The Effect of Nicotine on Reproduction and Attachment of Human Gingival Fibroblasts In Vitro

Mark E. Peacock, Donald E. Sutherland,* George S. Schuster,† William A. Brennan,* Robert B. O'Neal,‡ Scott L. Strong,‡ and Thomas E. Van Dyke§*

THE ABILITY OF FIBROBLASTS TO REPRODUCE and attach to teeth is of paramount importance in re-establishing the lost connective tissue attachment after periodontal therapy. This study examined the effect of nicotine, a major component of the particulate phase of tobacco smoke, on human gingival fibroblast (HGF) reproduction and attachment to tissue culture surfaces. Pooled HGF cultures made from explants of gingival biopsies were utilized between passages 5 and 10 and plated in 96-well plates at 1.0×10^4 cells per well. Cell numbers were determined using 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT), which is a reflection of mitochondrial dehydrogenase activity. The concentrations of nicotine used were 0.025, 0.05, 0.1, 0.2, and 0.4 μM, the average serum concentration for a smoker being approximately 0.1 μM. The effect of continuous nicotine exposure on HGF reproduction was determined by incubating cell cultures and media containing nicotine for up to 48 hours. Residual toxicity was determined by preincubating cells with nicotine for 1 or 6 hours. HGF suspensions and increasing concentrations of nicotine were added together to determine the effect on attachment. Results showed an enhanced effect of nicotine on HGF attachment, with increasing numbers of cells attaching with increasing nicotine concentrations, compared to the control. Low concentrations of nicotine had a stimulatory effect on cell replication, while higher concentrations of nicotine appear to have no significant effect on HGF reproduction. The responses of cells to some concentrations of nicotine may persist after its removal. *J Periodontol 1993;64:658–665.*

Key Words: Connective tissue attachment; fibroblasts; nicotine/adverse effects; smoking/adverse effects.

The role of tobacco use in the etiology of periodontal disease is not clear, however most practitioners believe there is a strong positive correlation between the use of tobacco and increased incidence and severity of periodontal disease.[1–3] Increased amounts of plaque and debris in smokers have been found in some studies,[4–6] while others have reported a decrease in plaque accumulation.[7–9] Smokers may have greater amounts of calculus [2,6,7,9] and a higher incidence of staining which can act as a nidus for subsequent calculus deposition.[10] Gingivitis and loss of attachment also appear to be more prevalent in tobacco users,[2,7,11] although some investigators question the degree of gingival inflammation present in smokers as compared to non-smokers.[8,9] Preber and Kant, in a study of 15-year-old school children, found no significant differences in gingival inflammation between non-smokers and smokers when subjects with the same level of oral hygiene were matched.[12] Others have reported higher incidences of acute necrotizing ulcerative gingivitis in smokers, where the smoke or its components seem to potentiate the effects of stress and oral sepsis.[13,14]

Similarly, wound healing is perceived by some to be influenced by the use of tobacco products. Some clinicians have experienced less success with certain surgical procedures, such as free gingival grafts, in smokers.[15] Preber and Bergström showed that the reduction of the periodontal probing depth following non-surgical therapy in smokers was less than that in non-smokers.[16] Smoking also impaired the healing response after periodontal surgery when compared to post-surgical healing in non-smokers.[17] Decreased healing associated with tobacco use also has been reported

*U.S. Army, Fort Gordon, GA.
†Medical College of Georgia, Augusta, GA.
‡University of Michigan, Ann Arbor, MI.
§Eastman Dental Center, Rochester, NY.
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after oral surgical procedures,[18] cosmetic surgery procedures for the removal of wrinkles,[19] head and neck skin grafting,[20] and duodenal ulcer treatment.[21] While much evidence suggests a detrimental relationship between tobacco use and periodontal health, the actual nature of the relationship between tobacco and its components and the tissue responses in periodontal therapy still remains unclear.[22]

Tobacco contains a complex mixture of substances including nicotine, various nitrosamines, trace-elements, and a variety of poorly characterized substances. Cellular responses to these substances vary widely and may relate to specific components of the smoke, the amount of such components, and the cell type. For example, the ability of oral polymorphonuclear leukocytes (PMNs) to survive in the presence of smoke is decreased, and their phagocytotic activity and chemotactic ability are also adversely affected.[23–25] In addition, degranulation of mast cells is stimulated,[26] lymphocyte viability decreased,[26] lymphocyte proliferation suppressed,[27] and antibody production reduced in the presence of cigarette smoke.[27] In a recent study, Fang et al. concluded that nicotine inhibits cellular growth and stimulates alkaline phosphatase activity in rat osteoblast-like cells.[28] Since nicotine is a significant component of tobacco, the aims of the current study were to determine the effect of nicotine on human gingival fibroblast reproduction after short- and long-term exposure, to ascertain whether any effect(s) seen upon human gingival fibroblast growth persist after removal of nicotine, and to determine if nicotine has any impact upon fibroblast attachment.

MATERIALS AND METHODS

Cell Cultures

Human gingival fibroblasts (HGFs) were obtained as primary cultures from the Medical College of Georgia (MCG) School of Dentistry, Department of Oral Biology, in passages 2 – 4. HGF cultures were procured from gingival biopsies taken during routine periodontal surgical procedures on adult patients, and consisted of pools of three to five patient samples. Subcultures were maintained in Eagle's minimum essential medium (EMEM) with Earle's salts and glutamine, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). The cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2 and utilized in the experiments in passages 5 – 10.

For experimental procedures, cells were seeded at a cell density of $1 - 1.4 \times 10^4$ cells/well in 96-well tissue culture plates after determination of cell viability with trypan blue and counting in a hemocytometer. Cell numbers were determined by reaction with 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT),[‖] which is a reflection of mitochondrial dehydrogenase activity in living cells.[29] Absorbance is directly proportional to the number of living cells present as determined by standard curves, which were prepared from serial dilutions of viable cells.

Effect of Nicotine on HGF Attachment to Tissue Culture Plates

HGFs were seeded into six 96-well plates with 0, 0.025, 0.05, 0.1, 0.2, and 0.4 μM nicotine in EMEM. The average serum concentration for a smoker has been previously determined to be approximately 0.1 μM for nicotine.[30–32] Nicotine[¶] 98–100%, in EMEM was prepared from a 1 mg/mL stock solution of pure nicotine in water, which was prepared fresh prior to each experiment. The plates were incubated at 37°C in a 95% air/5% CO_2 atmosphere for 10, 20, 30, 45, or 60 minutes. At the end of the incubation, the plates were emptied by inversion and blotting onto plastic-backed absorbent paper and assayed for attached cells with the MTT procedure.

In a separate confirmation experiment, sub-confluent HGFs were treated for 72 hours with 2 μCi/mL ^{3}H-thymidine. These cells were then seeded into a 96-well tissue culture plate as above. After attachment for one hour, the plate was rinsed twice with PBS. The attached cells were treated with trypsin overnight and then counted in a scintillation counter.

Effect of Nicotine on HGF Mitochondrial Enzyme Activity

HGFs were seeded into six 96-well tissue culture plates and incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. The plates were emptied and then washed once with sterile PBS, then re-incubated with medium containing 0 (control), 0.025, 0.05, 0.1, 0.2, or 0.4 μM nicotine. The plates were incubated at 37°C in 5% CO_2 for 0, 10, 20, 30, 45, or 60 minutes. Following incubation, the plates were emptied, blotted, washed twice with sterile PBS, and assayed with MTT.

Effect of Continuous Exposure of Nicotine on HGF Cell Growth

HGFs were seeded into four 96-well tissue culture plates and incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. The plates were then incubated with medium containing 0 (control), 0.025, 0.05, 0.1, 0.2, or 0.4 μM nicotine and incubated at 37°C in 5% CO_2 for 4, 20, 24, or 48 hours. Following incubation the plates were emptied and blotted as before, washed twice with sterile PBS, and assayed by the MTT procedure as described above.

Effect of Pre-Exposure to Nicotine on HGF Cell Growth

HGFs were seeded into ten 96-well tissue culture plates and incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. The plates were emptied, washed with PBS and re-incubated at 37°C in 5% CO_2 for 1 or 6 hours with 0 (control), 0.025, 0.05, 0.1, 0.2, or 0.4 μM nicotine

[‖]Promega, Madison, WI.

[¶]ICN Biomedicals, Inc., Irvine, CA.

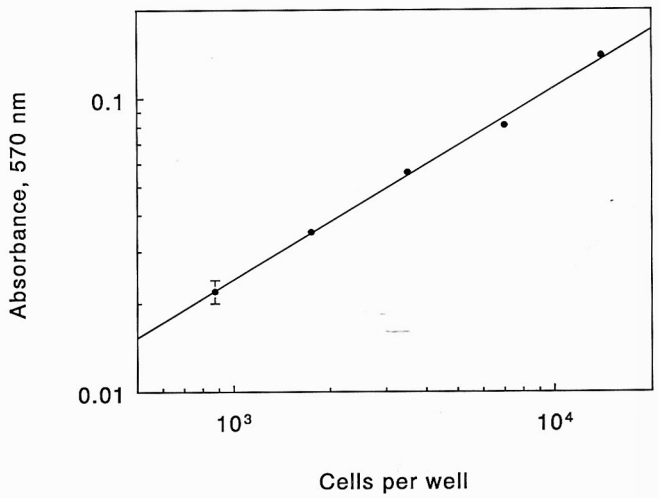

Figure 1. Standard curve for the MTT cell proliferation assay. Serial dilution of 1.4 × 10^4 cells/well. (r = 0.999).

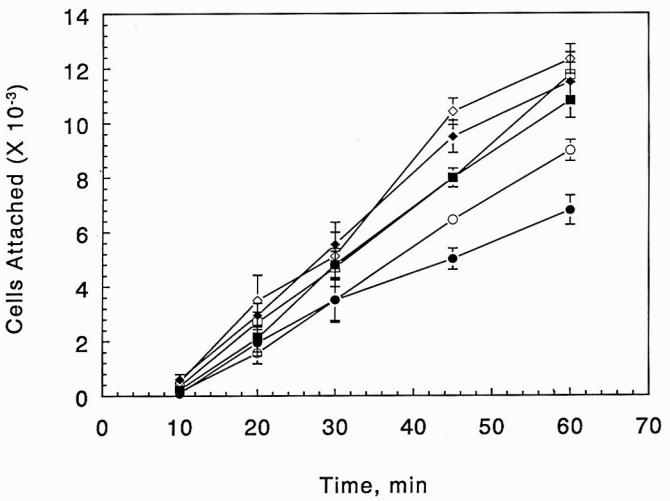

Figure 2A. Attachment of HGFs to tissue culture plates versus time with continuous exposure to nicotine.
● *Control (0 μM nicotine)*
○ *0.025 μM nicotine*
■ *0.05 μM nicotine*
□ *0.1 μM nicotine*
◆ *0.2 μM nicotine*
◇ *0.4 μM nicotine*

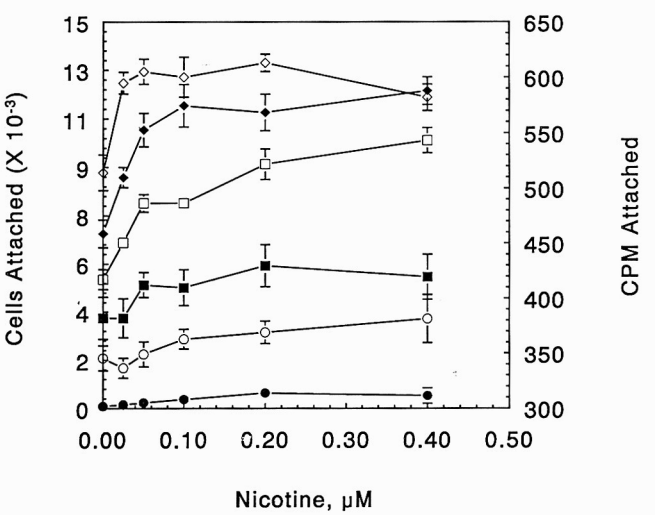

Figure 2B. Same data as Figure 2A plotted as attachment versus nicotine concentration.
● *10 minutes*
○ *20 minutes*
■ *30 minutes*
□ *45 minutes*
◆ *60 minutes*
◇ *60 minutes attachment (CPM) with ^{3}H-thymidine-labeled HGFs.*

in EMEM. Following incubation, the plates were emptied and washed twice with sterile PBS. Fresh medium was added to each well, and the plates were incubated for an additional 0, 1, 20, 24, or 48 hours. Following this incubation, the plates were assayed by the MTT procedure as described above.

Statistical Analysis

The data for each investigation were statistically evaluated using a univariate analysis by the one-way analysis of variance with replicates. An additional analysis employed Fisher's LSD test for each of the time periods. Further comparisons between individual concentrations of nicotine and the controls were evaluated with the Student *t*-test. For comparison between plates, MTT absorbance values were normalized to 100% for the controls (0 μM nicotine); however, all statistical procedures were carried out on the original absorbances.

RESULTS

MTT Standard Curves

To determine the relationship between the amount of formazan produced in the MTT assay and the number of viable cells present, viable HGFs were counted in a hemocytometer after Trypan blue exclusion and seeded into 96-well tissue culture plates as serial 1:2 dilutions of a working suspension. Following an overnight attachment period, the plates were assayed with the MTT procedure. Figure 1 illustrates a representative standard curve which was linear from 1.4 × 10^4 to 316 cells/well, with r = 0.999.

HGF Attachment in the Presence of Nicotine

Cells began to attach to the tissue culture plate surfaces very soon after being plated, whether in the presence or absence of nicotine. The number of cells attached was determined by the MTT assay at 10, 20, 30, 45, or 60 minutes after seeding into plates in the presence of varying concentrations of nicotine. The number of fibroblasts that attached upon exposure to the various concentrations of nicotine generally increased over time and with increasing nicotine concentrations up to 0.1 μM, after which they plateaued (Fig. 2A and B). For cells exposed to 0.4 μM nicotine, the number attached was nearly double after 1 hour as compared to the control cells. At concentrations of nicotine ≥ 0.1 μM the cell numbers were significantly greater than control cultures at all time periods ($P < 0.05$), and by 45 and 60 minutes, the numbers at all nicotine concentrations

were significantly greater than controls ($P < 0.0013$). A separate experiment performed with ^{3}H-thymidine-labeled HGFs (Fig. 2B) showed that at one hour, significantly more cells were attached at all nicotine concentrations compared to the control ($P = 0.0002 - 0.0057$), confirming the MTT results.

Phase contrast photomicrographs show that fibroblasts exposed to 0.4 μM nicotine tended to exhibit a more flattened appearance than the control cells, starting at the 30-minute time period (Figs. 3A and B). The margins appeared rough or crenated (somewhat suggestive of senescence). By 60 minutes the nicotine-exposed cells showed an even greater difference from control cells. The latter had a more raised appearance than the cells exposed to nicotine, which looked much flatter and appeared to be spread out over the culture plate surface (Figs. 3C and D). By 4 hours these differences disappeared and no morphologic differences were apparent between experimental and control cells.

Mitochondrial Enzyme Activity

To determine if the MTT assay reflected cell numbers and not altered enzyme activity, HGFs were plated, exposed to varying concentrations of nicotine in the range of 0 to 0.4 μM for up to 4 hours, and the dehydrogenase activity determined by the MTT assay. After 20 minutes, enzyme activity dropped rapidly, with the decrease directly proportional to the nicotine concentration, until 45 minutes, after which time activity began to rise, returning to approximately control levels by 4 hours. MTT absorbance values for all concentrations of nicotine were statistically different from control at 45 minutes ($P < 0.02$) except for 0.025 μM, and all concentrations were statistically identical to control by 4 hours except for 0.05 μM ($P = 0.0001$) (Fig. 4). These results emphasize the transient time/dose relationship between nicotine concentration and dehydrogenase activity and indicate that the increase in attachment seen was not due to increased mitochondrial enzyme activity.

Cell Reproduction Upon Continuous Nicotine Exposure

Since by 4 hours after nicotine exposure enzyme activity had returned to approximately 100% of control, indicating absorbance is a reflection of the number of cells present, nicotine exposure for periods of 4 hours and longer were examined. The lowest concentration of nicotine, 0.025 μM, produced a steady increase in fibroblast number over 48 hours and this was statistically significant at all times beyond 4 hours ($P < 0.001$ at 48 hours). The higher concen-

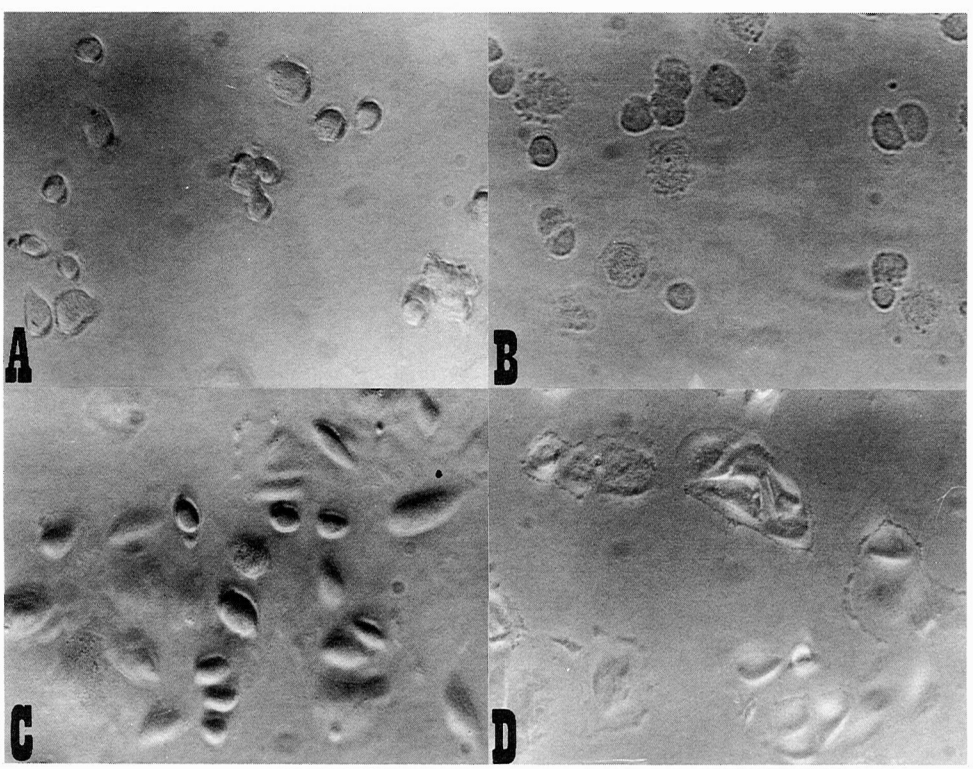

Figure 3 **A.** *Control cells 30 minutes after plating. Cells appear raised with very little spreading (original magnification × 400).* **B.** *0.4 μM nicotine-treated cells 30 minutes after plating. The cells appear more flattened than control cells (original magnification × 400).* **C.** *Control cells 60 minutes after plating. Cells appear more oval-shaped than at 30 minutes, but are still raised from the surface with less spreading than nicotine-treated cells (original magnification × 400).* **D.** *0.4 μM nicotine-treated cells 60 minutes after plating. There is almost complete flattening of the cells, and roughening of the margins is very evident (original magnification × 400).*

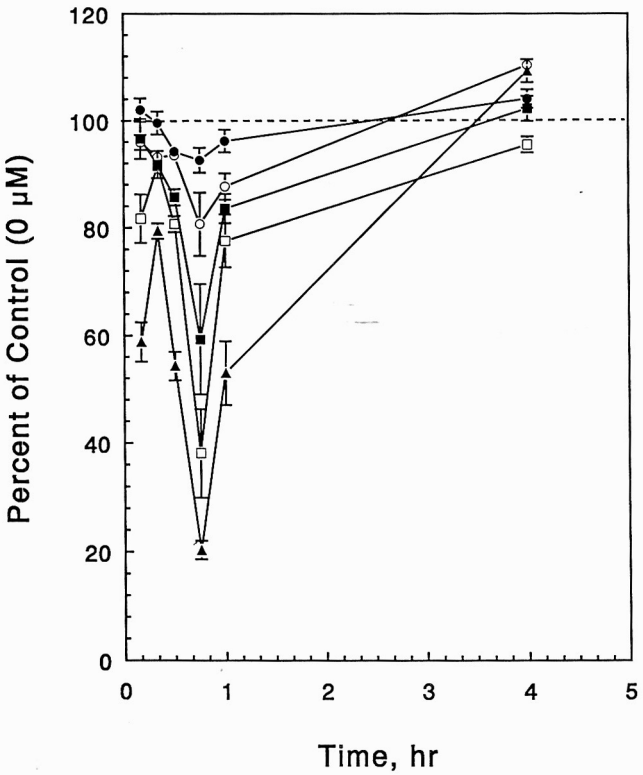

Figure 4. Enzyme activity/growth of HGF cultures exposed to nicotine for up to 4 hours.

● *0.025 µM nicotine* ○ *0.05 µM nicotine*
■ *0.1 µM nicotine* □ *0.2 µM nicotine*
▲ *0.4 µM nicotine*

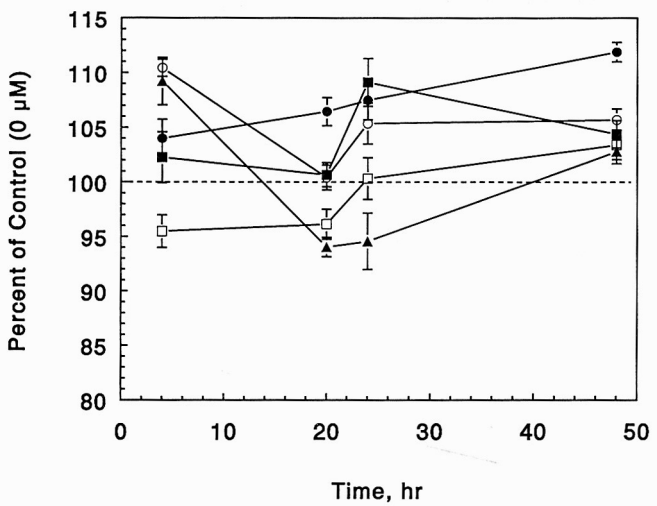

Figure 5. HGF reproduction with continuous nicotine exposure.

● *0.025 µM nicotine* ○ *0.05 µM nicotine*
■ *0.1 µM nicotine* □ *0.2 µM nicotine*
▲ *0.4 µM nicotine* --- *Control (0 µM nicotine)*

trations of nicotine generally resulted in decreased cell numbers after 20 hours compared to the levels at the start of the experiments, and this appeared to be concentration dependent (Fig. 5). However, by 24 hours, fibroblast numbers in cultures with nicotine began to increase although

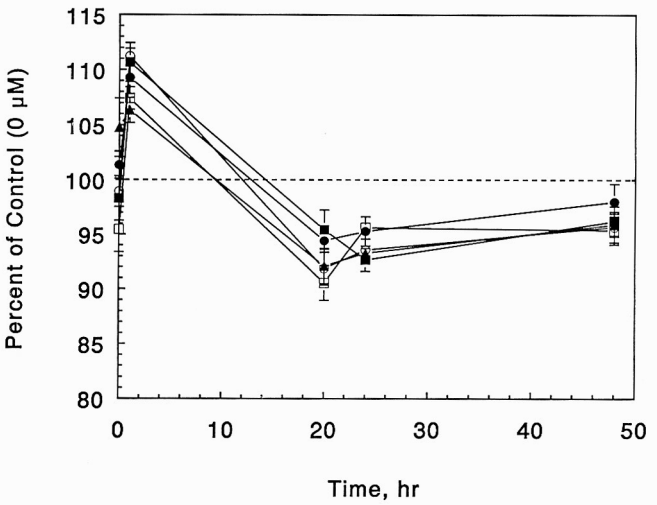

Figure 6. HGF reproduction after a 1-hour pre-exposure to nicotine. Zero time is immediately after the 1-hour exposure.

● *0.025 µM nicotine* ○ *0.05 µM nicotine*
■ *0.1 µM nicotine* □ *0.2 µM nicotine*
▲ *0.4 µM nicotine* --- *Control (0 µM nicotine)*

they were generally equal to control cultures. After 48 hours of exposure to nicotine, there was a statistically significant increase in cell number for all concentrations ($P < 0.008$). (Fig. 5).

Cell Growth Following a 1-Hour Pre-Incubation With Nicotine

Cells plated and allowed to attach overnight were refed with medium containing various concentrations of nicotine. After 1 hour the medium was removed and the cells refed with fresh medium lacking nicotine in order to determine whether any responses persisted following nicotine removal. One hour after removal of nicotine and refeeding there was an initial increase in absorbance, likely a result of a burst of enzyme activity due to refeeding with fresh medium ($P < 0.0001 - 0.0023$). Following the initial increase, the number of cells fell significantly below those in control cultures (Fig. 6) ($P = 0.0001 - 0.0386$). By 24 hours post-nicotine removal, cell numbers started to increase toward control values, but, by 48 hours, had not yet achieved that level for 0.2 and 0.4 µM ($P < 0.028$). At all nicotine concentrations at 1, 20, and 24 hours after removal of nicotine, the cell numbers as determined by the MTT assay were significantly different from control values ($P < 0.0386$). Cells that were pre-exposed to the 0.025 µM nicotine tended to rebound back toward control levels earlier than the other concentrations.

Reproduction Upon a 6-Hour Pre-Incubation With Nicotine

When cells were treated as above but with a 6-hour pre-incubation, the initial burst of activity was not detected. Rather, a general decline which continued until about 20 hours post-removal was noted. This was followed generally

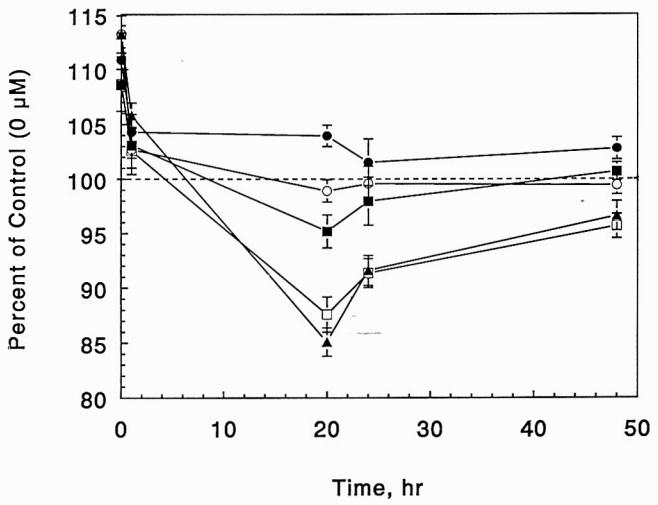

Figure 7. HGF reproduction after a 6-hour pre-exposure to nicotine. Zero time is immediately after the 6-hour exposure.

● *0.025 μM nicotine* ○ *0.05 μM nicotine*
■ *0.1 μM nicotine* □ *0.2 μM nicotine*
▲ *0.4 μM nicotine* --- *Control (0 μM nicotine)*

by some recovery (Fig. 7). At 0 time at all nicotine concentrations the increase over control cultures was statistically significant ($P < 0.024$). At 1 hour post-refeeding, cells exposed to all concentrations of nicotine showed activity above control values, but only those exposed to 0.4 μM were significantly elevated ($P = 0.0298$). By 20 hours cells exposed to the lowest concentration of nicotine (0.025 μM) were still significantly elevated, while at other levels there was a decrease, with the higher concentrations (0.1, 0.2, and 0.4 μM) being significantly less than control values ($P < 0.0189$). By 24 hours only the two highest concentrations were significantly different from control values, and this pattern was still evident at 48 hours ($P < 0.0152$).

DISCUSSION

Subsequent to the destruction caused by periodontal disease, the ability of cells to attach to root surfaces is a critical event. Indeed, one of the major goals of periodontal therapy is the re-establishment of lost connective tissue attachment to the root surface. Recent studies have suggested a harmful effect of nicotine on fibroblasts, especially in regard to the reattachment and wound healing responses following periodontal treatment. In the current study we demonstrate a positive effect of nicotine on gingival fibroblast attachment to a substrate; and, in concentrations to which light smokers are generally exposed, an enhanced rate of reproduction of gingival fibroblasts. Also noted in this study was that fibroblast adherence and growth may be differentially affected by the presence of nicotine, dependent upon time and concentration of nicotine outside and possibly inside the cells. Mitochondrial enzymatic activity was transiently depressed by nicotine, while simultaneously, increasing numbers of cells were seen attaching to the plastic tissue culture plates.

Human gingival fibroblast attachment and reproduction in the presence of tobacco components have not previously been done, although non-specific binding and uptake of nicotine by HGF have been demonstrated.[33] The present study shows that exposure to nicotine enhances gingival fibroblast attachment to a plastic surface, and that this enhanced effect is concentration dependent. Using morphologic criteria to study foreskin fibroblasts, Raulin et al.[34] concluded that exposure of such cells to nicotine resulted in structural alterations which might possibly prevent the cells from becoming "firmly attached" to root surfaces. Foreskin fibroblasts are from neonatal tissue and may behave differently than cells of periodontal origin from adults. Our attachment study was quantitative, in addition to using morphologic criteria. It showed a significant increase in HGF attachment with increasing nicotine concentration ($P < 0.0001$ for 45 and 60 minutes). The colorimetric assay used in the present study counts viable cells by measuring mitochondrial dehydrogenases, primarily succinic dehydrogenase. Such activity is not present in non-viable cells. The transient decrease in mitochondrial dehydrogenase activity, which disappeared by 4 hours, can be accounted for by changes in enzyme activity, as opposed to changes in cell numbers. This further strengthens the interpretation of results of the attachment studies, since the numbers of attached cells were increasing at the same time that the assay was measuring decreased enzyme activity. Thus the reported numbers of attached cells at these times may actually be underestimated.

It must be realized that, in this experiment, attachment was occurring to tissue culture-treated plastic. It is not known whether or how nicotine may bind to the plastic substrate. Such binding could create a positively charged surface effect, resulting in increased binding of cells by electrostatic forces. Further experiments will be required to determine the differences in binding to collagen, fibronectin, or other extra-cellular matrix proteins.

This study also demonstrated a modest but statistically significant stimulatory effect of nicotine on fibroblast proliferation, especially at low nicotine concentrations (0.025 μM), levels similar to those seen in light tobacco users. Over a 48-hour period the cells continuously exposed to a single dose of 0.025 μM nicotine steadily increased in number, exhibiting a near linear growth pattern. As the nicotine concentration increased, however, the cells decreased in number until 20 hours, and then started to increase up to 48 hours. After 48 hours of exposure to nicotine, the higher concentrations all produced a statistically significant increase in cell number compared to the control cultures. These results seem to suggest that, except for the very low nicotine concentrations perhaps equivalent to amounts seen in light smokers, the metabolism of cells that are continuously exposed to high levels of nicotine may become transiently impaired by these levels (or some cells may be killed by it). As the cells recover, cellular metabolic activity is once again stimulated, resulting in an increased prolifera-

tion rate. A metabolic byproduct could be stimulatory. Another possible explanation for the decreased numbers is that replication in the presence of nicotine may be altered due to changes in the cell cycle. Also, as cells divide they may become more rounded, and in general become less adherent. This effect may be enhanced in the presence of nicotine and possibly lead to cell loss during cell manipulation.

The fibroblasts that were pre-incubated with nicotine for one hour showed a burst of metabolic activity after refeeding, but this stimulation did not seem to persist upon removal of the nicotine, although the cells were approaching control values 48 hours later. The decreased numbers at 20 and 24 hours may be an expression of cell loss due to residual toxicity by internalized nicotine. This possibility is further enhanced by the decreases seen at these same time points in cells continuously exposed to nicotine. The recovery by this latter group (continuously exposed cells) then could result from the cells avoiding the toxicity of internalized nicotine, perhaps by sequestering internalized nicotine within vesicles. Raulin et al.[34] observed "vacuolization" in human foreskin fibroblasts upon long-term exposure to nicotine, and the degree of vacuolization seen increased as the nicotine concentration increased. These may actually have been vesicles containing nicotine.

The cells that underwent a six-hour pre-exposure period did not show the same initial burst of activity or increase in number as did those that were pre-exposed to nicotine for only one hour. These cells initially decreased in number, but after 20 hours post-removal of nicotine their numbers gradually increased toward control values. It is possible that initially, while the cells were exposed to nicotine, there was some stimulation of division until internal concentrations accumulated to an inhibitory level. This is analogous to the stimulation seen with continuous exposure to 0.025 μM nicotine, but a negative effect occurs as internal concentrations increase. Gradually the cells' metabolic activity may return as the nicotine is sequestered into vesicles and they again begin to multiply.

There is renewed interest in the role of nicotine in conditions such as Parkinson's disease and a possible use of this agent in treatment.[35–37] Drug therapy for Parkinson's disease is aimed at correcting or modifying neurotransmitter defects by either inhibiting the effects of acetylcholine or enhancing the effects of dopamine.[38] It is known that nicotine acts as a stimulus to bring about the circulation of dopamine. Thus, these, and other possible effects of nicotine, lend credence to the opinion that it may not be the culprit in various negative tissue responses to tobacco. Rather, other agents present in tobacco may be responsible for the detrimental effects. A dose-response with toxicity due to high doses of nicotine or other tobacco constituents may also help explain the varied effects on the periodontal tissues mentioned earlier.

In summary, the results of this study indicate that: 1) exposure to nicotine enhances human gingival fibroblast attachment to a plastic substrate, and this appears to be concentration-dependent; 2) mitochondrial dehydrogenase activity is transiently decreased following nicotine exposure, but by 4 hours the enzyme activity is approximately equal to control; 3) exposure to low concentrations of nicotine has a statistically significant stimulatory effect on HGF reproduction, while the higher concentrations produce only a slight increase in HGF culture growth; and 4) some effects of nicotine upon HGF reproduction may persist following nicotine removal.

Acknowledgments

This study was approved and supported by the U. S. Army (DDEAMC 91-6) and the Medical College of Georgia School of Dentistry. The authors would like to thank Dr. Gretchen Caughman and Dr. Baldev Singh, Medical College of Georgia School of Dentistry, for their assistance in interpretation of results. We would also like to thank Mr. Jack Horner, D.D. Eisenhower Army Medical Center, Fort Gordon, for laboratory and photomicrograph assistance.

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Send reprint requests to: Director, Periodontal Residency Program, U.S. Army Dental Activity, Fort Gordon, GA 30905.

Accepted for publication January 15, 1993.