Abstract. Smoking has deleterious effects on osteoporosis and periodontitis both characterized by bone loss. Smoking also interferes with the protective effect that hormone replacement therapy (HRT) has on bone loss. Our study investigated two mechanisms by which smoking may affect bone metabolism: nicotine-induced proliferation and nicotine-induced cytokine secretion in osteoblasts. Two osteoblastic cell models were used: mouse osteoblasts derived from mouse calvaria and human osteoblasts. Thymidine incorporation and immunoassays were used to evaluate proliferation, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) secretion. Parametric and nonparametric statistical analyses were used for comparisons. The results showed that nicotine induced stimulation and inhibition of proliferation in both osteoblastic cell models. In human osteoblasts, the proliferative and inhibitory effects were also donor dependent. IL-6 secretion showed different patterns in mouse and human osteoblasts. In mouse osteoblasts, nicotine significantly increased IL-6 secretion and estradiol significantly inhibited the nicotine-induced IL-6 release. In human osteoblasts, cells derived from one subject did not respond to nicotine. However, in the second sample, nicotine increased secretion of IL-6 but estradiol did not oppose this effect. In human osteoblasts, nicotine also induced an increase in the TNF-α secretion and estradiol opposed this increase. These results suggest that nicotine affects bone metabolism by modulating proliferation, and IL-6 and TNF-α secretion. These studies provide a possible explanation for differences in bone loss among subjects who smoke and offer a possible mechanism for the oppositional effect of smoking on HRT in subjects with bone loss.

Introduction

Smoking is one of the most important risk factors for a number of diseases, including osteoporosis, a specific bone disease (1). Daniell (2) observed that middle-aged men and women with symptomatic osteoporosis were almost exclusively heavy cigarette smokers. In a further study, it was shown that postmenopausal women who smoked exhibited more bone loss than postmenopausal women who did not smoke (3). Furthermore, hormone replacement therapy (HRT) had less beneficial effect on osteoporosis of postmenopausal women who smoked compared to woman who did not smoke (4), further suggesting the important role of smoking on bone metabolism.

Smoking also has a deleterious effect on periodontal disease. Periodontal disease is an oral disease characterized by the destruction of the tissues supporting the teeth, in which the bone surrounding the teeth, alveolar bone, is frequently involved. There are several lines of evidence supporting the effect of smoking on periodontal disease and consequently alveolar bone loss: this disease is more prevalent in smokers than in nonsmokers (5,6); the prevalence of periodontal disease correlates with the amount of smoking (7,8); smokers have significantly more severe disease than nonsmokers (9); smokers respond less well to periodontal treatment than nonsmokers (10,11) and former smokers' periodontal conditions improve significantly compared to current smokers (12). The detrimental effect of smoking on alveolar bone is also reflected by results showing that the risk of more severe alveolar bone loss is greater in smokers than in non-smokers, ranging from 3.25 to 7.28 for light and heavy smokers, respectively (13). Within 20 years the marginal bone loss in smokers is 50% more than in nonsmokers (14).

One of the toxic components of smoking is nicotine and some studies suggest that bone integrity may be affected by this substance. In an animal study, a significant reduction in bone density and bone mineral content was found in nicotine-treated animals compared to untreated animals (15). Another study investigating bone healing in bone guided regeneration in dogs showed that nicotine significantly decreased the area of new bone formation in the created defects compared to the controls (16). Thus, these studies suggest that one of the common mechanisms by which smoking affects both alveolar
bone destruction and osteoporosis is the effect of nicotine on bone metabolism.

Bone is a dynamic tissue containing vital cells that produce and respond to various external and internal stimuli. In normal physiology, there is a coupling of resorption and formation in the bone remodeling sequence. The mechanisms which affect the balance of resorption and formation are not completely understood (17). However, important factors in this remodeling sequence are osteoblastic proliferation and cytokine secretion. Nicotine, one smoking compound, may affect bone metabolism by regulating both of these processes. Nicotine regulation of osteoblastic cell proliferation has been published by other authors but the studies showed contradictory results, possibly because they used different osteoblastic cell models, concentrations and culturing conditions (18,19). The present study shows that nicotine-induced proliferation is both concentration and cell-type dependent.

Another mechanism by which nicotine may affect bone diseases is by modulating the production of cytokines by osteoblastic cells. In previous studies (20), we found that HRT significantly decreased alveolar bone loss in post-menopausal women who had never smoked. However, HRT did not have any effect on postmenopausal women who smoked. These results suggested that smoking counterbalanced the beneficial effects of HRT. Since estrogen is the major component of HRT, the beneficial effect of HRT may be due to estrogen. Estrogen, an antiresorptive agent, exerts its effects on bone by modulating cytokine production, including that of IL-6 and TNF-α (21,22). A hypothesis put forward to explain how nicotine interferes with HRT’s beneficial effect on osteoporosis and alveolar bone loss is that nicotine may prevent the estrogen-induced decrease in cytokine production such as that of IL-6 and TNF-α. We tested this hypothesis by investigating the effect of nicotine and 17β-estradiol on the release of IL-6 and TNF-α in mice calvarial and human osteoblastic cells.

Materials and methods

Mouse osteoblast cell isolation. The calvaria of 48-h old unweaned fetal CD-1 mice (Charles River Labs Inc., Indianapolis, IN) were digested with collagenase Type 2 (Worthington Biochemical, NJ). Five sequential collagenase digestions were performed (23). The pooled 3, 4, and 5, osteoblast populations were washed twice with phenol-red free Dulbecco’s modified Eagle’s medium (DMEM) + 0.5% bovine serum albumin (BSA) and centrifuged for 15 min. The cells (1×10⁶ cells/ml) were then cultured in (Fitton-Jackson Modification) BGJB medium + 10% fetal calf serum (FCS) at dilutions of 1:4, 1:6, and 1:10, and grown to confluence in a 5% CO₂, 37°C incubator.

Human osteoblastic cell isolation. This procedure is well established in our laboratory (24). Normal human osteoblastic cells were obtained by a modification of the method of Beresford et al (25). The bone was obtained from healthy individuals with no known medical problems nor receiving any medication that might influence bone metabolism (as assessed by their medical history). Bone samples were minced into 1-mm² pieces, cultured in BGJB supplemented with 10% (v/v) FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Confluent monolayer cultures most typically formed after 4-6 weeks of culture under these conditions. All experimental procedures were performed on early passages (4-8), a time when the cells have the features typical of the osteoblastic phenotype, including 1,25(OH)²D₃ induced alteration in the osteocalcin levels and alkaline phosphatase activity. Our studies also showed that the cellular response to various hormonal stimulations did not change with the passage number.

Proliferation study. Mouse osteoblasts were cultured for 24 h in 24-well plates at a concentration of 1x10⁵/ml (0.5 ml/well) in BGJB + 10% FCS at 37°C and 5% CO₂. Then, the media was replaced with DMEM + 2% stripped serum (SS) for an additional 24 h, after which the media was replaced again with DMEM + 2% SS in the absence (control) or presence (experimental groups) of nicotine, estradiol, or a combination of both, for 24 h. The nicotine and estradiol concentrations used for this study were of a comparable concentration to those used by other researchers investigating the nicotine-induced proliferation in other cell models (19). Nicotine was used at between 35 ng and 1 μg/ml and estradiol at 10⁻⁷ and 10⁻⁸ M. During the last 2 h of incubation, tritiated [3H]-thymidine (Amersham Life Sciences, Arlington Heights, IL) was added to the wells at a final concentration of 1 μCi/ml. The wells were washed once with DMEM + 2% SS followed by a 10-min ice-cold precipitation with 10% trichloroacetic acid (TCA) (1 ml). After additional washes (4x) with 10% TCA (0.5 ml), the cells were solubilized with 0.5 N KOH for 2 h at 23°C. Finally, the plates were neutralized with 1 N HCl and the samples counted using a Rack Beta counter. An identical procedure was used for human osteoblastic cells. Nicotine was used at concentrations of between 2x10⁻⁴ M and 6x10⁻⁴ M.

IL-6 and TNF-α assessment. Mouse and human osteoblasts were subcultured into 24-well tissue plates at a concentration of 1x10⁶ cells/ml in BGJB medium + 10% FCS at 5% CO₂ and 37°C. After 24 h, the media was replaced with phenol-red free DMEM + 2% SS and the cells were incubated for an additional 24 h. Then, fresh DMEM + 2% SS containing the test agents was added for an additional 24 h. The media was
then removed and assessed for IL-6 and TNF-α levels using specific immunoassays for IL-6 and TNF-α (R&D Systems, Minneapolis, MN). The directions and procedures of the IL-6 and TNF-α assays were followed according to the manufacturer’s instructions.

Statistical analysis. The data obtained was analyzed for homogeneity of variance and normality of distribution using the statistical package SPSS. Then, the differences in significance among dependent variables (CPM in mouse model, IL-6 production) were determined using one-way ANOVA followed by post-hoc tests (t-test) with p-value corrected according to the number of statistical tests used (Bonferroni correction). However, if the data did not fulfill these conditions, the differences between dependant variables (CPM in human model, TNF-α production) was determined using nonparametric statistics (Kruskal-Wallis followed by Mann-Whitney U test).

Results

Nicotine and estradiol-induced proliferation in mouse osteoblastic cells. Fig. 1 shows the effect of various concentrations of estradiol and nicotine on mouse osteoblastic cell proliferation. Estradiol (10⁻⁷ M and 10⁻⁸ M) significantly increased (p<0.0001) cell proliferation compared to the control alone (5830 and 4506 vs 2521 cpm/well, respectively). Low concentrations of nicotine [500 ng/ml (3x10⁻⁶ M) and 1 μg/ml (6x10⁻⁶ M)] significantly increased (p<0.0001) proliferation compared to control (5417 and 4291 vs 2521 cpm/well, respectively) and high nicotine concentrations (300 and 600 μg/ml) significantly decreased osteoblastic cell proliferation.
When nicotine \((2 \times 10^{-7} \text{ M})\) was added in the presence of estradiol \((10^{-7} \text{ M})\) to the mouse osteoblastic cells, the estradiol-induced proliferation was completely abolished by the nicotine \((p<0.001)\). The results of this study show that, in mouse osteoblastic cells, estradiol increases proliferation, nicotine-induced proliferation is concentration-dependent, and nicotine reverses the estradiol-induced proliferation.

### Nicotine-induced proliferation in human osteoblastic cells.

The effect of nicotine on human osteoblast proliferation was investigated in samples collected from 5 different subjects (2 males and 3 females). The means and standard deviations are derived from 3-4 experiments that were performed on cells derived from the same donor. As Fig. 2 shows, the osteoblasts from the first two subjects (hosts 1 and 3) responded through both stimulation and inhibition of proliferation. Nicotine concentrations of \(6 \times 10^{-4} \text{ M}\) significantly inhibited proliferation by approximately 30 and 40\% respectively \((p<0.05)\) and nicotine concentrations of \(6 \times 10^{-6} \text{ M}\) and \(2 \times 10^{-6} \text{ M}\) significantly stimulated proliferation of osteoblastic cells \((p<0.05)\). The osteoblasts derived from samples collected from the third subject (host 4) responded only with increased proliferation when the cells were incubated with nicotine \(2 \times 10^{-4} \text{ M}\) \((\text{mean} \pm \text{SD}, 111 \pm 11)\) \((p<0.05)\). By comparison, when osteoblasts derived from the next two subjects (hosts 5 and 2) were incubated with nicotine \(6 \times 10^{-4} \text{ M}\), their proliferation was inhibited by 42 and 15\% \((\text{mean} \pm \text{SD}, 58 \pm 15; 85 \pm 15)\) \((p<0.05)\). These results show that, in human osteoblasts, nicotine modulates human osteoblastic cell proliferation, and nicotine’s effect on cell proliferation is concentration-dependent and donor-cell dependent.

### Nicotine and estradiol-induced IL-6 secretion in mouse osteoblastic cells.

 Estradiol significantly inhibited \((p<0.001)\) the release of IL-6 in a dose-response manner \((\text{Fig. 3})\), while nicotine significantly increased \((p<0.001)\) the release of IL-6 in mouse osteoblasts \((\text{Fig. 4})\). A nicotine concentration of \(6 \times 10^{-4} \text{ M} (100 \mu\text{g/ml})\) significantly increased IL-6 production compared to controls \((1298 \text{ vs } 730 \text{ pg/ml, respectively})\) and the mean was comparable to that observed in the presence of TNF-\(\alpha\) at a concentration of \(10^{-8} \text{ M}\) \((1298 \text{ vs } 1342 \text{ pg/ml, respectively})\).

Fig. 5 shows the combined effect of estradiol and nicotine on the release of IL-6. Nicotine significantly increased \((p=0.0001)\) the release of IL-6 compared to controls but the
addition of estradiol significantly inhibited this release (p<0.01). In this experiment, the values are expressed as pg IL-6/ml of media and are not normalized for the number of cells/well present at the end of the incubation period. Fig. 6 shows that when the results are normalized in this manner, nicotine still significantly increased (p=0.0011) the IL-6 release compared to the controls and cells incubated with estradiol alone.

Fig. 7 shows that TNF at 10^-4 M significantly increased (p=0.0011) the IL-6 release compared to the controls and to cells incubated with TNF + estradiol suggesting that estradiol reversed the TNF-α-induced increase in IL-6 secretion. TNF-α and nicotine slightly increased the IL-6 release compared to TNF-α alone but the increase was not statistically significant.

Nicotine and estradiol-induced changes in the secretion of TNF-α in human osteoblastic cells. Two samples collected from 2 different subjects were investigated. As Fig. 9 shows, nicotine and estradiol-induced changes in the secretion of TNF-α in human osteoblastic cells were investigated in two cell lines derived from two different subjects. Incubation of the cells with increasing concentrations of nicotine, estradiol or a combination of nicotine and estradiol for 24 h in 2% calf serum (CS) or 0.01% CS, did not significantly modify the secretion of IL-6 (data not shown). The results of this study show that, in human osteoblastic cells, nicotine modulates IL-6 production, nicotine-induced IL-6 production is donor-cell-line dependent, and estradiol does not affect nicotine-induced IL-6 production, at least in some cell lines.
nicotine 6x10^{-4} M significantly (p<0.001) increased the secretion of TNF-α in the osteoblasts from both subjects (hosts 1 and 2). Estradiol did not affect the TNF-α secretion in either sample. When estradiol was added to nicotine 6x10^{-4} M, it completely eliminated the nicotine-induced secretion. These results show that, at least in some osteoblastic cells derived from human samples, high concentrations of nicotine stimulate TNF-α secretion and that estradiol opposes this secretion.

Discussion

Smoking is a risk factor for several diseases, including diseases characterized by bone loss, such as osteoporosis and periodontal diseases. The beneficial effects of HRT on osteoporosis and alveolar bone height are decreased by smoking. Therefore, identifying the mechanism by which smoking induces its deleterious effects on bone as well as the mechanism by which smoking opposes HRT has significant implications. Our study identified two mechanisms by which smoking affects bone metabolism: proliferation and cytokine secretion. It may also provide a potential explanation for the oppositional effects of smoking on HRT.

Nicotine is a major component of tobacco smoke. Its serum concentrations during smoking range from 0.1-0.45 µM (4.5x10^{-5} M) but its concentrations in saliva can reach 9.6 µM (10^{-3} M) in chronic snuff users (26) suggesting that nicotine may have an important role in the mechanism by which smoking induces bone loss. Some of the nicotine concentrations used in this study are higher but, as previously suggested, it is possible that nicotine accumulates in bone and is then released during the remodeling process, giving rise to significantly higher local concentrations than those reached in plasma (27).

Bone is a dynamic tissue that, under normal physiological conditions, is in continuous remodeling with a balance between bone formation and bone resorption. Osteoporosis and alveolar bone loss can result from an imbalance between these two processes. Osteoblasts are bone-forming cells whose proliferative activity is important in the maintenance of the balance between bone resorption and bone formation. Nicotine has been found to increase proliferation in osteoblasts derived from chick calvaria in a dose-dependent manner at concentrations ranging from 4x10^{-5} to 6x10^{-4} M (18). In a rat osteosarcoma cell line, UMR 106-01, nicotine appeared to inhibit proliferation at concentrations ranging from 10^{-6} to 10^{-3} M (18). Our study showed that, in osteoblasts derived from mice calvaria, there was increased proliferation at concentrations of 10^{-4} M but decreased proliferation at higher nicotine concentrations (10^{-3} M). The differences in results between our study and other studies may be explained by the differences in methodology used but also by the differences in cell line responses to nicotine. The effect of nicotine on human osteoblastic cell proliferation has also been reported. Walker (27) showed that nicotine increased osteoblast proliferation at concentrations of 10^{-3} to 10^{-2} M but decreased proliferation at concentrations of 10^{-1} M. Our study showed that the effect of nicotine on human osteoblastic cells depended on the host cell cultures. In two cell cultures, the effect of nicotine was bimodal, inducing both proliferation and inhibition; in one cell culture, nicotine had only proliferative effects; and in the other two cell cultures, nicotine had only proliferative effects. The proliferative and inhibitory effects of nicotine are exerted at approximately the same concentrations that Walker's study used. The pattern of these responses, however, appeared differently and this may be due to differences in methodology as well as data presentation. Walker's study presented the proliferative data compiled from 3 patients while our data is presented separately for each subject. It is known that the effect of smoking on osteoporosis and alveolar bone loss varies from patient to patient. Some patients are more susceptible than others to the harmful effects of smoking. Our data showing the individual patient response to nicotine may at least in part explain these differences.

Estrogen is an antiresorptive agent. Estrogen deficiency leads to accelerated bone loss and estrogen replacement therapy is effective in preventing this bone loss. Receptors for estrogens have been demonstrated in both osteoblasts and osteoclasts, indicating that estrogens exert their beneficial influence on skeletal homeostasis by direct actions on bone (28). The anti-osteoporotic effect of estradiol may be explained by its ability to interact with bone cells and regulate the cytokine circuitry that controls bone remodeling. In the estrogen replete state, estrogen functions to reduce cytokine production and thereby temper the rate or extent of osteoclast formation and activity. In the estrogen deficient state, there is an increased cytokine secretion, leading to more osteoclast formation and increased bone resorption (28). The mouse osteoblastic cells significantly decreased the production of IL-6 in response to estradiol but the cells derived from humans did not show the same effect. IL-6 was increased by nicotine in both cell models, mouse and humans. However, in humans, the response was cell dependent. The cells derived from the first subject increased IL-6 secretion in response to nicotine but the cells derived from the second subject did not increase the IL-6 secretion upon nicotine stimulation. Estrogen significantly inhibited the nicotine-induced secretion of IL-6 in mouse osteoblasts but did not inhibit the nicotine-induced secretion of IL-6 in human osteoblasts. These results provide a possible explanation for the differences in susceptibility to the effects of smoking of individual subjects as well as an explanation for the decreased beneficial effect of HRT on osteoporosis and alveolar bone loss in smokers (20). The TNF secretion in response to nicotine was only examined in humans and the data appeared more homogenous. However, the reduced number of subjects used in this study precludes us from generalizing the results.

Mouse osteoblastic cells are often used as a model to study various aspects of bone metabolism. Our study used mouse and human osteoblastic cells under comparable experimental conditions to determine similarities and differences between their responses to nicotine and estradiol stimulation. The results of our study suggest that, although many similarities exist between mouse and human osteoblasts, there are also differences and caution should be exercised when extrapolating the results obtained from mouse models to humans.

The biological basis for the effects of smoking on bone is not completely understood. Decreased intestinal absorption of calcium (30), inhibition of oxidative metabolism and collagen synthesis (19), and lowered tissue levels of ascorbic acid (31) have been reported as mechanisms. Our studies showed that another mechanism by which smoking may affect bone loss
is by increasing the cytokine secretion, such as that of TNF and IL-6. Our studies also showed that, at least in selective cases, the nicotine-induced secretion of cytokines may not be opposed by estrogen and, as such, the protective effect of estrogen against bone loss may be lost. We recognize that the number of subjects used in this study is limited and there is a need to be cautious in generalizing the results. However, the results obtained from each subject were consistent within the 3–4 experiments suggesting that the variability found between subjects may be genuine.

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References

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