Prilocaine induces apoptosis in osteoblastic cells

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Purpose: To determine whether prilocaine, a local anesthetic, induces apoptosis in osteoblastic cells.

Methods: After reaching subconfluence, human osteoblastic Saos-2 and MG63 cells and mouse osteoblastic MC3T3-E1 cells were exposed for 48 hr to varying concentrations of prilocaine up to 10 mM and the cytotoxicity of the cells was analyzed by phase-contrast microscopy and WST-1 assay. Saos-2 cells treated for 48 hr with 5 mM prilocaine were stained with Hoechst 33342 and nuclear fragmentation was examined under a fluorescence microscope. DNA was extracted from the cells treated with 5 mM prilocaine and DNA ladder formation (a hallmark of apoptosis) was analyzed by agarose gel electrophoresis.

Result: Prilocaine induced cell death in Saos-2 cells in a dose- and time-dependent manner up to the concentration of 10 mM. Marked nuclear condensation and fragmentation of chromatin were observed in the prilocaine-treated cells. DNA ladder formation also was induced by prilocaine treatment. Prilocaine-induced DNA ladder formation was dose-dependent with maximal effect at a concentration of 5 mM and was time-dependent from 12 to 48 hr. DNA ladder formation was also induced by prilocaine treatment in human osteoblastic MG63 cells and mouse osteoblastic MC3T3-E1 cells. Cycloheximide prevented prilocaine-induced apoptosis in Saos-2 cells in a dose-dependent fashion up to 20 μM as determined by WST-1 assay and DNA ladder formation in agarose gel electrophoresis.

Conclusion: Osteoblastic cells treated with prilocaine exhibit both morphological and biochemical features indicative of apoptosis. The apoptotic mechanisms involve transcriptional regulation of specific proteins or protein synthesis.

Objectif : Déterminer si la prilocaïne, un anesthésique local, a induit l'apoptose de cellules ostéoblastiques.

Méthode : Après avoir atteint le stade de sous-confluence, des cellules ostéoblastiques humaines Saos-2 et MG63 et ostéoblastiques de souris MC3T3-E1 ont été exposées pendant 48 h à des concentrations variables de prilocaïne allant jusqu'à 10 mM et la cytotoxicité des cellules a été analysée par microscopie en contraste de phase et par dosage WST-1. Les cellules Saos-2 traitées pendant 48 h avec 5 mM de prilocaïne ont été colorées avec le Hoechst 33342 et la fragmentation nucléaire a été examinée sous microscopie en fluorescence. L'ADN a été extrait et la formation d'échelle d'ADN (signe cardinal de l'apoptose) a été analysée par électrophorèse sur gel d'agarose.

Résultats : La prilocaïne a induit la mort des cellules Saos-2 d'une manière dépendante de la dose et du temps jusqu'à une concentration de 10 mM. En effet, la prilocaïne a induit dans les cellules traitées une condensation nucléaire marquée et la fragmentation de chromatine de même que la formation d'échelle d'ADN. La formation d'ADN, dépendante de la dose, a connu son effet maximal avec une concentration de 5 mM et était dépendante du temps entre 12 et 48 h. La formation d'ADN a été aussi induite par la prilocaïne dans les cellules humaines MG63 et dans les cellules de souris MC3T3-E1. La cycloheximide a empêché l'apoptose induite par la prilocaïne de se produire dans les cellules Saos-2 d'une manière dépendante de la dose pour une concentration jusqu'à 20 μM comme l'ont déterminé le dosage WST-1 et la formation d'échelle d'ADN dans électrophorèse sur gel d'agarose.

Conclusion : Les cellules ostéoblastiques traitées avec de la prilocaïne ont présenté des caractéristiques morphologiques et biochimiques indicatrices d'apoptose. Les mécanismes de l'apoptose impliquent la régulation de la transcription de protéines spécifiques ou de synthèse protéique.

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LOCAL anesthetics inhibit membrane excitability and affect various cellular activities.[1] Examples of processes inhibited by several local anesthetics include cellular exocytosis and cell fusion,[2] platelet aggregation,[3] and membrane transport of calcium.[4] Local anesthetics are reported to inhibit cell adhesion and spreading,[5] cell motility,[6] and phagocytosis.[7] They also cause cytotoxicity of gingival fibroblasts,[8] hepatoma cells,[9] squamous carcinoma cells,[10] and neuroblastoma cells.[11] These findings indicate that local anesthetics affect cell proliferation and differentiation, possibly including a biological end point, apoptosis.

Apoptosis is an essential feature of the maintenance of cell population renewal in adult mammals.[12] The cytologically apparent stages of apoptosis are rapid condensation of chromatin and fragmentation of the cells, with membrane-enclosed apoptotic bodies that are phagocytosed and digested by nearby resident cells.[13] A characteristic biochemical feature of the process is double-strand breaks of nuclear DNA at the linker regions between nucleosomes, leading to the production of oligonucleosomal fragments with 185-200 base pairs.[14]

Local anesthetics are widely used in clinical practice but their effects on cultured cells are not well understood. In particular, the role of local anesthetics in the induction of apoptosis in bone-related cells is obscure. To study whether apoptosis is inducible in osteoblastic cells by treatment with local anesthetics, human osteosarcoma Saos-2 and MG63 cells, and mouse osteoblastic MC3T3-E1 cells were treated with prilocaine and apoptosis was studied by morphological and biochemical means.

Material and methods

Materials

Prilocaine hydrochloride, RNase, and Hoechst 33342 dye were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Proteinase K was obtained from Merck (Darmstadt, Germany). Stock solution of prilocaine (1 M), prepared in phosphate-buffered saline (PBS), was diluted to the appropriate concentrations with medium. Alpha modification of Eagle's minimum essential medium (α-MEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Plastic dishes were from Falcon Plastics (Los Angeles, CA, USA). Other materials used were of the highest grade commercially available.

Cells and culture conditions

Human osteosarcoma cell lines, Saos-2[15] and MG63,[16] were provided by Dr. Noriyoshi Kurihara. Mouse osteoblastic MC3T3-E1 cells[17] were provided by Dr. Masayoshi Kumegawa. The cells were grown in plastic dishes containing α-MEM supplemented with FBS 10% at 37°C in a humidified atmosphere of CO_2 5% and air 95%. The cells were subcultured every five days using trypsin 0.25% together with 1 mM EDTA in PBS. For the experiments, subconfluent cells were treated with the agents by adding small volumes of sterile stock solution to the medium at appropriate concentrations and culturing for indicated periods. The number of cells was determined by hemocytometer chamber counting. Cell modification was monitored by an Olympus IMT-2 phase contrast microscope equipped for photomicroscopy.

Cell death counting using WST-1 assay

The cytotoxicity of osteoblastic cells was measured by the WST-1 quantitative colorimetric assay for cell survival.[18] The assay detects living, but not dead, cells and the signal generated is dependent on the degree of activation of the cells. Subconfluent cells grown in 96-well culture plates were treated with the agents to be examined for 48 hr. The cells were incubated with 10 μl of the reaction solution (10 mM WST-1 and 0.2 mM 1-methoxy-5-methylphenazinium methylsufate, Cell counting kit, Dojindo Laboratory, Kumamoto, Japan) in 100 μl of the medium for four hours at 37°C in a humidified atmosphere of CO_2 5% and air 95%. The absorbance of the lysate was measured at 415 nm with an ImmunoMini NJ-2300 microplate reader (Japan Intermed, Tokyo, Japan).

Nuclear fragmentation assay with Hoechst staining

Morphologically, changes characteristic of apoptosis were monitored by staining cell nuclei with Hoechst 33342. The cells were plated on sterile 18-mm round coverslips placed in 60-mm plastic dishes and cultured for indicated periods. After appropriate incubation, the coverslips were removed from the dishes and placed directly into formalin 10% in PBS for 10 min at room temperature. After rinsing with PBS, the coverslips were incubated for 10 min at room temperature with Hoechst 33342 (10 $\mu g \cdot ml^{-1}$). The cells were examined under an Olympus BX-50 microscope equipped for epifluorescence illumination (BX-FLA) and for photomicroscopy (PM-30). Fluorescent photomicrographs were taken on Fuji Presto 400 films using the automatic exposure.

DNA isolation and agarose gel electrophoresis of DNA

DNA was prepared from cultured cells as follows. Cells were washed twice in PBS followed by lysis in cold 10

mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, and Triton X-100 0.5%. After cell lysis, debris was removed by centrifugation at 15,000 g for 20 min. DNase-free RNase was added to the lysates to a final concentration of 40 $\mu g \cdot ml^{-1}$, and incubated for one hour at 37°C with gentle shaking. Proteinase K was added to the RNase-treated lysates to a final concentration of 40 $\mu g \cdot ml^{-1}$. The lysates were further incubated for one hour at 37°C with gentle shaking. The DNA in the supernatant was precipitated with 2-Propanol and NaCl overnight at -20°C. After centrifugation and drying, DNA was dissolved in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Agarose gel electrophoresis of DNA was performed through a agarose gel 2% containing 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide. A molecular weight standard (Pharmacia Biotech, Uppsala, Sweden) was run in the same gel. To visualize apoptotic alterations to DNA integrity, DNA bands were observed on a transilluminator. Photographs were taken with a Polaroid DS-300 camera.

Results

Effects of prilocaine on viability of Saos-2 cells by WST-1 assay

Figure 1 shows that incubation with prilocaine led to a decrease in cell viability of human osteoblastic Saos-2 cells in a dose-dependent manner up to 10 mM. The oxidative activity measured by the WST-1 assay in the 10 mM prilocaine-treated cells was one-fifth that of the untreated control cells. Morphological changes and cell death of Saos-2 cells were demonstrated by phase-contrast microscopy. Also the effect of prilocaine on the viability of MG63 cells, another human osteoblastic cell line, was studied. As with Saos-2 cells, the morphological changes and cell death of MG63 cells increased significantly in a dose-dependent manner up to 10 mM of prilocaine, the highest dose studied (data not shown).

After reaching subconfluence, the Saos-2 cells were cultured for indicated periods up to 48 hr in medium with or without 5 mM prilocaine and its effect on cell viability was measured by WST-1 assay. Changes in cell viability were not observed until seven hours of prilocaine treatment. With time, the cells began to exhibit morphological changes and loss of cell viability. At 48 hr, the cell viability, as measured by the WST-1 assay, reached in the minimum level (data not shown).

Prilocaine induced nuclear fragmentation in Saos-2 cells

To determine whether the prilocaine-induced loss of Saos-2 cell viability was due to apoptosis, we looked, microscopically, for nuclear fragmentation and condensation of Saos-2 cells. Figure 2 shows that nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent, condensed chromatin (Figure 2B). The control cultures of Saos-2 cells did not show any apoptotic features (Figure 2A).

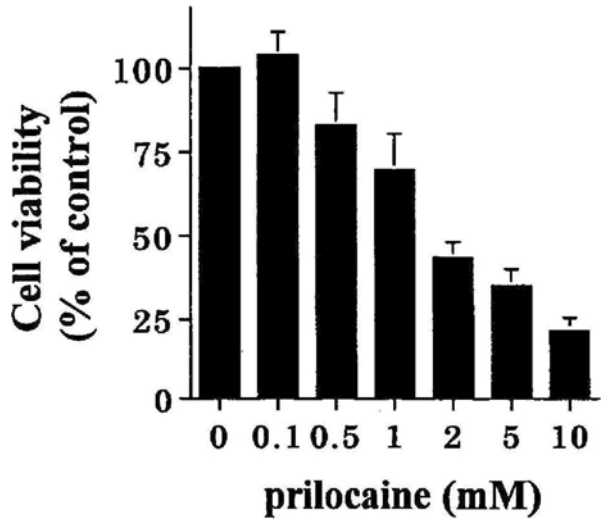


FIGURE 1 Effects of prilocaine on viability of Saos-2 cells. Saos-2 cells grown in 96-well plates and exposed to the varying concentrations of prilocaine as indicated for 48 hr. At the end points of incubation, the cultures were treated with tetrazolium salt for 4 hr and the mitochondrial activity of the cultures determined by WST-1 assay. The activity was compared to the control well (no prilocaine treatment) of the same cell line and results are expressed as a percentage of the control (means ± SEM). The experiments were done four times with 4-6 separate samples. The absorbance at 415 nm of the control cultures was 1.049 ± 0.245.

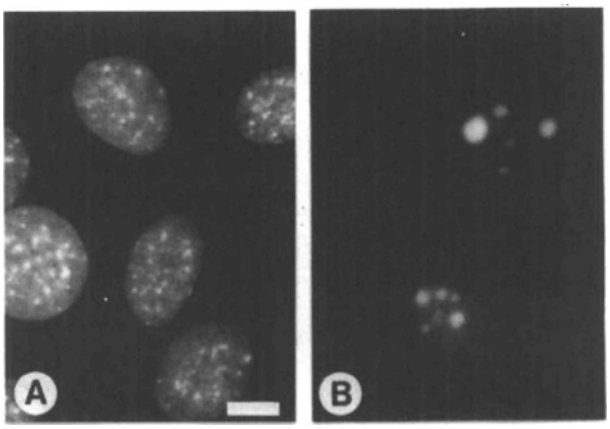


FIGURE 2 Nuclear morphology of untreated human osteoblastic Saos-2 cells and Saos-2 cells treated with 5 mM prilocaine for 48 hr. Cells treated with or without 5 mM were stained with Hoechst 33342 and observed under a fluorescent microscope. A, cells treated with the control medium; B, cells treated with 5 mM prilocaine. Bar represents 10 μm.

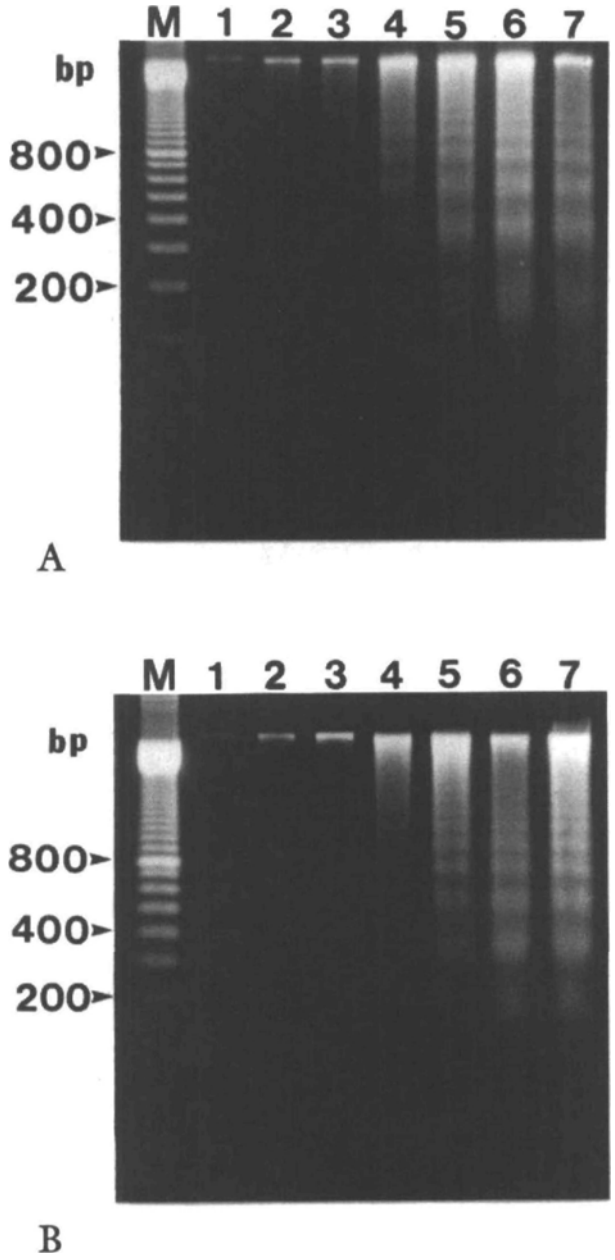


FIGURE 3 DNA ladder formation in prilocaine-treated Saos-2 cells. (A), Dose-dependent induction of DNA ladder formation in prilocaine-treated Saos-2 cells. The cells were exposed to prilocaine at various concentrations for 48 hr, and DNA extracted and electrophoresed through an agarose gel. Lane M, standard DNA markers (bp); lane 1, untreated control cells; lane 2, 0.1 mM; lane 3, 0.5 mM; lane 4, 1 mM; lane 5, 2 mM; lane 6, 5 mM and lane 7, 10 mM prilocaine-treated cells. (B), Time course of DNA ladder formation in prilocaine-treated Saos-2 cells. The cells were treated with 5 mM prilocaine for periods up to 48 hr, and DNA extracted and electrophoresed through an agarose gel. lane M, standard DNA markers (bp); lane 1, 0 ; lane 2, 4 ; lane 3, 8 ; lane 4, 12 ; lane 5, 24 ; lane 6, 36 and lane 7, 48 hr.

Prilocaine induced DNA fragmentation in Saos-2, MG63, and MC3T3-E1 cells

To further characterize prilocaine-induced apoptosis in Saos-2 cells, the extracted DNA was analyzed by agarose gel electrophoresis. In prilocaine-treated cells, a DNA fragmentation pattern forming a ladder of multiples of 185-200 bp was observed (Figure 3A). The DNA laddering pattern of prilocaine-treated cells reached a maximal level at 5 mM prilocaine. The time-dependence of prilocaine-induced DNA fragmentation of Saos-2 cells also was examined. When the cells were treated with 5 mM prilocaine, DNA laddering occurred by 12 hr sampling point after treatment. An increase in DNA laddering in relation to the time course was observed from 12 h to 48 hr (Figure 3B).

Because prilocaine induced a decrease in the viability of human osteosarcoma MG63 cells and mouse osteoblastic MC3T3-E1 cells, the effects of prilocaine on DNA fragmentation in both cell lines also were studied. DNA ladder formation also was observed in MG63 and MC3T3-E1 cells treated with 5 mM prilocaine (Figure 4).

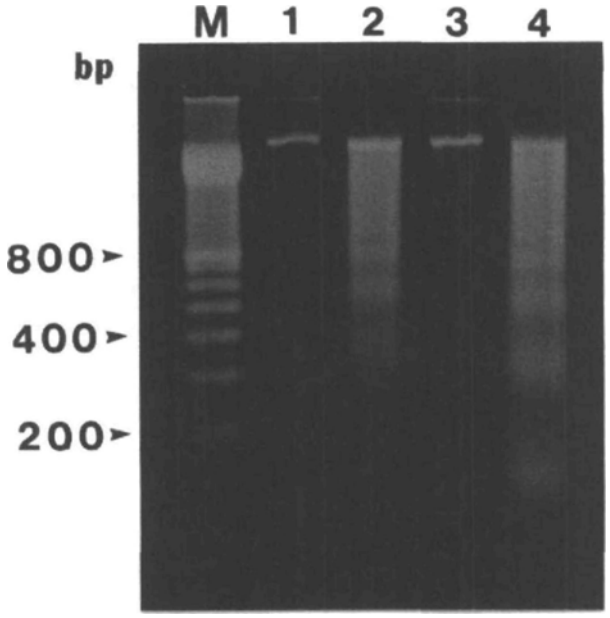
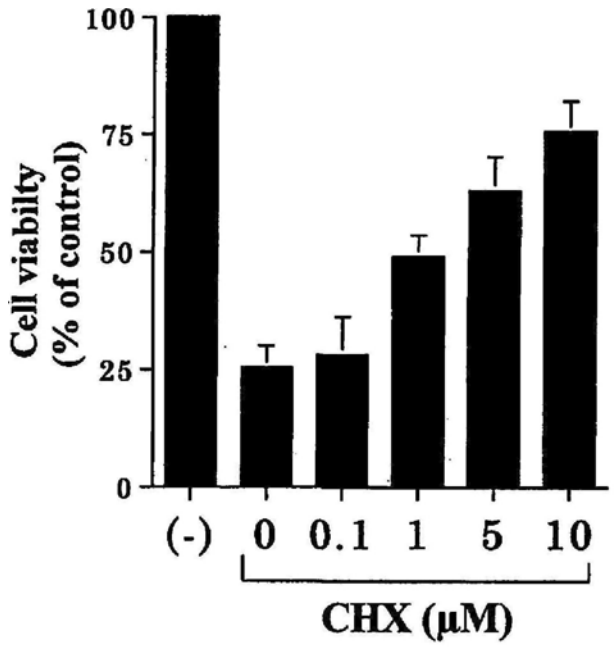
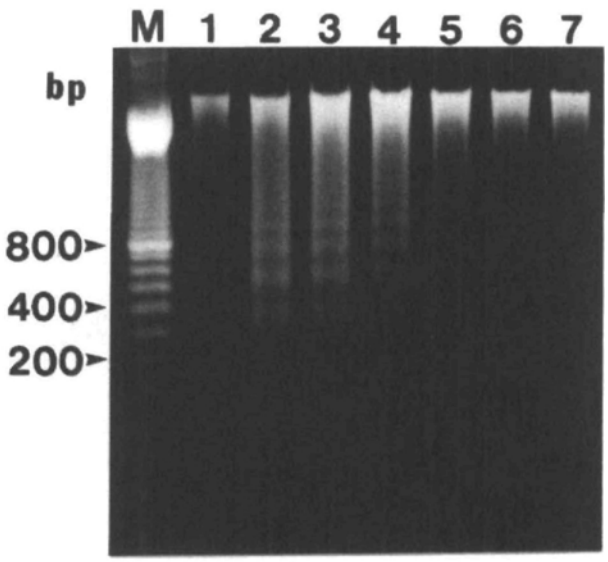


FIGURE 4 DNA ladder formation induced by prilocaine-treatment in MG63 and MC3T3-E1 cells. Lane M, standard DNA markers (bp); lane 1, untreated MG63 cells; lane 2, MG63 cells treated with 5 mM prilocaine, lane 3, control MC3T3-E1 cells; lane 4, MC3T3-E1 cells treated with 5 mM prilocaine.



5A



5B

FIGURE 5 Effects of cycloheximide on the prilocaine-induced apoptosis in Saos-2 cells. (A), Cycloheximide protection of the prilocaine-induced cell viability in Saos-2 cells. Cells grown in 96-well plates were treated with various concentrations of cycloheximide under a fixed dose of prilocaine (5 mM) for 48 hr and cell viability was determined by WST-1 assay. The activity of the living cells was compared with that in the control well and is expressed as a percentage of control (means ± SEM). The experiments were done three times with four separate samples. The absorbance at 415 nm of the control cultures was 1.267 ± 0.252. (-), Cultures without prilocaine and cycloheximide. (B), Cycloheximide protection of prilocaine-induced DNA ladder formation in Saos-2 cells. Saos-2 cells were exposed to 5 mM prilocaine and various concentrations of cycloheximide for 48 hr, and DNA was extracted and run in the agarose gel. Lane M, standard DNA markers (bp); lane 1, untreated cells; lane 2, 5 mM prilocaine alone; lane 3, prilocaine and 0.1 μM cycloheximide; lane 4, prilocaine and 1 μM cycloheximide; lane 5, prilocaine and 2 μM cycloheximide; lane 6, prilocaine and 5 μM cycloheximide, and lane7, prilocaine and 10 μM cycloheximide

Cycloheximide protected prilocaine-induced apoptosis in Saos-2 cells

Cycloheximide increased the cell viability of prilocaine-treated cells in a dose-dependent manner up to 20 μM (Figure 5A). Cell viability after co-incubation with cycloheximide was up to three times higher than that of the prilocaine-only treated cells. Figure 5B shows an agarose gel of electrophoresed DNA isolated from cells treated with 5 mM prilocaine and with varying concentrations of cycloheximide. In prilocaine-treated cells, DNA ladder formation was obvious. However, the degree of DNA fragmentation decreased as the concentration of cycloheximide increased. The same concentrations of cycloheximide alone did not induce DNA ladder formation in the treated cells.

Discussion

Human osteosarcoma cell lines, Saos-2 cells, are widely used as osteoblastic models because they retain many osteoblastic properties including high levels of alkaline phosphatase activity and binding to parathyroid hormone and 1α, 25-dihydroxyvitamin D_3 to induce osteonectin synthesis.[15] MG63 cells and MC3T3-E1 cells also show osteoblastic phenotype with high alkaline phosphatase activity and 1α, 25-dihydroxyvitamin D_3 induction of fibronectin, osteocalcin, and interleukin-6.[16,17,19-22] In the present study, we cultured these cells in mediums containing varying concentrations of prilocaine, a local anesthetic, for various time periods to examine the relationship between osteoblast apoptosis and local anesthetics. Cell viability in Saos-2 cells was determined by the WST-1 assay

and phase-contrast microscopy. By using the Hoechst 33342 staining, marked nuclear condensation and fragmentation into spherical bodies characteristics of apoptosis were demonstrated. Moreover, DNA ladder formation, a hallmark of apoptosis, also was detected in Saos-2 cells. Prilocaine also induced a loss of cell viability and DNA fragmentation in MG63 cells and MC3T3-E1 cells, implicating apoptosis in the mechanism of cell viability loss in prilocaine-treated cells. The stimulatory effect of prilocaine on the induction of apoptosis in Saos-2 cells was dose-dependent with a maximum effective concentration of 5 mM. This is in agreement with published data concerning its cytotoxic effects on rat fibroblasts.[8] Apoptosis induced by 5 mM prilocaine also was time-dependent. Apoptosis could be induced in these cells to varying degrees by treatment with other local anesthetics: procaine, lidocaine, tetracaine, and dibucaine. Reports on the effects of cycloheximide on apoptosis indicate that the inhibition of protein synthesis may delay, enhance, or have no effect on induction of apoptosis, depending upon the system used.[23] In the present study, cycloheximide decreased the prilocaine-induced cytotoxicity and DNA laddering. The prevention of DNA ladder formation by cycloheximide-treatment is consistent with its protection against loss of cell viability induced by prilocaine. These findings suggest that transcriptional regulation of specific genes is needed to induce apoptosis in Saos-2 cells. Recently, it has been reported that apoptosis is related to the p53 oncosuppressor protein, which induces redox-related genes and formation of reactive oxygen species.[24] However, in our preliminary results, the antioxidants, ascorbic acid and cysteine, did not protect against prilocaine-induced apoptosis in Saos-2 cells (in preparation). Prilocaine-induced apoptosis in Saos-2 cells demonstrated in the present results may play a role in at least one pathway unrelated to reactive oxygen species.

There are only a few reports of cytotoxicity induced by local anesthetics in mammalian cells.[8-10] However, whether cytotoxicity induced by local anesthetics is due to apoptosis was not investigated in these earlier studies. It has been shown that treatment of human neuroblastoma cells (SK-N-MC) with local anesthetics including dibucaine, tetracaine, procaine, and lidocaine induced morphological changes characteristic of apoptosis and DNA ladder formation.[11] However, to our knowledge, the effects of prilocaine on apoptosis in mammalian cells has not been reported.

In clinical situations, prilocaine, Citanest®, appears to be a safe drug and is a commonly used local anesthetic especially in dental practice.[25-27] Prilocaine is used as Citanest® 2-4% solution which is more than 20 times higher than the concentration used in the present study. Cellular damage could occur at an injection site including gingiva and alveolar bone. Because our present data were collected from cultured osteosarcoma cells and immortal mouse osteoblastic cells, it is important to study the effects of prilocaine further on isolated osteoblasts *in vitro* and *in vivo*. Extrapolation of the results to the clinical situation should be done with caution.

In conclusion, our findings indicate that human and mouse osteoblastic cells treated with prilocaine exhibit both morphological and biochemical features indicative of apoptosis. Our present results also indicate that new protein synthesis is involved in prilocaine-induced apoptosis in osteoblastic Saos-2 cells.

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