Nicotine Inhibits Collagen Synthesis and Alkaline Phosphatase Activity, but Stimulates DNA Synthesis in Osteoblast-Like Cells[1] (43221)

WARREN K. RAMP,*,†,[2] LOIS G. LENZ,* AND RACHELLE J. S. GALVIN†
Departments of Oral Health and Pharmacology and Toxicology,† Health Sciences Center, University of Louisville, Louisville, Kentucky 40292*

Abstract. **Use of smokeless tobacco is associated with various oral lesions including periodontal damage and alveolar bone loss. This study was performed to test the effects of nicotine on bone-forming cells at concentrations that occur in the saliva of smokeless tobacco users. Confluent cultures of osteoblast-like cells isolated from chick embryo calvariae were incubated for 2 days with nicotine added to the culture medium (25–600 μg/ml). Nicotine inhibited alkaline phosphatase in the cell layer and released to the medium, whereas glycolysis (as indexed by lactate production) was unaffected or slightly elevated. The effects on medium and cell layer alkaline phosphatase were concentration dependent with maximal inhibition occurring at 600 μg nicotine/ml. Nicotine essentially did not affect the noncollagenous protein content of the cell layer, but did inhibit collagen synthesis (hydroxylation of [^{3}H]proline and collagenase-digestible protein) at 100, 300, and 600 μg/ml. Release of [^{3}H]hydroxyproline to the medium was also decreased in a dose-dependent manner, as was the collagenase-digestible protein for both the medium and cell layer. In contrast, DNA synthesis (incorporation of [^{3}H] thymidine) was more than doubled by the alkaloid, whereas total DNA content was slightly inhibited at 600 μg/ml, suggesting stimulated cell turnover. Morphologic changes occurred in nicotine-treated cells including rounding up, detachment, and the occurrence of numerous large vacuoles. These results suggest that steps to reduce the salivary concentration of nicotine in smokeless tobacco users might diminish damaging effects of this product on alveolar bone.** [P.S.E.B.M. 1991, Vol 197]

Use of smokeless tobacco in the United States is increasing, particularly among young persons. It is estimated that 12 million Americans use smokeless tobacco and that one fourth of these are under age 21 (1). The most common forms of smokeless tobacco are leaf or chewing tobacco and moist snuff. Use involves chewing the leaf or placing a small amount of moist snuff in the gingival-buccal fold and sucking on the quid. The use of smokeless tobacco has been related to lesions in the oral cavity including soft tissue irritation and damage, leukoplakia, oral cancer (2, 3), periodontal inflammation (4), and alveolar bone loss (3, 5).

One of the primary reasons for using smokeless tobacco is for the stimulatory or euphoric effects of nicotine, which is readily absorbed through the oral mucosa. Blood concentrations of nicotine obtained with smokeless tobacco and cigarette smoking are comparable (ca. 15 ng/ml), but, because of prolonged exposure, are sustained twice as long with smokeless tobacco (6). However, Hoffmann and Adams (2) have shown that snuff users have saliva concentrations of nicotine as high as 1.56 mg/ml, which is more than 100,000 times higher than the blood level. The concentration could be even higher in the local area directly under the quid. We have reported that high concentrations of nicotine profoundly inhibit oxidative metabolism and collagen synthesis in cultured embryonic chick

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[2] To whom requests for reprints should be addressed.

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bones (7), suggesting that nicotine could be a contributing factor to alveolar bone loss associated with use of smokeless tobacco.

The study presented here was undertaken to determine whether the previous findings regarding inhibition of bone metabolism by nicotine (7) are also observed as effects of the alkaloid on isolated, bone-forming cells. These cells produce mineralized nodules containing type I collagen, contain and release high alkaline phosphatase (AlPase) activity, and show a marked increase in cAMP content in response to parathyroid hormone (unpublished data). In addition to confirming inhibition of collagen synthesis by cultured osteoblast-like cells, this model was used to evaluate effects of nicotine on noncollagenous protein synthesis, cell proliferation and morphology, and glycolytic and AlPase activities.

Materials and Methods

Cell Culture. Osteoblast-like cells were harvested from 17-day chick embryo calvariae (White Leghorn; Heartland Hatchery, Portland, IN) according to the method of Hefley *et al.* (8). Each isolation involved collecting approximately 50 calvariae, removing the periostea, and releasing cells from frontal bones by sequential enzymatic digestion using a purified collagenase-protease mixture. Bones were held in isolation medium containing 25 m*M* Hepes (pH 7.4), 10 m*M* $NaHCO_3$, 100 m*M* NaCl, 60 m*M* sorbitol, 3 m*M* K_2HPO_4, 1 m*M* $CaCl_2$, 1 mg/ml bovine serum albumin (BSA), and 5 mg/ml glucose, during collection and dissection. Purified collagenase (375 units/ml, Type VII; Sigma), purified protease (75 units/ml, Type XIV; Sigma), and tosyl lysine chloromethyl ketone (7 n*M*; Sigma) were added to the isolation medium. The frontal bones were incubated in 10 ml of isolation medium (with enzymes) in a shaker bath at 38°C for 10 min; this medium and cells were discarded. Three 30-min digestion periods followed. Released cells and medium were removed and 10 ml of fresh isolation medium were added to the bones at the end of each interval. Cells were recovered by centrifugation (800*g*), rinsed three times in Hanks' balanced salt solution buffered with 25 m*M* Hepes (pH 7.4), and resuspended in growth medium consisting of degassed Dulbecco's modified Eagle's medium (Sigma) containing 25 m*M* Hepes (pH 7.4), 10% Nu-Serum IV (Collaborative Research), 0.292 mg/ml glutamine, 0.1 mg/ml ascorbic acid, 0.04 μg/ml B_{12}, 2 μg/ml *p*-aminobenzoic acid, 0.2 μg/ml biotin, and 100 units/ml-100 μg/ml-0.25 μg/ml penicillin-streptomycin-Fungizone. Suspended cells were held at room temperature until all were collected and rinsed. The three suspensions were pooled, adjusted to a density of 2.5×10^5 cells/ml, and used to seed 12-well cluster plates (2 ml/well; Costar). The plates were placed in a humidified incubator at 38°C and left undisturbed for 24 hr to allow the cells to attach, after which they were placed on a rocker platform. The growth medium was changed and increased to 4 ml/well at 24 hr and was changed every 48 hr thereafter. Incubation was continued until cell layers reached confluence (7–8 days).

Confluent cultures were used to test effects of nicotine (free base; Sigma) added directly to the growth medium. Culturing was continued for 2 days with either L-[5-^{3}H]proline (1 μCi/ml, sp act 33 Ci/mmol; Amersham) or [*methyl*-^{3}H]thymidine (1 μCi/ml, sp act 86 Ci/mmol; Amersham) added to the medium. The medium was changed daily throughout the experimental phase of culture.

Analytical Methods. Cell layers were rinsed briefly in 150 m*M* NaCl and prepared for analysis by one of two methods:

1. Rinsed cell layers were suspended in 200 m*M* NaOH (1 ml/well), transferred to 16- × 100-mm tubes, stoppered, and solubilized at 58°C in a shaker bath overnight. The solubilized cell layers were used for determination of [^{3}H]thymidine incorporation, collagenous protein content by collagenase digestion, and noncollagenous protein content by collagenase indigestibility and the Bio-Rad protein assay.

2. Rinsed cell layers were suspended in 1 ml of 1 *M* NaCl-0.1% Triton X-100-0.01% trypsin inhibitor (soybean Type II; Sigma), transferred to 16- × 100-mm tubes, stoppered, frozen three times at −20°C, thawed at room temperature, and vortexed. Aliquots were removed for AlPase analysis. The DNA was extracted by adding 200 μl of Proteinase K (20 mg/ml in 10 m*M* Tris-HCl buffer, pH 8.0, containing 1 m*M* $CaCl_2$) and incubating overnight at 60°C in a shaker bath. Following DNA analysis, an equal volume of 12 *N* HCl was added to the remaining samples, which were hydrolyzed by heating overnight at 110°C. The HCl was evaporated with air using a Pierce Reacti-Therm sample dryer.

The noncollagenous protein and collagenous protein in the cell layers and in the medium were determined by collagenase digestion according to the method of Peterkofsky and Dieglemann (9). BSA (200 μl, 20 mg/ml) was added to 0.6 ml of cell layers solubilized in NaOH and to 1.2 ml of culture medium (0.6 ml pooled from each treatment day). Samples were placed in an ice bath, and protein was precipitated with an equal volume of ice-cold 10% trichloroacetic acid (TCA) containing 0.5% tannic acid. Following a 1-hr incubation in the ice bath, precipitable protein was pelleted by centrifugation and rinsed twice in ice-cold 5% TCA, centrifuging after each rinse. Pellets were dissolved in 0.6 ml of 0.8 *M* NaOH. Collagen in a 0.4-ml aliquot of the dissolved precipitated protein was digested by adding 1 m*M* Hepes (500 μl), 1.0 *M* HCl (150 μl), 62.5 m*M* *N*-ethylmaleimide (100 μl), 25 m*M* $CaCl_2$ (50 μl), and 16 units/μl purified collagenase (10 μl) followed by incubation at 38°C in a shaker bath for

90 min. Samples were then placed in an ice bath and 100 μl of 20 mg/ml BSA were added. Proteins were precipitated with an equal volume (1.3 ml) of ice-cold 10% TCA containing 0.5% tannic acid. Following centrifugation, supernatants were collected and precipitable proteins were rinsed once in 1 ml of ice-cold 5% TCA. Protein pellets were dissolved in 0.4 ml of 0.8 *M* NaOH. After adding 0.2 ml of 7.5% hydrogen peroxide, samples were incubated for 30 min at 37°C in a shaker bath to remove color. Tritium contents of 250-μl aliquots of the first and second supernatants (collagenous protein) and of a 100-μl aliquot of the solubilized nondigestible protein pellet (noncollagenous protein) were determined by liquid scintillation spectrophotometry.

Noncollagenous protein in the cell layers was also measured by the Bio-Rad protein assay. Following solubilization of the cell layers in NaOH, protein content was determined colorimetrically by shift in absorbance of Coomassie brilliant blue G-250 at 595 nm caused by binding of the dye to protein. This technique is characterized by wide variability in sensitivity to various proteins and is insensitive to collagen (10). As further verification of this finding, a comparison with BSA alone of the absorbance obtained with purified collagen (human placenta, type I; Sigma) plus an equal amount of BSA (Bio-Rad Standard II) showed minimal contribution to the total absorbance by the collagen (Fig. 1). Thus, it can be assumed that in samples containing both collagenous and noncollagenous protein, protein determinations made by this method primarily represent noncollagenous protein.

Collagen synthesis was also indexed by the hydroxylation of [^{3}H]proline to [^{3}H]hydroxyproline. The imino acids from the cells and from the medium were separated by thin-layer chromatography. Medium from the first and second treatment days were pooled and precipitated in ice-cold 10% TCA containing 0.5% tannic acid and rinsed twice in ice-cold 5% TCA. The precipitated proteins were hydrolyzed in 6 *N* HCl as described for the cell layers. The dried hydrolysates of the medium and the cell layers were resuspended in 200 μl of 200 m*M* NaOH and amino acids were dansylated using 0.4 *M* $NaHCO_3$ (2 μl/μl hydrolysate), 1 *N* acetic acid (1 μl/μl hydrolysate), and dansyl chloride (9 mg dansyl chloride/ml acetone, 6 μl/μl hydrolysate). The dansyl imino acids were separated on silica gel-G plastic sheets (Kodak), which were developed in a benzene:pyridine:acetic acid (80:20:2) solvent system. Tritium in the proline and hydroxyproline spots was evaluated by liquid scintillation spectrometry. This method is detailed in an earlier publication (7).

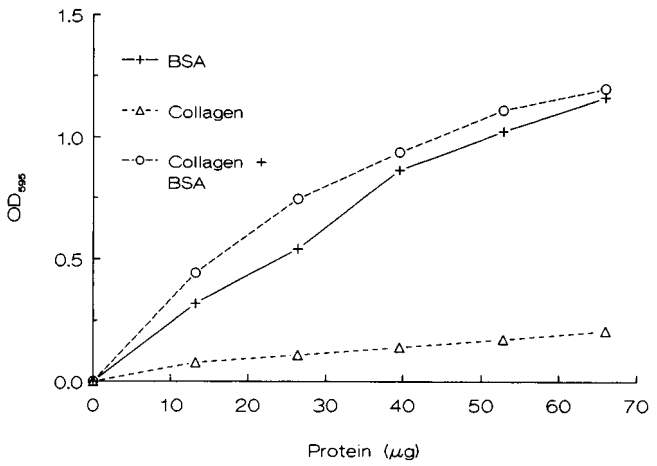

Figure 1. Absorbance of Coomassie brilliant blue G-250 after binding to collagen (human placental, Type I) and to BSA (Bio-Rad Protein Standard II). The analysis was performed according to procedures described in the manual of the Bio-Rad Protein Assay Kit II. Note that absorbance due to collagen is low when measured either alone or in combination with BSA. Each point represents a single determination.

The incorporation of [^{3}H]thymidine into DNA was used to evaluate cell proliferation. BSA was added to the NaOH-solubilized cell layers and DNA was precipitated with an equal volume of ice-cold 10% TCA. Precipitates were collected on glass fiber filters and washed with ice-cold 5% TCA, and radioactivity was measured by liquid scintillation spectrometry (11).

Total DNA was also determined according to the method of Lipman (12). Following extraction of DNA from the cell layers with Proteinase K, a 25-μl aliquot was added to a tube containing 0.154 *M* NaCl/0.015 *M* sodium citrate (2 ml), 10 m*M* Tris-HCl (75 μl, pH 8.0), and 1.5 μ*M* Hoechst 33258 (1 ml; Sigma). Tubes containing DNA standards (0–625 μg, bovine thymus; Sigma) were also prepared. Samples were incubated at room temperature in the dark for at least 15 min. Fluorescence was read on a fluorometer (model 112; Turner) equipped with a 310–390 n*M* general purpose UV lamp (no. 110-850), a 320–390 n*M* excitation filter (no. 110-811), and a >415 n*M* emission filter [no. 110-816(2A) +10% neutral density].

Lactic acid released to the culture medium was determined enzymatically (13) and served as an index of glycolytic activity. The activity of AlPase in the medium and in the freeze-thaw-extracted cell layers was assayed at pH 9.8 using *p*-nitrophenylphosphate as substrate (14). The enzyme activity is expressed as units, with 1 unit of activity representing 1 μmol of substrate hydrolyzed/hr at 38°C.

Statistical Analysis. All of the quantitative results are expressed on a per well basis. The data were evaluated using a one-way analysis of variance followed by the Tukey-Kramer honest significance difference multiple comparison analysis. These data are presented as the mean and 95% confidence interval with $P < 0.05$ considered significant. No statistical analyses were performed on the data for the protein assay (Fig. 1).

Results

The contributors to the total AlPase activity in the control cultures were the medium (42%, first day plus second day) and the cell layer (58%; Fig. 2A). The released, cell layer, and total activities were all significantly decreased ($P < 0.05$) in a concentration-related fashion at nicotine concentrations of 100, 300, and 600 μg/ml. The effect on release was more marked during the second day than during the first, showing maximal decreases of 87% and 66%, respectively, whereas the AlPase in the cell layer was diminished 62%. Control values of both medium AlPase activity and lactate production (Fig. 2B) were higher during the second day than during the first. Nicotine at 100 and 300 μg/ml of medium caused a small (12%), but significant ($P < 0.05$), increase in total lactate production and in lactate production during the first day of treatment.

The noncollagenous protein content of the cell layer was also only slightly affected by the higher concentrations of nicotine, demonstrating a small increase at 300 μg/ml by the Bio-Rad assay (Fig. 3B, insert) and a small decrease in collagenase-indigestible protein at 600 μg/ml (Fig. 3B). The latter trend was reflected in the [^{3}H]proline content of the cell layer, whereas release of [^{3}H]proline to the medium and, thus, the total [^{3}H] proline incorporation was more sensitive to inhibition by nicotine (Fig. 3A). Similar effects of nicotine were seen with respect to collagenase-indigestible protein (Fig. 3B).

Compared with its effects on proline uptake, nicotine had even greater inhibitory effects on the release of [^{3}H]hydroxyproline to the medium and on its content in the cell layer. These effects were concentration-dependent with 100, 300, and 600 μg/ml causing respective reductions of 40, 59, and 72% in release; 20 (not significant, $P \geq 0.05$), 44, and 76% in cell layer content; and 24, 47, and 75% in total [^{3}H]hydroxyproline. In the control cultures, the distribution of [^{3}H] hydroxyproline showed that 19% was released to the medium and 81% was incorporated into the cell layer. Figure 4B qualitatively confirms the [^{3}H]hydroxyproline results by showing similar inhibitory effects of nicotine on collagenase-digestible protein; however, a higher percentage of radioactivity was released to the medium (54% in the control cultures).

The DNA content of the cell layer was slightly decreased (20%) by nicotine at 600 μg/ml (Fig. 5A). In contrast, nicotine stimulated [^{3}H]thymidine incorporation (DNA synthesis; Fig. 5B). The increase was significant at 50 μg/ml (144%) and was maximal at 300 μg/ml (225%). None of the nicotine-treated groups that showed increased [^{3}H]thymidine incorporation compared with the control group was significantly different from the others ($P \geq 0.05$).

Compared with controls (Fig. 6A), a 2-day exposure to nicotine at 100 μg/ml resulted in vacuolation of osteoblast-like cells (Fig. 6B). This effect was more pronounced at 300 μg/ml (Fig. 6C). Other morphologic changes observed in nicotine-treated cells include loss of cell definition and a tendency to detach and round up, particularly at the higher nicotine concentration.

Discussion

Based on the results of this study, the inhibition of collagen synthesis by nicotine, which we previously

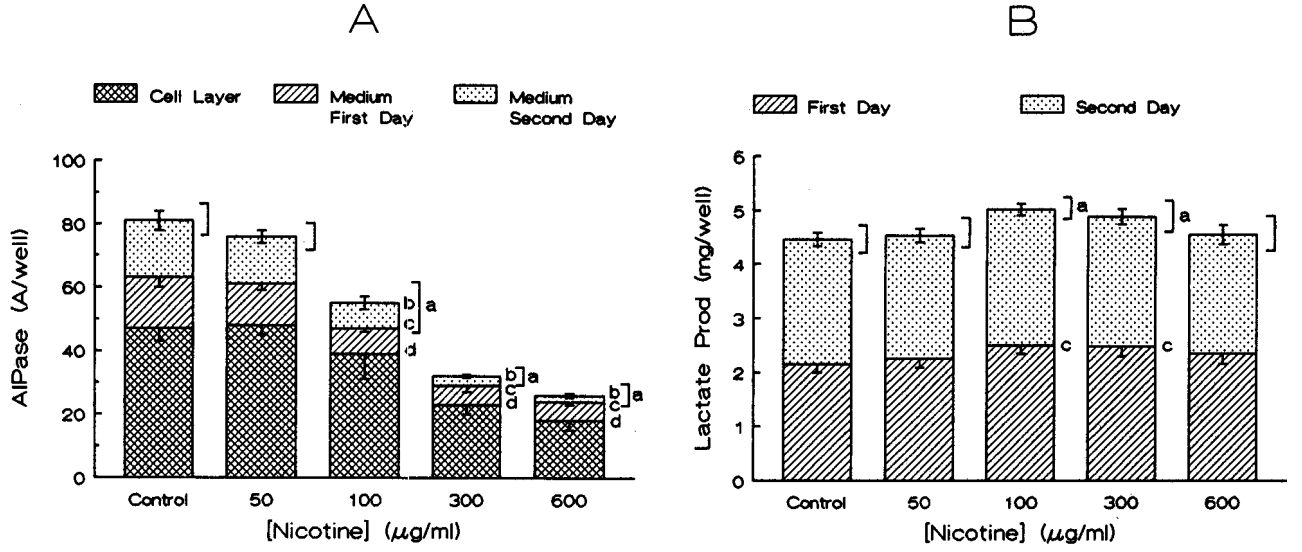

Figure 2. Effects of nicotine on AlPase release (A) and lactate production (B) by cultured osteoblast-like cells. The medium from confluent cultures was analyzed after the first (single cross-hatched bars) and second (stippled bars) day. Alkaline phosphatase activity in the cell layer was also determined (double cross-hatched bars). Each bar segment represents the mean of five wells. Vertical bars and brackets represent the 95% confidence intervals of segments and totals (cell layer + medium) at each nicotine concentration, respectively. Letters signify $P < 0.05$ compared with the appropriate control value.

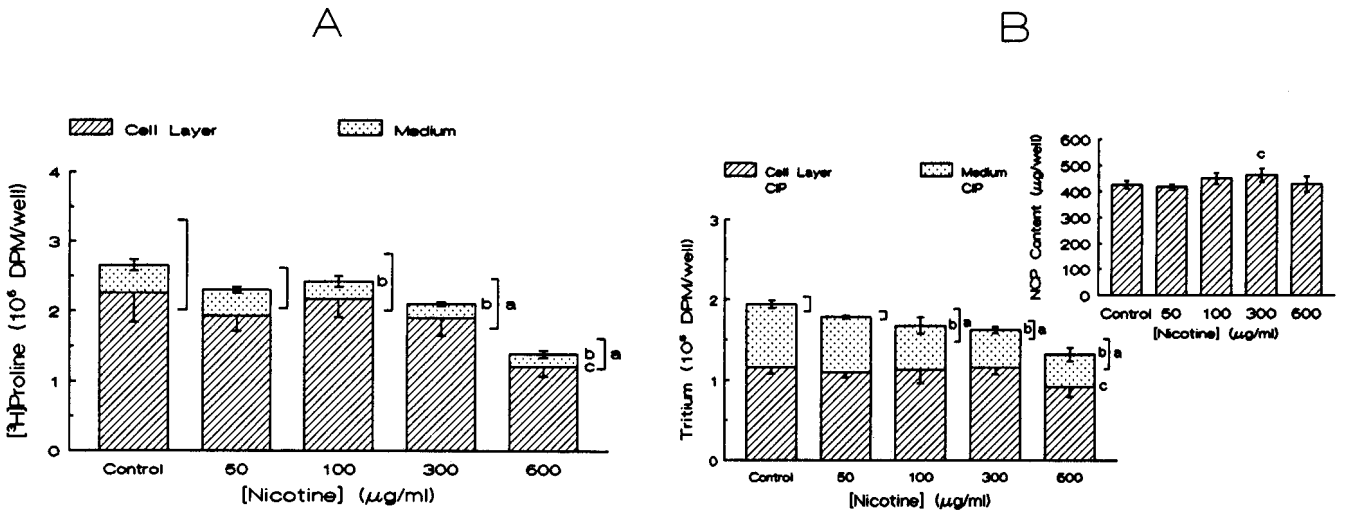

Figure 3. Effects of nicotine on proline incorporation (A), collagenase-indigestible protein (CIP, B), and noncollagenous protein (NCP, B insert) in osteoblast-like cells. Confluent cultures were incubated for 2 days with nicotine and [^{3}H]proline added to the medium. [^{3}H]Proline and collagenase-indigestible protein content of the cell layer (cross-hatched bars) and of the pooled first- and second-day medium (stippled bars) were determined. Each bar segment represents the mean of five wells. Vertical bars and brackets represent the 95% confidence intervals of segments and totals (cell layer + medium) at each medium nicotine concentration, respectively. Letters signify $P < 0.05$ compared with the appropriate control group.

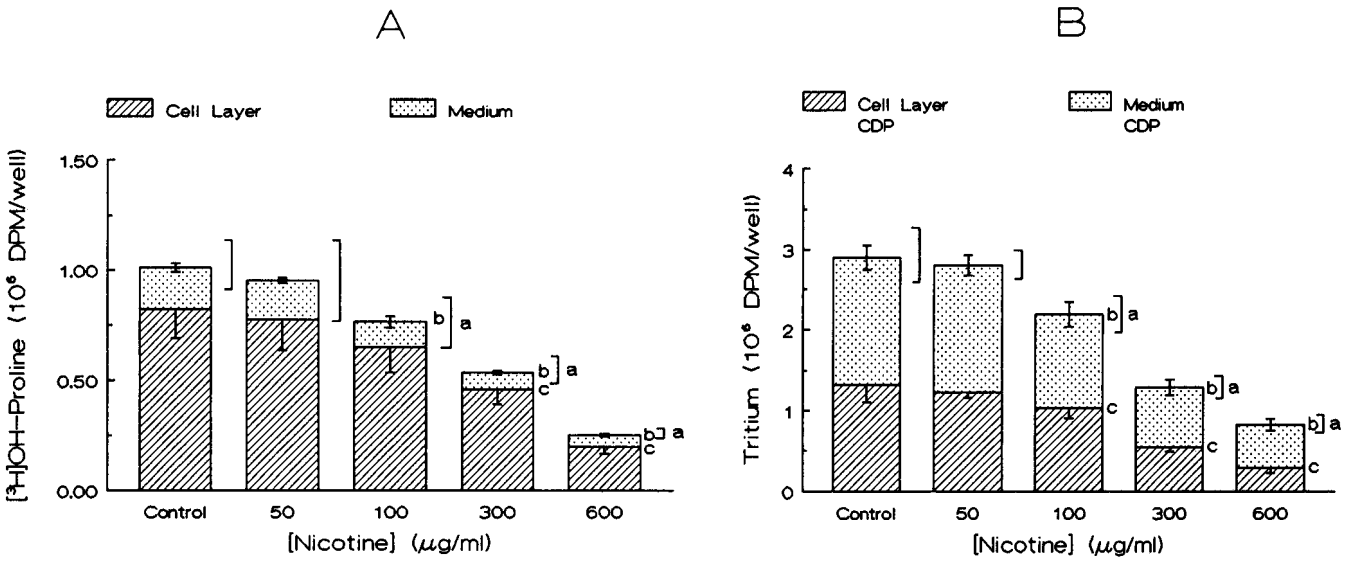

Figure 4. Effects of nicotine on hydroxylation of proline (A) and on collagenase-digestible protein (CDP, B) in osteoblast-like cells. Confluent cultures were incubated for 2 days with nicotine and [^{3}H]proline added to the medium. [^{3}H]Hydroxyproline and collagenase-digestible protein content of the cell layer (cross-hatched bars) and of the pooled first- and second-day medium (stippled bars) were determined. Each bar segment represents the mean of five wells. Vertical bars and brackets represent the 95% confidence intervals of segments and totals (cell layer + medium) at each medium nicotine concentration, respectively. Letters signify $P < 0.05$ compared with the appropriate control group.

reported using intact bones from chick embryos (7), was most likely due to an effect on osteoblasts. This concept is supported by the similarity of the concentration-response curves for inhibition of hydroxylation of [^{3}H]proline in tibiae (7) and in isolated osteoblast-like cells (Fig. 4A) and is confirmed by a similar effect on collagenase-digestible protein (Fig. 4B). The concentrations of nicotine at which this occurred were substantially less than the maximal concentration reported in the saliva of smokeless tobacco users (1560 μg/ml). Specificity of the inhibitory effect of nicotine for net collagen synthesis ([^{3}H]hydroxyproline content and collagenase digestibility) is shown by little effect on noncollagenous protein in the cell layer. The decreased release of [^{3}H]proline-rich and collagenase-indigestible proteins to the medium by nicotine-treated cells suggests that the alkaloid may have a general inhibitory effect on protein solubility or secretion by osteoblasts.

The increased proportion of tritium released to the medium using collagenase digestion versus hydroxyproline separation suggests that collagen released from the cells may be underhydroxylated. However, this obser-

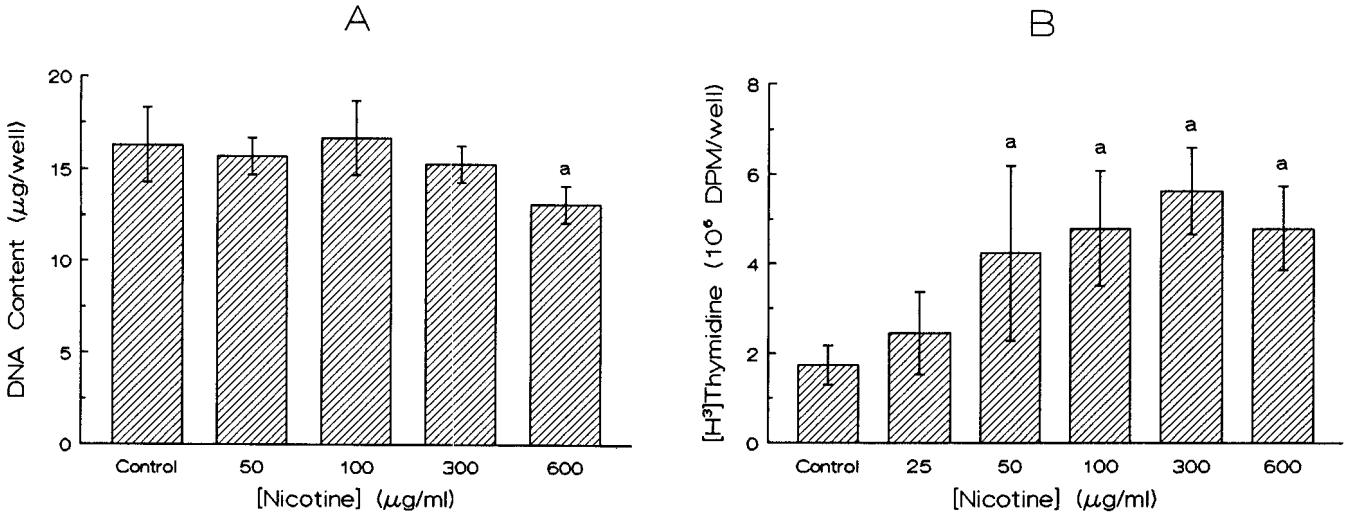

Figure 5. Effects of nicotine on DNA content (A) and DNA synthesis (B) in osteoblast-like cells. Confluent cultures were incubated for 2 days with nicotine and [^{3}H]thymidine added to the medium. Each bar represents the mean of five wells. Vertical bars represent the 95% confidence interval. a, $P < 0.05$ compared with the control group.

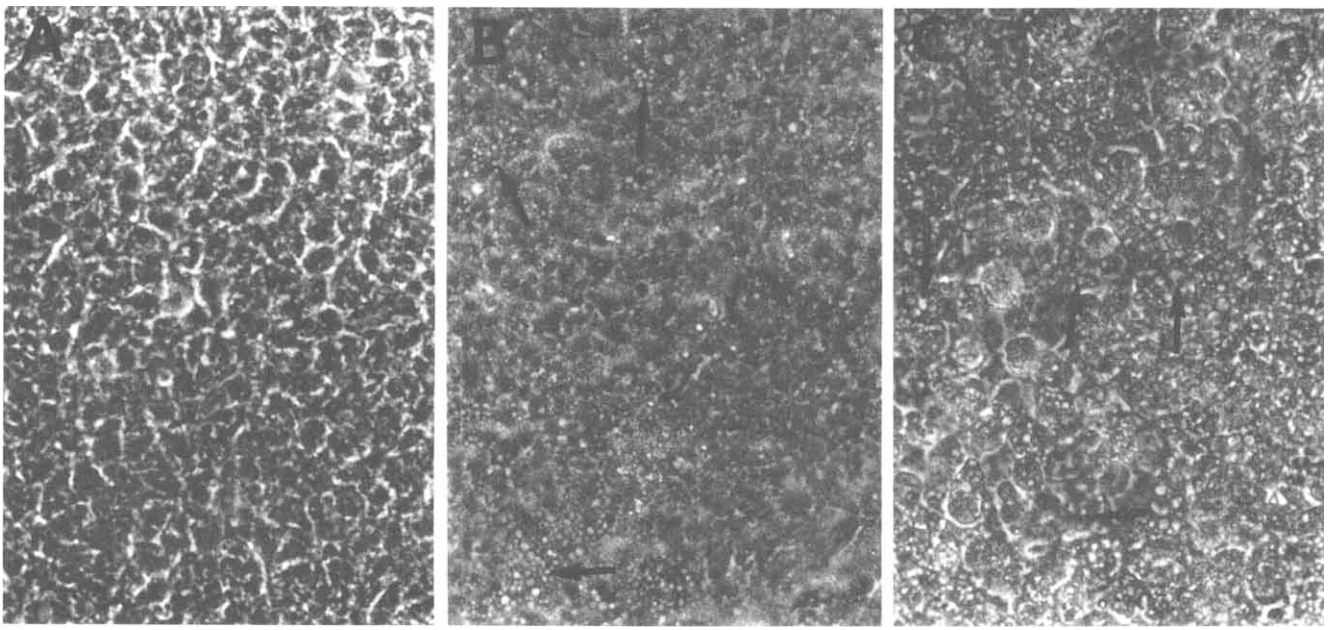

Figure 6. Vacuolation in confluent osteoblast-like cell cultures exposed to nicotine for 2 days. Control culture appears as a dense multicell layer of cells and matrix (A). Cultures exposed to nicotine (100 μg/ml) appear poorly organized and show numerous vacuoles (arrows, B). A higher concentration of nicotine (300 μg/ml) caused extensive vacuolization with an increase in the number and size of the vacuoles (arrows, C) (Phase contrast; original magnification ×200).

vation was not dependent upon nicotine and may be a characteristic of cultured bone cells. Nevertheless, at least half of the newly synthesized collagen was incorporated into the cell layer and in this regard the cultured osteoblast-like cells produced results very similar to those reported for cultured embryonic chick-bone explants (15).

Depression of protein secretion from cells treated with nicotine is consistent with the decreased AlPase activity seen in the medium of these cells, but does not explain the decreased AlPase activity in the cell layer. It should be noted that nicotine-dependent, AlPase inhibition in the cell layer was reflected by similar decreases in the medium. Thus, depressed enzyme activity in the medium did not result from elevated AlPase storage in cell membranes or extracellular membrane vesicles. The marked inhibition of AlPase activity in osteoblast-like cells further suggests an effect of nicotine on synthetic functions, since this enzyme is often associated with bone accretion and osteoblastic activity (16). Because increased AlPase activity precedes bone mineral deposition (17) and inhibition of AlPase has

been associated with decreased bone mineralization (18), it can be speculated that nicotine may impede mineral deposition in bone; however, this idea was not tested in the present study.

The inhibitory effects of nicotine on collagen synthesis and AlPase cannot be attributed to generalized toxicity, since glycolysis, a primary pathway for glucose metabolism and ATP production in osteoblasts, was either not affected or was slightly increased by these concentrations of the alkaloid. Furthermore, DNA synthesis, as indexed by elevated [^{3}H]thymidine incorporation, was substantially increased by nicotine without an increase in cell number (DNA content) in the cell layer. Nicotine has been shown to have diverse effects on DNA synthesis and cell multiplication in other types of cells. For example, increases in DNA synthesis have been reported in arterial smooth muscle cells (19) and lung epithelium (20) of rats; decreases have occurred in rabbit blastocysts (21), rat brain cells (22), and human leukemia cells (23); whereas no effect of nicotine was noted on DNA metabolism in HeLa cells or human lung fibroblasts (24). The importance of increased DNA turnover in response to nicotine is not known and, based upon the morphologic changes caused by nicotine, may reflect loss of contact inhibition of the cells and increased cell death and detachment from the cell layer. Nevertheless, these findings suggest an abnormal rate of proliferation of bone cells exposed to nicotine with possible neoplastic consequences.

As has been demonstrated in mouse macrophages (25, 26) and human skin fibroblasts (27) in response to nicotine, osteoblast-like cells in the present study showed marked vacuolation. Weak organic bases, such as nicotine, are known to accumulate in lysosomes by membrane permeation and trapping by protonation (28). As the base concentration within the lysosome increases, water enters osmotically and the lysosomes swell into large vacuoles. Endocytosis and intralysosomal degradation of substances are normal functions for macrophages and other specialized cells including osteoclasts (29). Vacuolation of this type has not been reported for osteoblasts and may represent a protective mechanism against toxic effects of nicotine.

It is not known if the effects of nicotine on bone cells are direct. For example, it is possible that nicotine alters the activity, production, or binding of growth factors either produced by the cells or supplied in the medium by Nu-Serum. It is also not known if effects of nicotine seen with bone cells cultured from chick embryos can be extrapolated to effects on osteoblasts in the human bone of smokeless tobacco users. Nevertheless, this study does allow the following speculations and suggestions regarding smokeless tobacco.

The circulating concentrations of nicotine attained through tobacco use (ca. 15 ng/ml) are probably too low to cause acute effects on osteoblast function; however, this is not true for possible local damaging effects of the high nicotine concentrations that can occur in the saliva of smokeless tobacco users. Impaired osteoblastic function (collagen synthesis and AlPase activity) could contribute to the periodontal damage known to occur with smokeless tobacco use (3–5) and to the net alveolar bone loss associated with this disease. Although completely removing nicotine from smokeless tobacco is not practical or desirable, steps to decrease the salivary concentration of nicotine would make a safer product. This might be accomplished by manufacturing smokeless tobacco with low-nicotine tobacco or by removing most of the endogenous nicotine from the tobacco and replacing it with nicotine in a slow-release form.

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