Carbonyl Levels and Survival Rates in Oral Cancer Cells Exposed to Cigarette Smoke

ELLA KRAYZLER and RAFAEL M. NAGLER

Department of Molecular Pharmacology, Medical Faculty, Technion-Israel Institute of Technology, Haifa, Israel

Abstract. *Background: Cigarette smoke (CS) is the main inducer of oral cancer, increasing prevalence 4-7 times. Materials and Methods: We examined SCC-25 and SCC-15 suitability for studying CS effects on oral cancer cells, measuring carbonyl levels for free radical-mediated CS effect on survival and time/CS dependence. Results: Protein oxidation increased significantly during CS exposure. At all time points, carbonyl levels increased six-fold ($p<0.001$) in both cell lines. Cell viability decrease was time-dependent. Longer CS exposure led to higher cell mortality. At 120 min, SCC-25 cell survival reduction was 43.7%, ($p<0.01$). Propidium iodide (PI) assay results matched the Trypan blue assay showing a time-dependent cell viability decrease following CS exposure. At 120 min, cell survival reduction was 37% ($p<0.05$). Conclusion: Cell death is mediated by CS free radicals with pathological process occurring first. Oral cancer cell models SCC-25 and SCC-15 are suitable for studying CS-induced free radical-related damage, potentially leading to the pathogenesis of oral cancer.*

Oral cancer-squamous cell carcinoma (SCC) is the sixth most frequent cancer in the world with approximately 30,000 new cases diagnosed annually in the United States. The overall 5-year survival rate for patients is 50%, among the lowest for major cancers, and has not changed during the last half century. Despite therapeutic and diagnostic progress related to this cancer, the mortality rate remains unchanged and the disease is characterized by a high rate of morbidity. Thus, a better understanding over the biological nature of this aggressive disease is mandatory. In has been well-established that cigarette smoke (CS) is the main inducer of this cancer, increasing its prevalence by 4-7 times (1-3).

Correspondence to: Professor Rafael Nagler, Department of Molecular Pharmacology, Rappaport Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Bat Galim, Haifa, 31096 Israel. Tel: +972 77886687, Fax: +972 46541295, e-mail: nagler@tx.technion.ac.il

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The purpose of the current study was to examine the suitability of two possible cell models for studying the effects of CS on oral cancer cells. We intended to examine whether CS significantly affects the survival rate of oral cancer cells in two widely used but different oral cancer cell lines, SCC-25 and SCC-15, by using 2 different viability assays: Trypan blue and Propidium iodide (PI) exclusion assays. By measuring the carbonyl levels in both cell lines, we examined whether the CS effect on survival is mediated by a free-radical attack and whether the effect is time/CS-dependent, along a 2-hour period of exposure to CS.

Materials and Methods

Cancer cell lines. In this study, we used SCC-25 and SCC-15 human squamous cells, which are epithelial carcinoma cells from the tongue. These SCC-25 and SCC-15 cell lines (SCC-15 ATCC # CRL-1623 and SCC-25 ATCC # CRL-1628, respectively, both from American Type Culture Collection, Rockville, MD, USA:) were grown in 90% Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 media. Cultures also contained 10% heat-inactivated fetal bovine serum (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% CO_2.

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 SCC-25 or SCC-15 cells in 5-10 ml medium (depending on dish size). 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.

Detection of protein oxidation-carbonyl levels examination. In the current paper, we used the ELISA method (protein carbonyl enzyme immuno-assay kit biocell Corp., City, New Zeeland), which enables carbonyl to be measured quantitatively with microgram quantities of protein. Control and CS exposed cells were scraped from their dishes, centrifuged (1,000 ×g for 10 min at 4˚C) and the pellets suspended

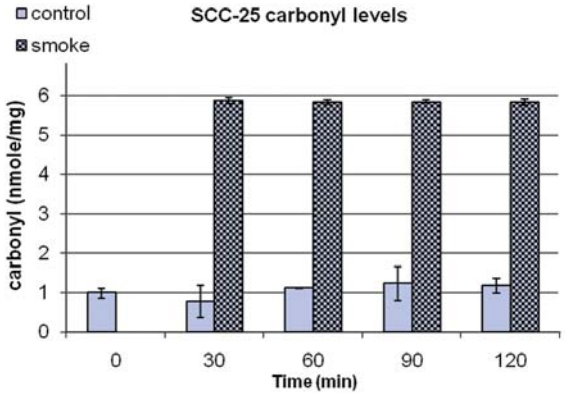

Figure 1. *Carbonyl levels measured by Biocell PC test (protein carbonyl enzyme immuno-assay kit). p<0.0001 SCC-25 control cells versus not CS exposed cells (n=3).*

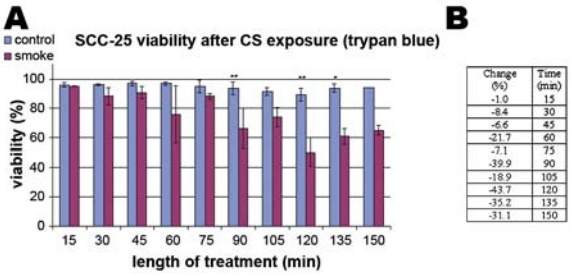

Figure 2. *SCC-25 cell viability after CS exposure (pressure of 0.2 bar) as measured by the Trypan blue exclusion. Percent of live cells (panel A), change in viability of the treated cells versus control cells (panel B). Values shown are the mean of 2-5 separate experiments±STDEV. *p<0.05 and **p<0.01 versus cells not exposed to CS.*

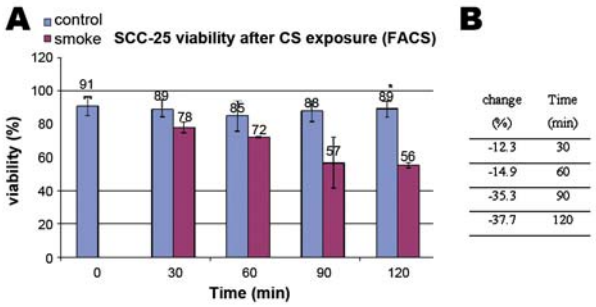

Figure 3. *SCC-25 cell viability after CS exposure (pressure of 0.2 bar) as measured by PI exclusion using FACS. Percentage of live cells (panel A), change in viability of the treated SCC-25 cells versus control cells (panel B). Values shown are the mean of 4 separate experiments±STDEV. *p<0.05 versus cells not exposed to CS.*

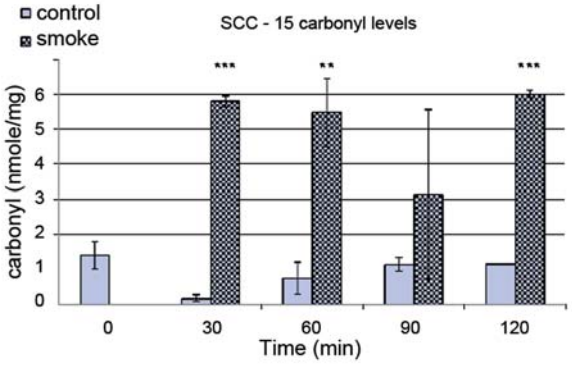

Figure 4. *Carbonyl level measured by the Biocell PC test (protein carbonyl enzyme immuno-assay kit). **p<0.01, ***p<0.0001 SCC-15 control cells versus not CS exposed cells (n=3).*

in 150 μl lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 10% glycerol, pH=7.8). After 30 min incubation at room temperature, the samples were centrifuged (11,000 ×g for 10 min at 4˚C) and their supernatants were frozen at –20˚C. On the day of carbonyl analysis, the supernatants were thawed and their protein concentrations were determined by the Bradford method (4) using bovine serum albumin (BSA) as a standard. A volume representing 20 μg was transferred to a 1.5 ml microvial and with the addition of water high pressure liquid chromatography grade (HPLC) all samples were brought to the same volume of 100 μl. We added 0.8 volumes of ice cold 28% trichloroacetic acid (TCA), mixed well and after 10 min incubation on ice the tubes were centrifuged (10,000 ×g, 3 min, 4˚C). Supernatants were carefully aspirated without disturbing the pellet. Five μl of EIA buffer (1 M phosphate solution containing 1% BSA, 4 M NaCl, 10 mM EDTA and 0.1% sodium azide) and 15 μl diluted 2,4-dinitrophenol (DNP) solution (according to the manufacture's instructions) added to samples as instructed by the manufacturer. After 45 min incubation at room temperature, 5 μl was taken to a parallel set of 1.5 ml microvials containing 1 ml EIA buffer. The solutions were mixed well and 200 μl of each sample was added to assigned ELISA-plate wells. The plate was covered and left overnight at 4˚C. The next day, the plate was washed with EIA buffer (3×250 μl per well) and 250 μl of diluted blocking solution (provided by manufacturer) was added per well. After 30 min incubation at room temperature, the wells were washed as described above and 200 μl of diluted anti-DNP-biotin-antibody was added per well and incubated for 1 hour at 37˚C. The plate was washed as described above and 200 μl of diluted streptavidin-HRP was added per well. After 1 hour of incubation at room temperature, the plate was washed as described above. In order to obtain color development, 200 μl of chromatin reagent (provided by manufacturer) per well and after 5 min 100 μl per well of stopping reagent were added. Absorbencies of the samples were measured at the wavelength 450 nm directly after the addition of the stopping reagent using a (model) plate reader (supplier and address).

The same procedure was followed for standards and controls provided by the manufacturer in order to create a standard curve. Carbonyl content of the samples (nmole/mg protein) was calculated from a standard curve made of 5 oxidized protein standards also provided by the manufacturer.

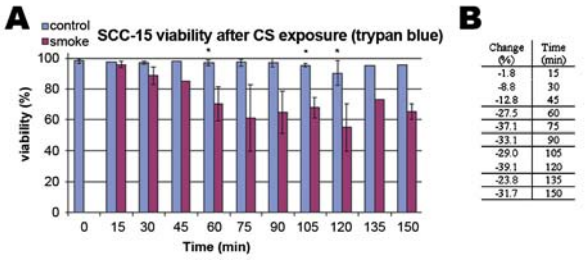

Figure 5. *CS effects (pressure 0.2 bar) on the SCC-15 cell line viability by Trypan blue exclusion. Percentage of live cells (Panel A), change in viability of the CS exposed cells versus control cells (Panel B). Values shown are the mean of 2-5 separate experiments ±STDEV. *p<0.05 control SCC-15 versus cells not exposed to CS.*

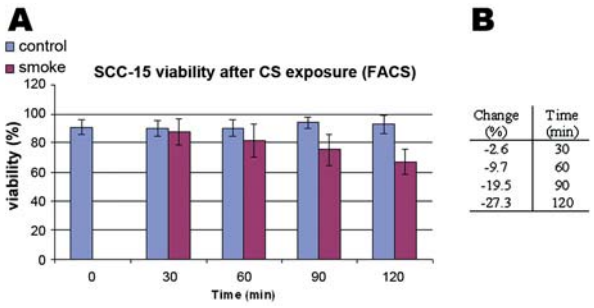

Figure 6. *CS effect (pressure 0.2 bar) on cells viability by PI exclusion with the aid of FACS analysis. Percentage of live cells (panel A), changes in viability of the treated cells versus control cells (panel B). Values shown are the means of 4-6 separate experiments±STDEV.*

Viability measurements-Trypan blue and Propidium iodide (PI) exclusion assays. For cell viability, control and treated cells were harvested by trypsinization, centrifuged (1,000×g, 10 min, 4˚C) and re-suspended in 0.5 ml PBS containing 8 μg/ml PI and 10 μg/ml RNase. After 10 min incubation at 4˚C, routinely 10,000-30,000 cells were collected per assay and analyzed in a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Fluorescence-assisted cell sorting (FACS). Cells were trypsinized and centrifuged (1,000 ×g for 5 min at 4˚C) and the pellets re-suspended in 4 ml PBS and centrifuged (1,000×g for 5 min at 4˚C). The pellets were re-suspended in 475 μl of a solution containing 0.3% saponin, 50 μg/ml RNase, 5 mM EDTA pH=8; all the components dissolved in PBS (pH=7.4). After 30 min incubation at room temperature in the dark, 50 μl PI was added (0.5 μg/ml). After 10 min incubation in the dark, cells were vortexed briefly before FACS analysis. The flow cytometer used was as described above. For each assay, 15,000 cells were randomly collected.

Statistical analysis. For statistical analysis, experimental and control groups were at n≥5. Results are presented as means±standard deviation (STDEV). Determination of statistical significance was performed 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

Carbonyl levels in SCC-25 cell line following CS exposure. Protein oxidation, as measured by protein carbonyl content, increased significantly during the CS exposure. The results shown in Figure 1 are highly significant and they substantiate the assumption that CS mutagenic effects are mediated via reactive oxygen species (ROS). At all time points measured along the 2-h experiment, following the start of CS exposure, carbonyl levels increased by approximately six times ($p<0.0001$).

Trypan blue and PI exclusion results in SCC-25 cells following CS exposure. A time-dependent decrease in cell viability was observed (Figure 2). The longer the CS exposure took place, the higher the SCC-25 cell line mortality was noted. After 60 min of exposure, the difference become substantial and at 90 and at 120 min exposures exhibited highly significant changes ($p<0.01$) between exposed and control cells. At 120 min, the reduction in the SCC-25 cell survival was 43.7% ($p<0.01$). The results obtained with the PI assay were in accordance with the Trypan blue assay showing a time-dependent decrease in cell viability following CS exposure and at 120 min when the reduction in cell survival was 37% ($p<0.05$) (Figure 3).

Carbonyl levels in SCC-15 cell line following CS exposure. Protein oxidation, as measured by protein carbonyl content, increased significantly during the CS exposure. The results shown in Figure 4 are highly significant and they substantiate the assumption that CS mutagenic effects are mediated via reactive oxygen species (ROS). At all time points measured along the 2-h experiment, following the start of CS exposure, carbonyl levels increased by approximately six times ($p<0.001$).

Trypan blue and PI exclusion results in SCC-15 cells following CS exposure. A significant time-dependent decrease in cell viability following exposure to CS was noted. The longer the CS exposure took place the higher the SCC-15 cell line mortality was noted (Figure 5). After 60 min of exposure, the difference became significant and at 120 min the reduction in the SCC-15 cell survival was 39% ($p<0.05$). The results obtained with the PI assay were in accordance with the Trypan blue assay showing a time-dependent decrease in cell viability following CS exposure and at 120 min when the reduction in cell survival was 27%, although it did not reach statistical significance (Figure 6).

Discussion

Oral cancer-SCC is the sixth most common malignancy worldwide and kills one person every hour (5). Oral cancer originates from oral epithelium and represents 2-3% of all malignancies worldwide. Despite therapeutic and diagnostic progress related to this type of cancer, the mortality rate remains unchanged and the disease is characterized by a high rate of morbidity (6-9). Thus, a better understanding of the biological nature of this aggressive disease is imperative. We performed cell survival analysis in two widely-used oral cancer cell lines exposed to CS, using two different methods (10-12).

The determination of cell viability is important in tissue cultures, especially following aggressive and non-aggressive treatments. There exist several accepted assays of viability that utilize the exclusion of certain dyes by live cell membranes (13). However, there are problems with these routine staining procedures that limit their usefulness. Accordingly, we chose to use the Trypan blue exclusion, which is the most common assay for cell viability measurement, although it may be inaccurate in the identification of dead cells. Cells must be counted within 3-5 min because the number of blue-stained cells increases with time (14). Where large number of samples have to be counted, it may be inconvenient to perform the assay using this method. Instead, certain fluorescent dyes appear more reliable indicators of cell viability than the traditional dyes. Propidium iodide (PI) is known to pass through only the membranes of dead or dying cells (13-21). Thus, the additional method chosen to evaluate cell mortality was PI exclusion, assayed by a computerized method with the aid of FACS. This method is based on the same principle as the Trypan blue assay. It binds DNA by intercalating with the bases with little or no sequence preference and with a stoichiometry of one dye molecule per 4-5 base pairs of DNA and it is, thus, used to differentiate necrotic and apoptotic cells from healthy cells.

We used cigarette smoke as a free radical model. Oxidative damage to proteins is known to occur *via* conversion of side chain amino groups to corresponding carbonyl derivatives. Carbonyl derivatives of some amino acid residues are among the products of oxygen radical damage (20-24). Total carbonyl contents in SCC-25 and CSS-15 cell lines were used as a biomarker of protein oxidation.

The conclusion from both viability assays is that CS causes significant cell mortality in both oral cancer cell lines employed. The longer the exposure time the more cells die from this exposure as, within 2 h, the survival rate of the cells dropped by approximately 40%. There was a good match between the results from both the Trypan blue exclusion and the PI exclusion assays. This time-dependent reduction in cell viability corroborates effects seen in other cells exposed to CS (25). Interestingly, a very profound increase in carbonyl levels, by 6-fold, was demonstrated already at 30 min, which was the first point examined. Accordingly, one may assume that this increase occurred long before the first examination point, possibly immediately as the cells were exposed for the first time to CS. However, the survival loss of the cells was noted for the first time only at 60 min of exposure to CS and gradually increased with the time of exposure. In this regard, the Trypan blue assay seems to be slightly more sensitive than PI exclusion assay for measuring the death toll of the cells.

In summary, the presented results suggest that cell death is mediated by free radicals born in the cigarette smoke, while the pathological process leading to cell death must first take place before it occurs or, at least, before it can be noted. Furthermore, our results certainly support the suitability of the 2 oral cancer cell models, SCC-25 and SCC-15, for free radical-related damage induced by CS, potentially leading to the pathogenesis of oral cancer.

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