Genotoxic and Antiapoptotic Effect of Nicotine on Human Gingival Fibroblasts

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Growing evidence suggests that nicotine, the addictive component of cigarettes, can have a direct role in tumor development by enhancing cell proliferation and impairing apoptotic process in certain types of human cancer cell lines. Since the correlation between apoptosis and DNA damage is already well documented, we investigated the response of human gingival fibroblasts (HGFs) to nicotine exposure by examining its effect on DNA damage induction and apoptotic process in parallel. To assess the genotoxicity of this drug, the cytokinesis-block micronucleus (CBMN) test was performed. Treatment of HGFs with nicotine, at a concentration of 1 μM, caused a statistically significant increase of micronucleus (MN) frequency at the tested time intervals, while no change was detected in cell growth under the same conditions. Furthermore, we found that preincubation of HGFs with 1 μM nicotine strongly attenuated staurosporine (STP)-induced apoptosis. Finally, we found that cultures exposed to nicotine showed an increase of reactive oxygen species, as determined by increased levels of 2,7-dichlorofluorescein (DCF). When cells were prelabeled with N-acetyl-cysteine (NAC), a substrate for glutathione synthesis, and catalase (CAT), the oxygen free radical scavenger, a significant reduction in cytogenetic damage was observed. Thus, for the first time, we report a concomitant genotoxic and antiapoptotic effect of nicotine in HGFs.

Key Words: HGFs, nicotine, apoptosis, DNA damage.

Nicotine is the major alkaloid in tobacco and has been considered, at least partly, responsible for some deleterious effects of smoking, such as cardiovascular diseases (McPhail et al., 1998) and impairment of the immune system (Singh et al., 2000). Reports indicate that nicotine affects a variety of cellular processes ranging from altered gene expression (Zhang, 2000) to secretion of hormones (Sano et al., 1999) and modulation of enzymatic activity (Yildiz et al., 1999).

While it has been clearly demonstrated that smoking is a potent inducer of genetic damage in human and rodent cells (Hopkins and Evans, 1980; Jones et al., 1991), studies on the genotoxic effect of nicotine are limited and contradictory. Some authors have reported that nicotine and its major metabolites do not represent a genotoxic hazard (Doolittle et al., 1995) and that DNA alteration induced by tobacco is not related to nicotine content (Mizusaki et al., 1977); others (Trivedi et al., 1990) have indicated that this alkaloid slightly increased the frequency of chromosome aberrations and sister chromatid exchanges in mammalian cells.

Although nicotine has been long identified as a noncarcinogenic component of smoke, growing evidence suggests that this drug could be a potential factor in carcinogenesis by acting as a cancer growth promoter. Studies have indicated that the alkaloid, at levels similar to those found by Russell et al. (1980) in the blood of habitual smokers (0.4 μM, measured 2 min after smoking), shows a mitogen effect in several cell types (Nylen et al., 1993; Villablanca, 1998) including cancer cell lines (Schuller, 1989). Recently, Heeschen et al. (2001) showed that nicotine, by inducing endothelial cell proliferation, can stimulate angiogenesis, a complex multistep process associated not only with the neoplastic growth but also with the metastatic potential of many tumors.

Furthermore, another important effect of nicotine is the impairment of apoptosis, a morphologically distinct process of cell death that involves the activation of a cell-intrinsic suicide program. Apoptosis is an important defense mechanism to prevent and eliminate cancer cells (Kerr et al., 1994) and, therefore, its deregulation can contribute to tumorigenic development and growth and to a decrease in the efficacy of cancer therapies. Much data collected over the last decade indicate that nicotine exerts a suppressive effect on the signaling of the death pathway, including in cancer cell lines (Wright et al., 1993), which contributes to tumor growth.

It is well known that cigarette smoke is the most common oxidant stress in daily life, but it is still debatable whether nicotine is responsible for the effects, due to free radical generation, associated with tobacco use. Studies in vitro and in vivo on rodent cells showed that exposure to nicotine produces oxidative tissue injuries in Chinese hamster, rat, and mouse, often resulting in a depletion of glutathione content and a decrease in the activity of some oxygen free radical scav-
engers, such as catalase (CAT) and superoxide dismutase (Bhagwat et al., 1998; Wetscher et al., 1995). To date, no examinations of the oxidative properties of nicotine on human-derived cells have been reported.

Among the known target tissues for the nicotine effect, cells of the oral cavity certainly represent the first tissue to be exposed to this drug, both during cigarette smoking and in widespread use of nicotine to treat smoking addiction. Gingival fibroblasts are fundamental components of the periodontal tissue and their role is critical for maintenance of the oral connective tissue and for optimal wound healing responses. Nicotine readily penetrates the epithelial barriers of skin and oral mucosa (Hanes et al., 1991), where it is able to affect immunological defense mechanisms (Neher, 1974), to alter oral mucosa (Hanes et al., 1991), and to modulate some fibroblast functions in vitro, such as collagen production and secretion (Giannopoulou et al., 2001) and the growth and attachment of cells to substrates (Lahmouzi et al., 2000). Thus, this drug can be considered as a risk factor in the etiology and progression of inflammatory periodontal diseases, whose incidence is increased by tobacco use (Haber, 1994).

In this study, we investigated the in vitro response of human gingival fibroblasts (HGFs) to nicotine, taking into account the DNA damage induction, assessed by the cytokinesis-block micronucleus (CBMN) test, and the possible interference of the drug with the apoptotic process. We found that 1 μM nicotine induced DNA damage, revealed by increased micronucleus (MN) frequency; nicotine affected the activation of the apoptotic pathway; and, finally, nicotine’s genotoxicity can be modulated by the antioxidants N-acetyl-cysteine (NAC) and CAT. To our knowledge, this is the first study in which a dramatic combination of effects of nicotine has been demonstrated on human gingival fibroblasts.

MATERIALS AND METHODS

**Cell lines and reagents.** HGF (not immortalized) line was purchased from Coriell Cell Repository (Camden, NJ) (AG09429). Cells were cultured in Eagle’s MEM, supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/ml of antibiotics, in plastic culture flasks and maintained in an atmosphere of 5% CO₂ in air at 37°C. Upon confluence, HGFs were detached by incubation with trypsin in EDTA solution for 10 min at 37°C. Then, cells were plated on 8-well tissue culture plates or into sterile Slide Flask (Nunc AS, Roskilde, Denmark) at a density of 1 × 10⁵ cells/well and cultured in growth medium at 3 ml/well. Immediately before each experiment, the growth medium was removed and fresh medium was added to the cells. Unless specified, all chemicals were of cell culture grade and obtained from Sigma Chemical Co. (St. Louis, MO).

**Treatments.** The doses of nicotine were selected through preliminary experiments performed with the drug at a final concentration of 1 μM, 10 μM, and 1 mM. Nicotine dilution was obtained from stock solution with serum-free culture medium and was freshly prepared before each test. All experiments with nicotine were performed in the dark. Control cells were treated with the same volume as that used to dissolve nicotine. For the antioxidant experiments, NAC (1 μM) and CAT (50 μg/ml ) were added to the cells 2 h prior to the nicotine addition.

**Evaluation of cytogenetic parameters.** The DNA damage was evaluated with CBMN test, which uses cytochalasin B to block cytokiderosis. Thus, the cells that divide in its presence become binucleated (Fenech, 1993). Micronuclei (MNs) were scored only in binucleated cells (BNCs) that had undergone at least one division after treatment. The MNs in fact originate from centric or acentric DNA fragments not incorporated into the main nucleus at telophase.

To determine MN frequency, cytochalasin B (cyt-B), in the presence or absence of chemicals according to the experimental protocols, was added to the wells 24 h before harvest at the final concentration of 6 μg/ml (stock solution 1 mg/ml in dimethyl sulphoxide, DMSO). Following exposure at four different times (24, 36, 48, and 72 h), HGFs were harvested and subjected to hypotonic treatment (0.075 M KCl) for 10 min, fixed in 3:1 methanol/acetic acid for 30 min, and washed once in the same fixative. Cell suspension was dropped into slides. After air drying, the slides were stained with 7% Giemsa in phosphate buffer for 20 min. The MNs were scored in 1000 BNCs for each experimental point in three independent cultures and their frequency was calculated as number of MNs on 1000 BNCs. Slides were scored at ×100 magnification.

**Cell growth test.** HGFs were incubated in the presence and absence of nicotine and antioxidants, alone or in combination. The cells were harvested at the culture times as described above and resuspended in medium containing 50% vol/vol trypan blue. Cell counts were performed using a Nautauer counting chamber. Nonviable fibroblasts were identified by trypan blue staining and excluded from counting.

**Intracellular redox assay.** For in situ detection of intracellular reactive oxygen species (ROS) accumulation, the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF) was used. Aliquots of 10 mM DCF were prepared in DMSO and stored at −20°C. After pretreatment (2 h) with NAC and CAT, cells were exposed to nicotine for 24 h and then were incubated for 1 h in the presence of 10 μM DCF. ROS accumulation was qualitatively monitored by a Nikon fluorescence microscope (Nikon Corporation, Tokyo, Japan) using excitation and emission wavelengths of 485 and 530 nm, respectively, and images were collected.

**Induction of cell death and detection of apoptotic cells.** For the induction of apoptosis in HGFs, staurosporine (STP) was added to the culture medium for a final concentration of 1 μM in DMSO. Nicotine was added 2 h before STP exposure. Controls received vehicle only. Apoptotic cells were visualized using the DNA fluorochrome Hoechst 33258. After 24 h exposure to chemicals, the cells were fixed in 3:1 methanol/acetic acid and incubated for 30 min with Hoechst 33258 (1 μg/ml in PBS) at 37°C. As such, the cells were washed twice with 0.1 M PBS, and nuclear morphology was observed under a Nikon fluorescence microscope. The apoptosis percentage was determined by counting apoptotic and nonapoptotic nuclei in at least 200 cells for each experimental point in triplicate cultures.

**DNA fragmentation.** DNA fragmentation was analyzed by gel electrophoresis following the Eastman method (Eastman, 1995), with minor modifications. Briefly, the top part above the wells of a 2% agarose gel was removed. DNA was quantified using the DNA fluorochrome Hoechst 33258. After 24 h exposure to chemicals, the cells were fixed in 3:1 methanol/acetic acid and incubated for 30 min with Hoechst 33258 (1 μg/ml in PBS) at 37°C. As such, the gel was washed twice with 0.1 M PBS, and nuclear morphology was observed under a Nikon fluorescence microscope. The apoptosis percentage was determined by counting apoptotic and nonapoptotic nuclei in at least 200 cells for each experimental point in triplicate cultures.

**Statistical analyses.** All statistical analyses were performed using Student’s t-test, after assessing the normality of the distribution and homogeneity of the variance.

RESULTS

To examine the effects of nicotine in HGFs, a preliminary test on dose response was performed exposing cultures to three concentrations of the drug (1 μM, 10 μM, and 1 mM) for 24 h. After the exposure period, induction of MNs and cell viability were assessed. The genetic damage was revealed by CBMN test, which used cytochalasin B to block cytoplasmic division. In its presence, cells became binucleated and MNs appeared as
small additional nuclei. The results of the dose-response test are shown in Figure 1. Compared to untreated cultures, treatment with nicotine at 1 \( \mu \text{M} \) significantly enhanced the frequency of MNs (\( p = 0.0004 \)), with no influence on the HGF viability. In contrast, 10 \( \mu \text{M} \) induced MNs with minor efficiency (\( p = 0.04 \)), affecting also cell survival (\( p = 0.005 \)); and the 1 mM dose did not result in a significant increase of DNA damage, owing to its strong cytotoxicity, as indicated by the pronounced decrease in cell number (\( p = 0.0005 \)). This loss was consistent with cell death without typical apoptosis morphological changes, including cell shrinkage and apoptotic bodies.

Therefore, all subsequent experiments were performed using nicotine at the concentration of 1 \( \mu \text{M} \), similar to the dose found in the plasma of smokers (Heusch and Maneckjee, 1998; Russell et al., 1980).

The time-dependent genotoxicity of the drug was evaluated by continuous exposure of cells to nicotine from 24 to 72 h. As shown in Figure 2, a statistically significant increase in MN frequencies was observed in nicotine-treated cultures compared to spontaneous values at all time intervals tested (\( p_{24} < 0.0001; \ p_{36} = 0.006; \ p_{48} = 0.0001; \ p_{72} = 0.0007 \)). The DNA damage induction reached the highest level within 24 h, significantly declined by 36 h, and persisted without significant differences for the last two exposure periods.

In a second set of experiments, cell growth in the absence or presence of nicotine was determined. For these studies HGFs were seeded at a density of \( 1 \times 10^5 \), treated with 1 \( \mu \text{M} \) nicotine for 24–72 h, and, following each incubation, cell growth was assessed as described in Materials and Methods. The results indicated that nicotine neither inhibited nor stimulated cell growth at any time intervals (Fig. 3). Also, statistical analysis of the binucleated/mononucleated cell ratio, a marker of in vitro proliferation, did not show significant changes between untreated and treated cultures (data not shown).

Since previous studies have suggested that nicotine inhibited or attenuated apoptosis induced by various stimuli, another investigation was performed. Figure 4 shows the experiment in which the effect of nicotine on apoptotic process was tested. STP, a protein kinase inhibitor able to arrest the cell cycle progression (Bertrand et al., 1994), was used as a cell death inducer. HGF cultures were exposed to 1 \( \mu \text{M} \) STP for 24 h, with or without nicotine pretreatment for 2 h, and typical
morphological and biochemical changes correlated to apoptosis were analyzed. When stained by Hoechst 33258, viable cells showed normal nuclei with characteristic, diffuse granular staining, while condensed chromatin or nuclear fragmentation with a reduction in nuclear size were considered features of apoptosis (Figs. 4A [untreated cells] and 4B [STP-treated cells]). In Figure 4B we show the percentages of apoptotic cells. As expected, the STP-treated cells presented a strongly significant increase in proportion of apoptotic nuclei compared with control ($p < 0.0001$), while treatment with nicotine alone did not induce a significant difference. When nicotine was added to cultures 2 h before STP exposure, a significant decrease in the number of cells with apoptotic nuclear morphology was seen compared with those treated with STP alone ($p = 0.002$). This combined exposure exhibited higher values than control ones ($p = 0.04$). The morphological assessment was confirmed by DNA cleavage with a pattern of internucleosomal ladder characteristic of apoptosis (Fig. 4C).

Also, we determined the reactive oxygen species production in our cells in the presence of nicotine with or without the antioxidants NAC and CAT using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF), which mainly reflects intracellular peroxide level. In fact, this compound rapidly diffuses into cells where it is hydrolyzed to DCFH, and, in the presence of reactive oxygen intermediates, it is oxidized to the highly fluorescent DCF (Bass et al., 1983). Cells were treated as described in Materials and Methods, examined under a fluorescence microscope, and representative images were collected. Cultures exposed to $1 \mu M$ nicotine for 24 h and treated with the probe ($10 \mu M$) for 1 h showed increased intensity of DCF-labeled cells compared with untreated cells (Figs. 5A and 5B). Cultures pretreated with NAC or CAT for 2 h before nicotine addition had a strong reduction in the intensity of DCF fluorescence compared to cells exposed to nicotine alone (Fig. 5C).

Finally, we examined the effect of the two antioxidants on DNA damage induction caused by nicotine. The results are shown in Figure 6. When HGFs were preincubated for 2 h with NAC (1 mM) or CAT (50 $\mu g/ml$) before nicotine exposure, statistically significant reductions in MN frequencies were observed at 24 and 36 h for CAT and at 24, 36, and 48 h for NAC compared with the responses to nicotine alone. Thus,
NAC protects against nicotine-induced DNA damage for a longer time and in a more efficient way than CAT (\(p_{\text{NAC,24h}} = 0.003; p_{\text{CAT,24h}} = 0.01; p_{\text{NAC,36h}} = 0.003; p_{\text{CAT,36h}} = 0.02\)). The effect of NAC or CAT alone was not substantially different from the control.

**DISCUSSION**

In this study, we assessed the response of HGFs to nicotine exposure by analyzing the induction of DNA injury and the influence of the drug on cell viability and proliferation.

Data obtained in our laboratory on HGF cultures demonstrated that nicotine, at concentrations similar to those generally found in the blood of habitual smokers (Heusch and Maneckjee, 1998; Russell et al., 1980), was able to produce strong DNA damage, as measured by the increase in MN frequency. Our results indicated that, firstly, a long-term exposure to nicotine was not necessary for genotoxic activity to occur. HGFs showed high values of damage after incubation with nicotine for only 24 h, suggesting a fast transport of the drug through the plasma membrane and its rapid association with intracellular components. This is consistent with published data reporting that nicotine is rapidly absorbed by the oral cavity, it is able to bind to fibroblast membranes, and it readily penetrates within the cells (Hanes et al., 1991). Secondly, nicotine exerted a prolonged effect on HGF cultures, as was clearly proved by its capability to raise significantly the MN frequencies by 36 h from the beginning of treatment. Nevertheless, the comparison of the different values of induced damage showed a significant decline in MN amount, which remained constant until the last evaluation, from 36 h.

We hypothesized that this decrease in the MN frequency could be due either to a slowing of proliferation rate or to a loss of cells that were strongly damaged. In any case, the cells could not reach mitotic telophase when MNs were scored in BNCs. The response to DNA damage is critical for cell viability and can lead to the activation of surveillance systems that either delay cell cycle progression until the DNA is repaired or induce apoptotic death, if damage caused is irreparable (Wang, 2001). We investigated whether the fall of nicotine-induced MNs from 36 h would be accompanied by a decrease in cell survival or growth rate of treated HGFs. Surprisingly, our results indicated that there was not any difference in proliferation index between untreated and treated cells as far as total cell number and frequency of binucleated fibroblasts. This implies that, despite the persistent DNA lesions, nicotine-exposed cultures presented a normal progression through cell cycle, maintaining proliferation ability, without undergoing either the expected mitotic delay or cell loss.

In the light of these considerations, the decrease in MN frequency observed from 36 h in our cells could likely be caused by partial loss of the nicotine activity and by metabolic process. It has been reported already that the taken-up nicotine is also, in time, released by cells, but a small amount remains within as a free and/or unmetabolized molecule (Hanes et al., 1991). The significant induction of MNs by nicotine with no change in proliferation rate even when the DNA damage was highest (i.e., at 24 h) raised concern about the possible ability of the drug to interfere with molecular pathways that underline the cell response to injury. It is known that nicotine affects apoptotic process induced by various stimuli including ultraviolet light (Sugano et al., 2001) and chemotherapeutic agents in cancer cell lines (Maneckjee and Minna, 1994; Onoda et al., 2001). The apoptotic assay we used with cell death inducer STP, an inhibitor of protein kinases, confirmed these previous investigations. The cell survival data indicated that pre-exposure to nicotine strongly decreased the percentage of apoptotic nuclei (18%) compared to treatment with STP alone (54%), suggesting that nicotine likely impaired the cytotoxicity of this drug. Also, the hallmark of the apoptotic process, DNA fragmentation, occurring in STP-treated cells was reduced by pre-incubation with nicotine.

Although at low levels of significance, a difference in values obtained from combined treatment and untreated cultures was found. This allows us to state that nicotine, rather than inhibit, markedly attenuated the apoptotic process.

The intracellular mechanisms involved in the suppression of apoptosis by nicotine are not yet well known, but they seem to interact with several signaling pathways. In mouse spinal cord neurons, the effect of nicotine occurs through the inhibition of caspase activation and the release of cytochrome c from mitochondria (Garrido et al., 2001), while in human lung cancer cells, nicotine leads to increased expression of antiapoptotic protein bcl-2 and the activation of protein kinase C (PKC), a pathway known to inhibit apoptosis (Heusch and Maneckjee, 1998). On this basis, our data suggest that, in HGF cultures,
nicotine could block the action of STP by reversing its inhibitory effect on the PKC pathway.

Although DNA-damaging agents have often been found to induce apoptosis, such as several antineoplastic drugs (Karpinich et al., 2002) and pesticides (Cicchetti and Argentin, 2003), we have demonstrated for the first time a concomitant genotoxic and antiapoptotic effect of nicotine in oral cells. In our opinion, this appears to dramatically support previous studies on the possible role of nicotine as a promoter in carcinogenesis. In fact, in our experiments, nicotine not only induced DNA damage, which may be the first step in the neoplastic process, but it also allowed the proliferation of aberrant cells, thus altering a crucial mechanism for the balance between cell survival and death. Furthermore, our data show the effect of a single-dose of nicotine for a short-term treatment; it is plausible that long-term exposure to nicotine can lead to continual DNA damage that can cause multiple genetic lesions.

Since data on oxidant ability of nicotine in human cells were not available, we also examined the induction of oxidative stress by nicotine in HGFs and the possible protective action of antioxidants NAC and CAT. NAC, a thiol-reducing agent, is a stress mechanism. NAC, and CAT was chosen because it is a well-known, free radical–scavenging enzyme. Our results demonstrated that nicotine is able to cause oxidative stress, as shown by an accumulation of fluorescent probe DCF in our treated cultures and its decrease in the presence of antioxidants.

Finally, to understand the causal link between the genetic damage observed in our treated cells and the production of redox-active substances, we analyzed the effect of the two antioxidants on genotoxic activity of nicotine. Our data indicated that both antioxidants were able to minimize the effect of nicotine, decreasing significantly the MN amount. Thus, the evidence that HGFs produced ROS in response to nicotine and the observation that NAC and CAT conferred protection against DNA injury caused by nicotine suggest that nicotine genotoxicity might be, at least in part, mediated by an oxidative stress mechanism.

The greater ability of NAC in protecting cells against the nicotine effect is likely due to the fact that it prevents damage in two ways: as a glutathione precursor, by increasing GSH stores depleted by nicotine, and as an antioxidant able to scavenge radicals and to inhibit the consequent genotoxicity (De Flora et al., 2001).

In conclusion, the present study indicates for the first time that nicotine leads to a complex pattern of changes in HGFs: (1) nicotine causes DNA damage; (2) nicotine affects the activation of the apoptotic pathway; and (3) nicotine induces oxidative stress, which contributes to DNA injury. These findings provide new information that is more important for the therapeutic use of nicotine in smoking cessation than for smoke risk. It is difficult to determine the effect of nicotine when it is inhaled with other substances present in tobacco. The cells of the oral cavity certainly represent the target of some products used to treat smoking addiction, and the evidence that DNA integrity, redox cell balance, and apoptotic process are impaired by nicotine indicates it is a risk factor in the etiology of periodontal diseases and in tumor development, suggesting that its use needs to be carefully evaluated.

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REFERENCES


