

DNA Fragmentation Induced by Cigarette Smoke in Oral Cancer Cells

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Abstract. *Background/Aim:* Cigarette smoke (CS) is the main inducer of oral cancer, increasing the prevalence by 4-7 times. We examined induction of apoptosis by CS exposure of SCC-25 and SCC-15 oral cancer cells. *Materials and Methods:* After controlled exposure to CS of various durations and at different time points we measured DNA fragmentation to assay apoptotic levels. *Results:* SCC-15 cells showed a 70% ($p < 0.05$) increase in apoptotic levels immediately after 30 min of exposure to CS. Twenty-four hours after 30-min CS exposure a further increase in apoptotic levels to 178% ($p < 0.05$) could be observed. However, SCC-15 cells showed a decrease in apoptotic levels immediately after 180-min exposure to CS. CS-exposed SCC-25 cells did not show such CS-related effects. *Conclusion:* SCC-15 and SCC-25 oral cancer cells respond differently to CS regarding apoptotic cell death levels. In this respect, SCC-15 cells are sensitive to CS, while SCC-25 cells are not. Further comparisons between these cells may give insight regarding relationships between CS, apoptosis and invasiveness of oral cancer.

Oral cancer - squamous cell carcinoma, which originates from oral epithelium, is the sixth most common cancer in the world, with approximately 30,000 new cases diagnosed annually in the United States and 200 in Israel. The overall 5-year survival rate for patients is 50% throughout the world, one of the worst for major cancers. Despite therapeutic and diagnostic progress related to this cancer, the disease is characterized by a high rate of morbidity that has not

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changed in the last half century (1-4). Thus, a better understanding of the biological nature of this aggressive disease is mandatory. It has been well established that cigarette smoke (CS) is the main inducer of this cancer, increasing its prevalence by 4-7 times (1-3).

Hence, a research effort focused on basic aspects of oral cancer is required, especially those related to CS (4-7). Previously, we and others have shown that CS exposure results in cellular death (8-10). Apoptosis is intimately related to carcinogenesis. Preliminary cell-cycle analysis suggested an increase in the pre-G₁ fraction of CS-exposed SCC-15 and SCC-25 cells. As the pre-G₁ fraction can be due to apoptosis (11), we were interested to know if the already demonstrated cellular death induced by CS in oral cancer cells is apoptotic in nature. For this purpose, we decided to use two different and widely-used oral cancer cellular models, the cell lines SCC-15 and the SCC-25. Apoptosis is a form of programmed cell death involving a series of biochemical events that lead to a variety of morphological changes, including blebbing, cell membrane changes, such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation leading into multiples of ~200 bp oligonucleosome-size fragments. In the present study, we measured DNA fragmentation (mono and oligonucleosomes) to assay changes in apoptosis rates of SCC-15 and SCC-25 cells exposed to CS in comparison to unexposed controls.

One purpose of the present study was to examine the suitability of these two cell models (SCC-15 and SCC-25 oral cancer cells exposed to CS) to studying the effects of CS on oral cancer cells. Therefore, we applied several durations of CS exposure and measured apoptosis immediately after and at 24 h after CS exposure. This would indicate whether apoptotic cell death induced by CS is a universal process regarding oral cancer cells.

Materials and Methods

Cancer cell lines. SCC-25 and SCC-15 human squamous cells, epithelial carcinoma cells from the tongue commonly used in oral cancer research (human oral squamous cell carcinoma cells from the

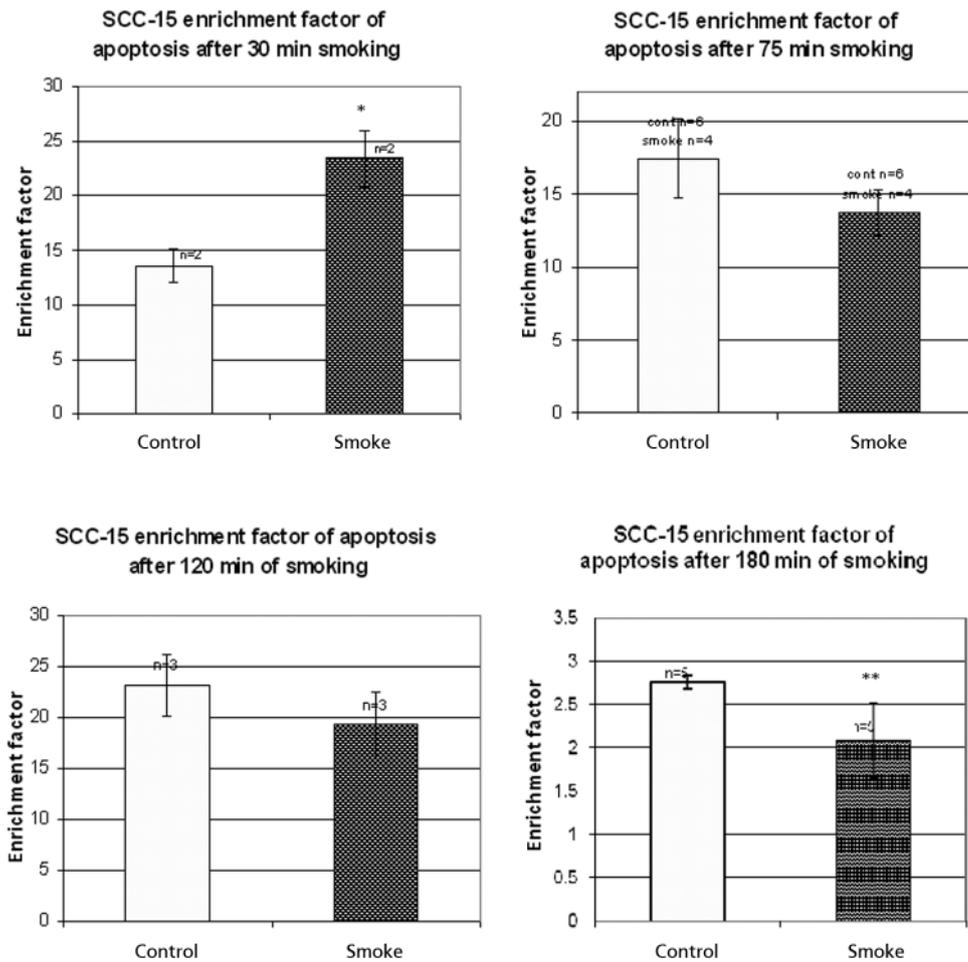


Figure 1. Enrichment factor of apoptosis levels in controls and in CS exposed SCC-15. Cells were exposed to CS (pressure 0.2 bar) for different periods of time (30 min, 75 min, 120 min, 180 min). Values presented are the mean of 6 separate experiments \pm STDEV * $p < 0.05$, ** $p < 0.01$.

American Type Culture Collection, Rockville, MD, USA), were grown in 90% Dulbecco's modified Eagle's medium: Nutrient Mixture (DMEM) Ham's F-12 media. Cultures also contained 10% heat-inactivated fasting blood sugar (FBS), 2.5 mM L-glutamine, penicillin-streptomycin solution (10,000 units/ml penicillin sodium salt and 10 mg/ml streptomycin sulfate) (1% v/v). Cells were grown at 37°C in 95% air and 5% CO₂.

Cigarette smoke exposure. In order to expose cells to CS, a cigarette was combined with a vacuum system to enable the inhalation of gas-phase cigarette smoke into a sealed apparatus containing a Petri dish with the cells in 5-10 ml medium (depending on dish size). The cells had been cultured previously for 3 days until they were confluent. A reproducible vacuum was created in the apparatus and, upon opening the vacuum, a cigarette was lit for approximately 5 seconds. In this way 80-100 ml of cigarette smoke 'puffs' were inhaled into the apparatus with a pressure of 0.2 bar (~150 mmHg). After part of the cigarette had been 'inhaled', the dishes were incubated with the smoke for 15 min at room temperature and then another inhalation was performed. This model mimics physiological

cigarette smoke exposure in smokers' mouths. Cells that were used for controls were subjected to the same procedure but exposed to fresh air rather than CS. The vacuum level was identical.

Detection of apoptosis levels. The Cell Death Kit (Cell Death Detection ELISA^{PLUS} Kit) was obtained from Roche Diagnostics GmbH, Mannheim, Germany, in order to determine the apoptosis levels of the SCC-25 cells. For this assay of DNA fragmentation, 10⁵ cells were seeded in a 24-well plate. After reaching confluent +1 day state, cells were scraped from the wells in their culture medium. The cells were centrifuged (1,000 × g, 10 min), then re-suspended in 1 ml ice cold PBS and transferred to 1.5 ml microvials. The cells were then centrifuged (10,000 × g, 10 min) and the pellets re-suspended with lysis buffer according to the manufacturer's instructions. After a 30-min incubation at room temperature, the lysates were centrifuged (200 × g, 10 min). A fraction of each supernatant (20 µl) was transferred to streptavidin-coated microtiter plate modules. Eighty µl of immunoreagent was added (anti-histone-biotin and anti-DNA-peroxidase in incubation buffer). After incubation with gentle shaking (100 rpm) for 2 h, the

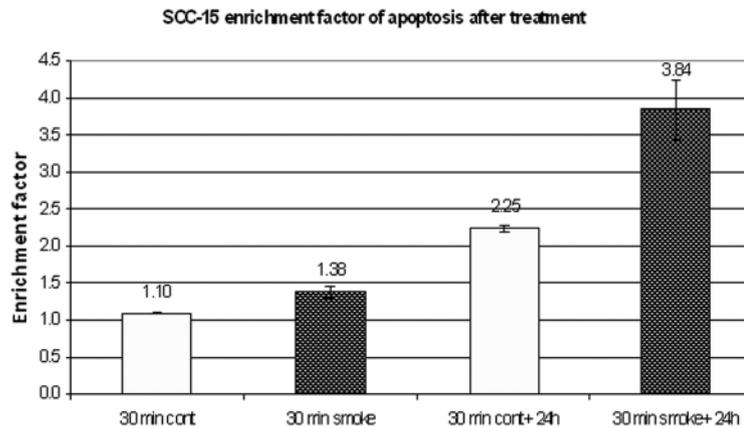


Figure 2. Enrichment factor of apoptosis levels in controls and in CS exposed SCC-15 cells. Cells were exposed to CS for 30 min with/without a following 24-h period of recovery. Values presented are the mean of three separate experiments \pm STDEV. * $p < 0.05$.

modules were washed three times with incubation buffer. The signal for apoptosis was measured following incubation with gentle shaking (50 rpm) for 20 min with 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid (ABTS) solution according to the manufacturer's instructions. The level of staining by ABTS substrate was determined with an ELISA reader (Model; supplier, address). Absorbance of the samples at a wavelength of 405 nm was measured. The reference absorbance (to measure non specific reading) was measured at a 490 nm wavelength.

We calculated the enrichment factor according to the manufacturer's instructions. Enrichment factor = (absorbance of the sample/absorbance of negative control).

Statistical analysis. For statistical analysis, experimental and control groups were at $n \geq 5$. Results are presented as means \pm standard deviation (STDEV). Determination of statistical significance was achieved by using a Student's *t*-test. When required, one-way analysis of variance (ANOVA) was carried-out and the appropriate tests performed. The criterion for statistical significance was $p < 0.05$.

Results

DNA fragmentation – apoptosis rate in SCC-15 cells exposed to CS. We observed a 70% ($p < 0.05$) increase in apoptotic levels following a short (30 min) exposure to CS. A decrease in apoptotic levels after an 180-min exposure to CS was observed (Figure 1) ($p < 0.05$). Figure 2 shows that at 24 h after 30 min of CS exposure there was a further increase in the apoptotic levels of the SCC-15 cells to 178% ($p < 0.01$), compared to the 70% increase seen immediately after 30 min of CS exposure to these SCC-15 cells. However, when compared to the unexposed control cells, the apoptotic level of the CS-exposed cells was still only higher by 70% ($p < 0.05$) at 24 h after 30-min of CS exposure. In this experiment, at the end of the 30 min CS exposure the apoptotic level in the

exposed cells was 25% higher than that of unexposed control cells, and this did not reach statistical significance.

DNA fragmentation – apoptosis rate in SCC-25 cells exposed to CS. The data presented in Figure 3 indicate that apoptotic levels of SCC-25 cells are not affected by CS exposure. Thus, as can be seen in Figure 4, we found that at 24 h after 30 min of CS exposure there was a 3-fold increase in the apoptotic level measured compared to the apoptotic level immediately after 30 min of CS exposure ($p < 0.01$) and the same phenomenon was observed in the controls as well. Accordingly, we concluded that this effect was induced by the delay period of the 24 h rather than by the CS exposure.

Discussion

Apoptosis is intimately related to carcinogenesis in general and we, thus, studied the potential effect of CS on apoptosis of oral cancer cells, as both healthy and diseased oral tissue of cigarette smokers by nature is continually exposed to CS. Our findings showed that 30 min of CS exposure can induce apoptosis in SCC-15 cells but not to a so great extent in SCC-25 cells. This induction of apoptosis is immediate and persists for 24 h after the 30-min CS exposure period. Thus, it appears that, at least in particular types of oral cancer cells, apoptosis can be indeed part of cell death induction by CS. Apoptosis is a form of programmed cell death that, in contrast to necrosis, prevents uncontrolled release of toxins from cells on a cell death pathway (12). As a longer exposure to CS of SCC-15 cells can present lower levels of apoptosis, this is probably due to a bigger proportion of cells undergoing necrosis. Indeed, preliminary unpublished data from our laboratory indicate that exposures of 75, 120 and 180 min to CS result in a decrease in cell viability.

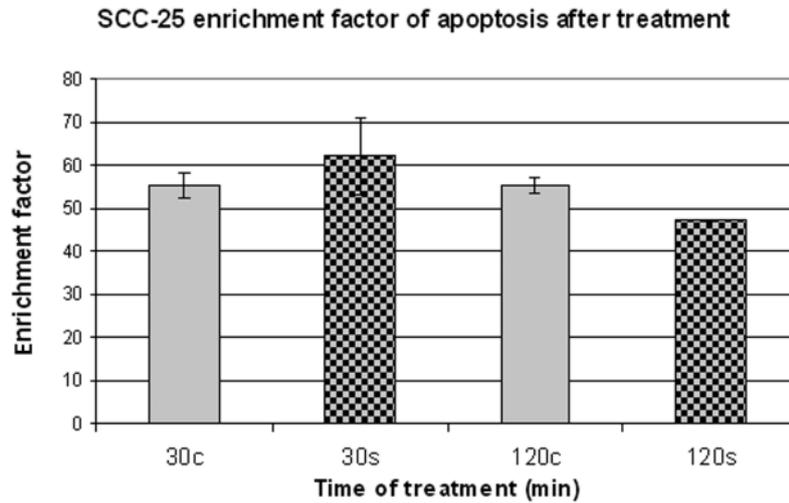


Figure 3. Enrichment factor of apoptosis levels in controls and in CS exposed SCC-25 cells. Cells were exposed to CS for 30 and 120 min (c=control, s=smoke).

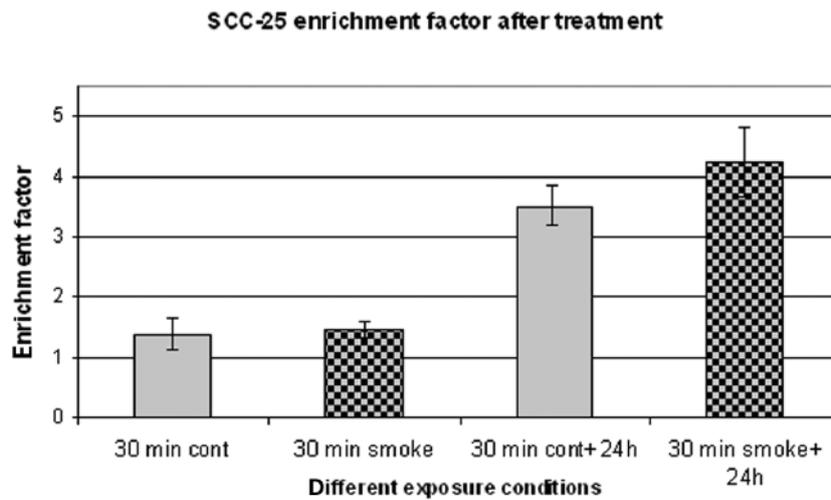


Figure 4. Enrichment factor of apoptosis levels in controls and in CS exposed SCC-25 cells. Cells were exposed to CS for 30 min with/without a following 24-h period of recovery, ** $p < 0.01$.

Our currently presented data demonstrating significant CS-induced increase in apoptosis in SCC-15 oral cancer cells are in accordance with other studies on other cell types. Thus, for example, Park *et al.* (13) reported apoptosis induced by CS in lung fibroblasts (MRC-5 cells), which was also manifested by an increase in caspase-3 and caspase-8. In another study, Xuan *et al.* (14) found that CS induced apoptosis in cells of the human bronchial epithelial cell line BEAS-2B. Furthermore, they reported that CS induced a Bax increase in the mitochondria, mitochondrial membrane potential loss, cytochrome *c* release and caspase-9 activation. Gao *et al.* (15),

who examined the effect of cigarette smoke total particulate matter on two oral squamous cell carcinoma cell lines (101A, 101B) and on normal human gingival epithelial cells, found that CS significantly activated the pro-apoptotic caspase-3 in all three cell types. Chang *et al.* (16) examined the effect of cigarette smoke extract on minimally-transformed oral keratinocytes and found that CS caused chronic depolarization of the mitochondrial membrane potential. They suggested that chronic tobacco exposure can induce carcinogenesis *via* selection of apoptosis resistance and mitochondrial mutation in addition to other genotoxic effects.

Regarding differences between SCC-15 and SCC-25 cells, previous studies have shown that SCC-15 cells are less invasive than SCC-25 cells (17). Possibly, the larger proportion of apoptotic cell death displayed by SCC-15 may contribute to the relatively reduced invasiveness. We suggest that necrotic cell death of SCC-25 cells, which are apparently resistant to CS-induced apoptosis, may be deleterious for surrounding healthy tissue, thus allowing for a relatively rapid expansion of an oral tumor.

Thus, comparisons between SCC-15 and SCC-25, regarding apoptosis and related processes, may give insights into essentials for oral cancer progression and development of tumor-type-specific treatments (one size does not fit all).

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