurosawa S, Horinouchi T, Kato M, Hashimoto Y: Inhalation anesthetics induce apoptosis in normal peripheral lymphocytes in vitro. ANESTHESIOLOGY 2001; 95:1467–72

29. Zhang W, Hashimoto K, Yu GY, Sakagami H: Decline of superoxide dismutase activity during antioxidant-induced apoptosis in HL-60 cells. Anticancer Res 2002; 22:219–24

30. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S: A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998; 391:43–50

31. Sakahira H, Enari M, Nagata S: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 1998; 391:96–9

32. Pan J, Xu G, Yeung SC: Cytochrome c release is upstream to activation of caspase-9, caspase-8, and caspase-3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel. J Clin Endocrinol Metab 2001; 86:4731–40

33. Ji C, Amarnath V, Pietenpol JA, Marnett LJ: 4-hydroxynonenal induces apoptosis via caspase-3 activation and cytochrome c release. Chem Res Toxicol 2001; 14:1090–6

34. Gilmore KJ, Quinn HE, Wilson MR: Pinocytic loading of cytochrome c into intact cells specifically induces caspase-dependent permeabilization of mitochondria: Evidence for a cytochrome c feedback loop. Cell Death Differ 2001; 8:631–9

35. Gilloteaux J, Jamison JM, Arnold D, Ervin E, Eckroat L, Docherty JJ, Neal D, Summer JL: Cancer cell necrosis by autoschizis: synergism of antitumor activity of vitamin C: Vitamin K_3 on human bladder carcinoma T24 cells. Scanning 1998; 20:564–75

36. O'Neal CL, Crouch DJ, Rollins DE, Fatah A, Cheever ML: Correlation of saliva codeine concentrations with plasma concentrations after oral codeine administration. J Anal Toxicol 1999; 23:452–9

37. Shah JC, Mason WD: Plasma codeine and morphine concentrations after a single oral dose of codeine phosphate. J Clin Pharmacol 1990; 30:764–6

38. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D, Ashkenazi A: Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 2002; 8:274–81

39. Butelman ER, Lewis JW, Woods JH: Methoclocinnamox: agonist and antagonist effects of a novel long-lasting opioid in rhesus monkeys. J Pharmac Exp Ther 1996; 279:934–8

40. Kam PC, Ferch NI: Apoptosis: mechanisms and clinical implications. Anaesthesia 2000; 55:1081–93

41. Heusch WL, Maneckjee R: Effects of bombesin on methadone-induced apoptosis of human lung cancer cells. Cancer Lett 1999; 136:177–85