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Oral cytology assessment by flow cytometry of DNA adducts, aneuploidy, proliferation and apoptosis shows differences between smokers and non-smokers☆

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Summary Oral cytology and morphometric staining is used to identify malignant keratinocytes in oral premalignant or malignant lesions. To detect and to begin to assess changes in oral keratinocytes exposed to tobacco-derived carcinogens, which are at risk for malignant transformation, a novel method is required. The approach uses oral cytology harvested oral keratinocytes analyzed using flow cytometry (FC) for changes in DNA content, damage, cell cycle and apoptosis. Six smoker and six non-smoker oral keratinocytes were evaluated using flow cytometry in the form of laser scanning cytometry (LSC) and laser microdissection (LMD). Among smokers compared to non-smokers, the method detected and assessed DNA damage from tobacco smoke exposure quantifying an enhanced formation of DNA adducts, such as, 8-hydroxy-2′-deoxyguanine (8-OHdG) which creates oxidation lesions and benzo[*a*]pyrene(B[*a*]P), which produces a B[*a*]P)-N2-dG bulky adduct. Increased DNA content, aneuploidy, percentage of cells in synthesis (S) and G_2+Mitosis (M), and apoptosis were recorded. Tissue and cell controls were used to verify these relationships. Data suggested healthy smokers were at increased risk for malignant transformation of oral keratinocytes because of the changes stated above. Using identical methods, keratinocytes exposed to the tobacco derived carcinogen, B[*a*]P parallel results obtained from smoke exposure indicating a direct link. Flow cytometric evaluation of oral cytology harvested keratinocytes can be used to measure exposure to tobacco carcinogens, and possibly establish a link to premalignant and malignant transformation before a lesion is noted.

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Introduction

A number of techniques are used to study the genetic and molecular abnormalities of oral keratinocytes in premalignant and malignant oral lesions but no oral cytology method has been validated to assess risk for oral cancer before a lesion is present. In this study an assessment of flow cytometric approaches were evaluated to determine utility for detecting important precursor biologic change associated with increased risk for malignant transformation among oral keratinocytes harvested from healthy smokers and non-smokers. For example, cytomorphometric analysis is used to determine changes in the cytoplasmic areas of oral keratinocytes obtained from premalignant or malignant oral lesions and the cells are evaluated for hyperchromatism, nuclear-cytoplasmic reversal, chromatin clumping, pleomorphism, and anaplasia.[1–5] The keratinocytes from lesions are also evaluated for metachromatic staining using nuclear dyes (e.g., dissociating keratotic cells derived from the stratum corium stain as more red than keratotic cells obtained from the proliferative zone which stain bluer).[6,8] Cytomorphometrics has been used to assess ''field change'',[6] and was reported to detect premalignant, dysplastic oral lesions. Other studies have used increased counts of nucleolar organizer region proteins associated with ribosomal genes (AgNOR) to detect the risk for malignant lesion presence and a correlation has been described between flow cytometry DNA index and identification of AgNOR in dysplastic and oral carcinomas.[7] Previous studies using oral cytology, analyzed nuclear, and metachromatic staining produced a wide variation in quantification among pathologists. Variability among prevalence or specificity of cytomorphometrics changes and risk for malignant change were noted.[3,7–9] One disadvantage to the visual assessment technique of oral cytology has been a high degree of subjectivity. Visual assessment is used to count usually fewer than 1000 morphometric, metachromatic stained cells, micronuclei or immunofluorescent stained cells.

A recent innovation and improvement has been the use of computerized-assisted analysis.[10,11] Computerized scanning of a cytology sample and analysis of the cytophotometric and nuclear staining increases precision and sensitivity detecting cytomorphometric and nuclear staining changes reducing visual identification disparity.

Other cytological approaches have used combined marker analysis to link responses to smoking and the presentation of malignant oral cancers. These evaluations have recorded increases in the rate of cellular proliferation, argyrophilic nucleolar organizer regions, and apoptosis.[12–16] In Asian or African populations with endemic use of smokeless tobacco the appearance among oral keratinocytes from lesions has included chromatin clumping, micronuclei formation, and nuclear dry mass determinations.[17] In healthy tobacco smokers and tobacco-areca nut chewers in India increased frequencies of micronuclei in the oral keratinocytes has also been recorded.[18–19] Another type of Indian tobacco, chutta (rolled tobacco), was associated with a high percentage of oral keratinocytes in lesions showing micronucleation and DNA adduct formation.[20] An elevation of micronuclei among nearly all tobacco chewers with a heavy keratotic lesion has been observed.[21] Despite these findings, the utility of such measurements for effective population-based pre-screening of at risk individuals has not been addressed.

Successful determination of patients at risk for malignant phenotype development requires the identification of non-apoptotic oral keratinocytes, which have the characteristics of increased cell proliferation, DNA adduct lesion formation, faulty repair, a diploid DNA content but with increasing numbers of cells with increased DNA content (aneuploid).[22] Exposure to tobacco carcinogens can initially produce ''non-genotoxic and aneuploidizing'' changes [23] and a damaged cell exhibiting bulky and oxidation DNA adduct.[24,25] Cells also appear to have faulty, depressed DNA repair enzyme activity (e.g., OGG1, and NTH1) and increased proliferation.[26,27]

Ploidy change precedes nuclear instability manifestations.[22,23,28] This precursor stage contains aneuploid and diploid cells but the aneuploid population is less capable of surviving the induction of apoptosis, division during nuclear instability, and is not likely to produce diploid progenitors.[29–31] Oral carcinomas have demonstrated a heterogeneous distribution of diploid and aneuploid cells. In oral squamous cell carcinoma the aneuploid cell is more commonly associated with metastatic spread.[32,33]

The precursor malignant cell, exhibiting nuclear instability (e.g., non-random cytogenetic aberrations, mutations in tumor suppressor genes, and changes in histone-chromatin associations [34,35]) is derived from a damaged diploid population containing also aneuploid cells.

Flow cytometry has measured DNA content and ploidy in cells from patients with oral premalignancy. [36,37] Aneuploidy was found among keratinocytes derived from dysplastic oral leukoplakias, which preceded and was predictive of subsequent oral cancer after several years of fol-

low-up. Similarly, aneuploidy was associated with poor prognosis in patients with oral cancer. [22]

In apparently healthy oral mucosa from smokers a single sample of oral keratinocytes can be evaluated using flow and laser cytometry. This study tested the hypothesis that continuous and chronic exposure to tobacco smoke will increase the percentage of oral keratinocyte containing DNA adducts; aneuploidy, DNA content; enhanced cell proliferation, and the induction of apoptosis. The presence of these changes was evaluated in parallel with identical methods to evaluate flow cytomeric changes in premalignant and malignant oral tissues and keratinocytes exposed to tobacco-derived carcinogen.

Materials and methods

Oral cytology method

Oral keratinocytes were harvested using a ''CytoSoft'' brush (Cytology Brush, Medical Packaging Corp., Camarillo, CA) from six smokers (of at least one pack per day) and six non-smokers. Each patient first rinsed with phosphate buffered saline (pH 7.4)(PBS) once to remove loose surface cells and decrease the level of bacteria. The initial brushing was also discarded because it contained primarily the superficial stratum corium oral keratinocytes which was found to be $>70\%$ apoptotic. This brush was selected because with continual brushing cells from deeper layers of the mucosa can be obtained without the appearance of an ulcer or any pain or discomfort to the patient. Cells from the right and left buccal mucosa were brushed at least 10 times in the identical site. After each brushing the cells were immediately placed into a 15 ml conical tube containing phosphate buffered saline, pH 7.4. (PBS, $1\times$). The right and left lateral borders of the tongue were also brushed to obtain tongue keratinocytes. These areas were selected because of the relatively high incidence of oral carcinoma identified in this site. To reduce the heterogeneity of cells from two anatomic sites the cells harvested from the tongue were separated from the buccal samples. Immediately following the last brushing the patient rinsed once with PBS and expelled the fluid into the identical tube containing the cells harvested by the brushing procedure. These cells were then centrifuged at 800 rpm at 23 °C. The pellet of cell was then used for the assays described. Cells were immediately fixed with 1% paraformaldehyde and processed. There was no storage of unfixed or processed cells for oral cytology analysis.

Cytomorphometric analysis of oral cytology

Following harvest the cells were gently centrifuged at 900 rpm and the supernatant was removed. One problem, which arose in some samples, was the lack of pellet formation because of the mucous characteristic to the saliva. In these instances, the cells were allowed to strand on ice for 10–20 min to allow for further compaction of the pellet. The saliva supernatant was removed by decanting in one single motion. About 96% of the supernatant was removed in these cases. The sample was then washed at least $2\times$ gently by adding PBS $1\times$. The cell pellet was finally suspended in 1.0 ml. of PBS $1\times$. The viability of the cells was initially determined using trypan blue dye exclusion (0.24%). The cells were counted using a hemocytometer and the mean number of viable cells counted for the smokers were 5.2×10^4 cells per 20 μl (2.6×10^6 cells/ml) The mean cell count for the non-smokers was 4.6×10^4 cells per 20 μl (9.2×10^6 cells/ml). The viability varied for the smokers from 75 to 84%, and for the non-smokers the viability was 88–95%. Among the nucleated cells from smokers 16–25% of the cells were non-viable. $>80\%$ of nucleated cells were viable cells. Nucleated cells are also non-viable in the non-smoker population but only 5–8%. The cytomorphometric analysis of harvested oral cells placed on to coated (lysine) cytospin slides after centrifugation at 900 rpm. Groups of 100 cells were counted for each nuclear staining. A percentage of these cells are calculated for staining positivity. One aliquot of cells were stained using the vital dye, Toluidine Blue or the metachromatic Papanicolaou stain. The metachormatic stain-ability of cells from non-smokers mucosa samples from the stratum spinosum (upper mucosa layers) in comparison to keratinocytes derived from stratum basilis layers (lower layers) was noted to produce upper region positive staining (80–90%, red hue stains) and lower region staining (10–20%, blue hue stains) (nucleated cells: 60–85%). Among samples obtained from smokers, 40–60% showed upper mucosa staining and 40–60% of the keratinocytes showed lower region staining (nucleated cells: 90–98%). Both stains provide similar morphologic results and are commonly used to assess the presence of abnormal, histomorphology of premalignant and the identification of micronucleus changes (250 cells counted) of the chromatin. Two investigators provided counts for the identical slides without knowledge of smoking status. The analysis for visual morphometric determination was assessed using kappa statistics differences. Histomorphometric analysis included the nuclear cytoplasmic reversal, hyperchromatism, pleomorphism,

anaplasia, bizarre mitoses and the number of keratotic lesions (e.g. stained red). To quantify these criteria a score is used and is composed of 0, 1 to 5 indicating 0 relative% of cells exhibiting the feature, 1=(1—20%), 2=(20—40%), 3=(40—60%), 4=(60—80%), and 5=(80—100%). Kappa statistics for histomorphometric analysis was 8—10%.

Detection of DNA adducts using immunohistochemistry

An aliquot of cells was counted as described above and stained using the antibodies (1:50) to the DNA adducts 8-hydroxy-2′-deoxyguanine (8-OHdG) (clone:MC-1001) and polyclonal antibody benzo[*a*]pyrene - 7,8 - dihydroxy - 9,10 - oxide(B[*a*]-P-adduct) (Trevigan,CA) for 1 h at 23 °C. The antibody bound to the adduct present in oral keratinocytes is detected with a secondary fluorescent antibody (FITC) (1:200) for 1 h at 23 °C. The numbers of positive stained cells (No. cells/100=%) are examined in an Olympus Fluorescent Microscope. Controls for this staining have included a primary antibody of the identical IgG isotype control and using another antibody to CD4, which is not present on oral keratinocytes. This method has been repeatedly reported to detect DNA-adducts following exposure to tobacco.[38] Previous studies using the identical antibodies have indicated antibody recognition of the number of bases per diploid genome (10^6 nucleotides for the 8-OhdG; 10^{10} nucleotides for B[*a*]P-DNA adduct). [39] The initial visual count of at least 100 cells was performed in a blinded manner. The kappa statistics difference was 12—18%. This method was repeated with laser scanning cytometry, which also provided a blind histometric analysis for DNA adduct formation but reduced the kappa statistic variance to 2—5%.

Flow cytometric analysis:DNA Content

The same aliquote of cells obtained for cytomorphic adduct analysis was analyzed for DNA content (>in the laser scanning cytometer following the immunoflouresecent staining. The procedure used was as follows: 10—20,000 cells were gated for each analysis. The cytometric data included nuclear content (e.g., DNA) staining with propidium iodide (PI) (1 μg/μl) with Triton X-100, 1 h at 23×C. This method stains isolated nuclei. Using laser scanning cytometry analysis the data was presented as a scatter plot with dual staining for DNA content (Sigma chemical, St. Louis, Co.) (PI, stains red) and (fluoresceinated isothiocynate conjugated anti mouse antibody (1:200), Sigma chemical, St. Louis) FITC, detection by the primary antibodies of either DNA adduct, 8-OHdG or B[*a*]P-adduct.

The DNA content is an assessment of the DNA amount 1N →4N. 2N is designated diploid and >2N is aneuploid. Inconjunction a ''DNA Index'', (D.I.) of 1.0 is indicated to be ''indistinguishable from diploid'' (MultiCycle for Windows, Phoenix Flow Systems, San Diego, CA) and a D.I. of 2.0 could be a G_2 cell, a tetraploid cell, or an aneuploid cell, which has abnormal chromosomes and is termed ''indistinguishable from tetraploid''. A determination of the G_2 diploid population is required to dissociate these cell populations. This is accomplished using cell cycle analysis (see below). The aneuploid ('DNA-aneuploid') designated cell is described here because aneuploidy is considered to be a marker for premalignant and malignant change and an indicator of DI differences in each cell cycle determination.

Cell cycle analysis

Using compatible computer language developed for us by Phoenix Flow Systems, San Diego, CA. The normalized DNA content data obtained from the laser scanning cytometer (Compucyte, Boston) was analyzed, and interpreted in the flow cytometer for cell cycle determinations (Beckman Coulter, Epics XL) (see Fig. 1). This analysis includes G_1, S, and G_{2+}Mitosis with the% of cells provided from at least 10,000 gated cells. The percent of cells that were apoptotic was also assessed from a hypodiploid wave which is pre-G_0/G_1. Fragmented nuclear DNA, number and sizes of DNA nuclei were evaluated and quantitated for apoptosis using a DNA fluorescence histogram. The cells for these discriminators are diploid and the G_2/G_1 ratio is fixed to reduce variability (below 1.9). The ''Background, Aggregate, and Debris (BAD)'' is eliminated from these calculations and is observed to be consistently, ⩽0.01% of the quantified population detected (Beckman Coulter, FL).

Control for flow cytometry: analysis of oral keratinocytes harvested from tissue sections

Two of the authors (WG, TX) provided paraffin sections from 13 patients (ages 31—82 years and all were smokers) with oral leukoplakia and/or squamous cell carcinoma lesions. The lesions were from the lateral border of the tongue (*n*=5) or floor of the mouth (*n*=8). Nine specimens contained carcinoma and 13 contained mild to moderate dysplasia. Paraffin embedded and hematoxylin stained tissues sections provided oral keratinocytes using a laser

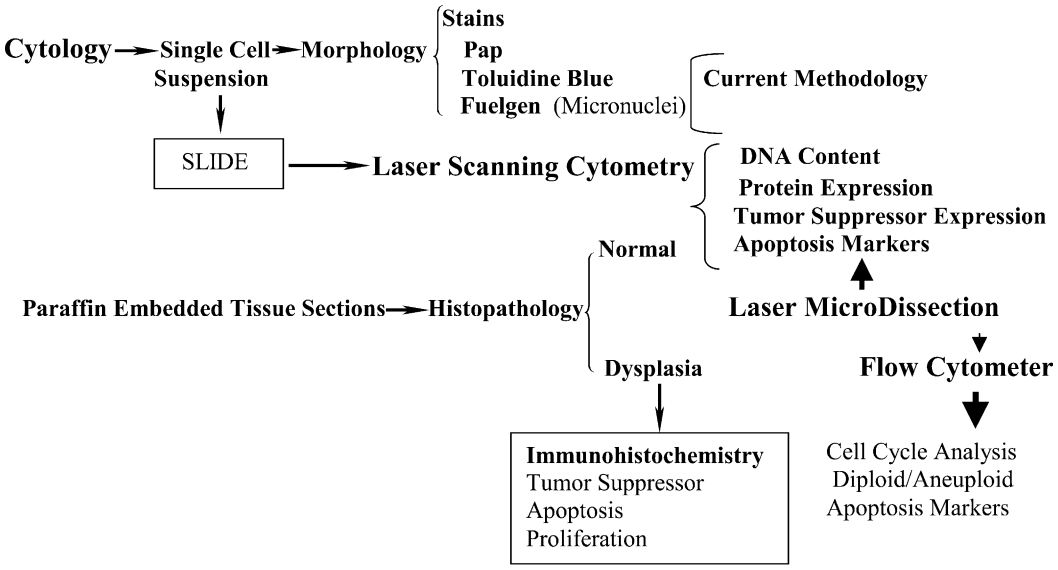

Figure 1 Scheme for the combined use of flow cytometry instrumentation to enhance the level of detection for early genetic and molecular changes found in the oral keratinocytes from smokers of tobacco.

microdissector (Arturus, CA). A single cell suspension of cells from dysplastic or normal sites was obtained. Cell suspensions from the microdissected tissue sections following treatment with trypsin+EDTA (0.25%), centrifugation in a cytocentrifuge tube (900 rpm) washing in phosphate buffered saline (pH 7.4), precipitation, resuspension were placed onto a cytospin slide and stained for immunohistochemical markers.

Immunohistochemical detection of markers for oral keratinocytes harvested from tissue sections

Slide preparations of single cell preparations of oral keratinocytes harvested from normal or dysplasia and carcinoma sites were stained for DNA content using propidium iodide (1 μg/ml) in Triton-X-100 solution, tumor suppressor [p.53, clone DO-7 (NeoMarkers, Fremont, CA), apoptosis markers (Apo2.7, Immunotech, Beckman Coulter, Hialeah, FL)] and differentiation (cytokeratin, clone: AE-1, NeoMarkers,Fremont,CA). A two-sample *T* test was used to determine the level of significance between calculated levels of staining between normal or dysplasia and carcinoma tissue sites. The number of sites recorded for each marker was at least 10 for each slide from each patient.

Immunohistochemistry of tissue sections

Paraffin embedded tissues sections were deparaffinized using xylene and 100 to 10% alcohol submersion for 1 min for each slide. The primary immunohistochemistry staining of the paraffin embedded tissue sections with normal, dysplastic and carcinoma sites were analyzed for cellular proliferation using proliferating cell nuclear antigen, PCNA (clone: PC10, NeoMarkers, Fremont, CA), tumor suppressor activity of p53 expression (clone: Pab240, NeoMarkers, Fremont, CA), apoptosis using TUNEL (ENZO Diagnostics, Farmingdale, NY) and inhibition of apoptosis using Bcl-2 (clone: 10CA). Horseradish peroxidase was used to detect changes in expression of the primary antibody using a secondary antibody of goat anti-mouse (1:1000). The software ''Image Pro Plus' was used to obtain values which reflect intensity and number of positive stained spots in the field. To quantify the number of positive stained objects and the total number of objects in the field (200×) expressing an intensity that is designated to be a positive stained object was assessed. All results were derived from at least three random sites from each slide.

Laser scanning cytometry analysis

Thirteen biopsy samples were prepared for cytospin slide, fixation, and staining with a chromogenic detector FITC (green) labeled goat anti-mouse antibody or phycoerythrin-labeled (red) anti-mouse antibody was applied. Double staining is normally performed for all cell preparations to reduce time, cost, and workload. Double staining also enhances analysis with scatter-plot distribution assessments. Laser scanning cytometry of stained cells cell staining used threshold contouring. Samples were performed in triplicate, and a coefficient of variation is assessed for each sample. A Student's T test was used to determine significance between normal and dysplasia and carcinoma

In vitro keratinocyte exposure to benzo[*a*]pyrene

To further evaluate the relationship between exposure to tobacco carcinogen in a non-malignant keratinocyte and flow cytometric analysis for adduct formation an in vitro assay was conducted. A non-tumorigenic, immortalized (human papilloma virus, E6/E7) keratinocyte cell was exposed to tobacco carcinogen, benzo[*a*]pyrene (B[*a*]P). The keratinocyte mucosa cell line, SK-ECTO [29] was treated with benzo[*a*]pyrene (B[*a*]P) or benzo[*a*]-diol epoxide (B[*a*]P-DE) (1.0 μM for 48 h). These chemicals were gifted from Dr. Shantilal Amin, American Health Foundation. The expression of hydroxylated DNA adducts such as 8-OhdG and B[*a*]P-DNA adduct, and DNA content, diploid/aneuploid percentage was ascertained using flow cytometry. All treatments and stains were performed in triplicate and compared to the untreated cell.

General scheme for oral cytology and flow cytometric analysis

In Fig. 1, the general scheme for the processing of oral cytologic samples obtained from healthy, non-lesion bearing (6) smokers and (6) non-smokers are presented. The initial histomorphologic assessment, current methodology uses classical nuclear stains to detect B[*a*]P-N2-dG bulky adduct and oxidation lesion 8-OHdG. An identical aliquot using a laser-scanning cytometry and/or flow cytometric approach verified these DNA lesions. This analysis as demonstrated below provides data for DNA damage, DNA adduct formation, DNA content, diploid/aneuploid and apoptosis markers. A variation of this analysis identified complementary flow cytometric changes in clinically diagnosed oral leukoplakias and oral squamous cell carcinomas obtained from smokers following laser microdissection. Standard immunohistochemistry for tumor suppressor, apoptosis, and proliferation was also obtained from tissue sections of these clinical specimens. Cell cycle analysis, diploid/aneuploid and hypodiploid apoptosis analysis was obtained using laser-scanning cytometry, and cell cycle conversion software (e.g., FSC file) available in the flow cytometer interpreted the digital information. This procedure was also accomplished for a non-tumorigenic keratinocyte cell line exposed to B[*a*]P or BPDE.

Table 1 Determination of histomorphology of oral keratinocytes

Stain	Nucleation (%)[a]	Binucleation (%)[b]	Micronuclei (%)[c]
Smokers[d]			
Pap	92.5±10.5	15.6±8.8	N.A.
Feulgen	N.A.	N.A.	62.7±9.0
Nonsmokers[e]			
Pap	65.4±15.0	5.4±3.0	N.A.
Feulgen	N.A.	N.A.	8.7±3.3

[a] Nucleation, the counting of percentage of cells per 100 showing increased nuclear hyperchromatism and/or the prsecence of nuclei.
[b] Binucleation, the counting of percentage of cells per 100 showing two nuclei.
[c] Micronuclei, the counting of percentage of cells per 100 showing a hyperchromatic piece of chromatin material in the nucleus.
[d] Smoker samples exhibited a higher percentage of cells with a nuclear cytoplasmic ratio reversal (2+), pleomorphism (3), hyperchromatism (4), anaplasia (1), and bizarre mitoses (1).
[e] Non-smokers which had 0 values except for hyperchromatism and pleomorphism, which were recorded as 1.

Results

Morphometric analysis

Table 1 compares the relative percentages of nucleated, binucleated, and micronucleated cells between the six smokers and nonsmokers. Smokers had higher levels of nucleation (92.5%±10.5 of cells/100 vs. 65.4%±15.0), binuceation (15.6%±8.8 vs. 5.4%±3.0) and micronucleation (62.7%±9.0 vs. 8.7%±3.3).

Smoker samples exhibited a higher percentage of cells with a nuclear cytoplasmic ratio reversal (2+), pleomorphism (3), hyperchromatism (4), anaplasia (1), and bizarre mitoses (1) compared to non-smokers which had 0 values except for hyperchromatism and pleomorphism, which were recorded as 1.

Immunofluorescent detection of DNA adduct formation

Fig. 2. depicts the lack of fluorescence in the nucleus of non-smokers for the 8-OHdG (a) and the high level of staining in the nucleus of smokers (b, c). The staining for B[*a*]P-DNA adduct was observed in both non-smoker and smoker nuclei (Ba,b). The staining in the non-smoker nuclei was more focal while the staining among the nuclei of the smoker

was more diffuse and contained small focal areas (see arrows). About 35.0% of the oral keratinocytes from smokers showed the presence of 8-OHdG DNA adduct. This adduct was found in less than 1% of the cells from nonsmokers. About 33% of cells from smokers expressed the B[*a*]P-DNA adduct, compared to 15.0% in nonsmokers.

Flow cytometric analysis for DNA adduct formation

Repeated analysis for the DNA adducts B[*a*]P-DNA adduct and 8-OHdG demonstrated a consistency among the smokers (37.5±2.5%; 41.3±3.3%) and non-smokers (8.1±1.8%; 2.6±0.6%) using the laser cytometric method. Repeated values did not significantly vary. For example, 8-OHdG values were within 5.5%±0.5% and B[*a*]P-DNA adduct values were within 6.0%±7.2%.

A comparison of DNA adduct formation and DNA content

This association describes a relationship between smoking exposure, DNA damage, adduct formation, and DNA content. Scatter-plot analysis separates into four quadrants the percentage of cells with a low to high level of DNA content and low to high levels of adducts. Quadrant III denotes high levels of DNA content and DNA adduct. A summary (mean+standard deviation of smokers or non-smokers) quantification showed smokers had higher levels of B[*a*]P-DNA adduct formation and DNA content (21.9±5.6%) compared to the non-smokers (0.3±0.2) (Table 2). Quadrant I records low DNA content and B[*a*]P adduct expression and smokers have 53.2±7.3% of oral keratinocytes in this quadrant. In contrast, 16.0±3.7% of the non-smokers are in this quadrant. High adduct expression but

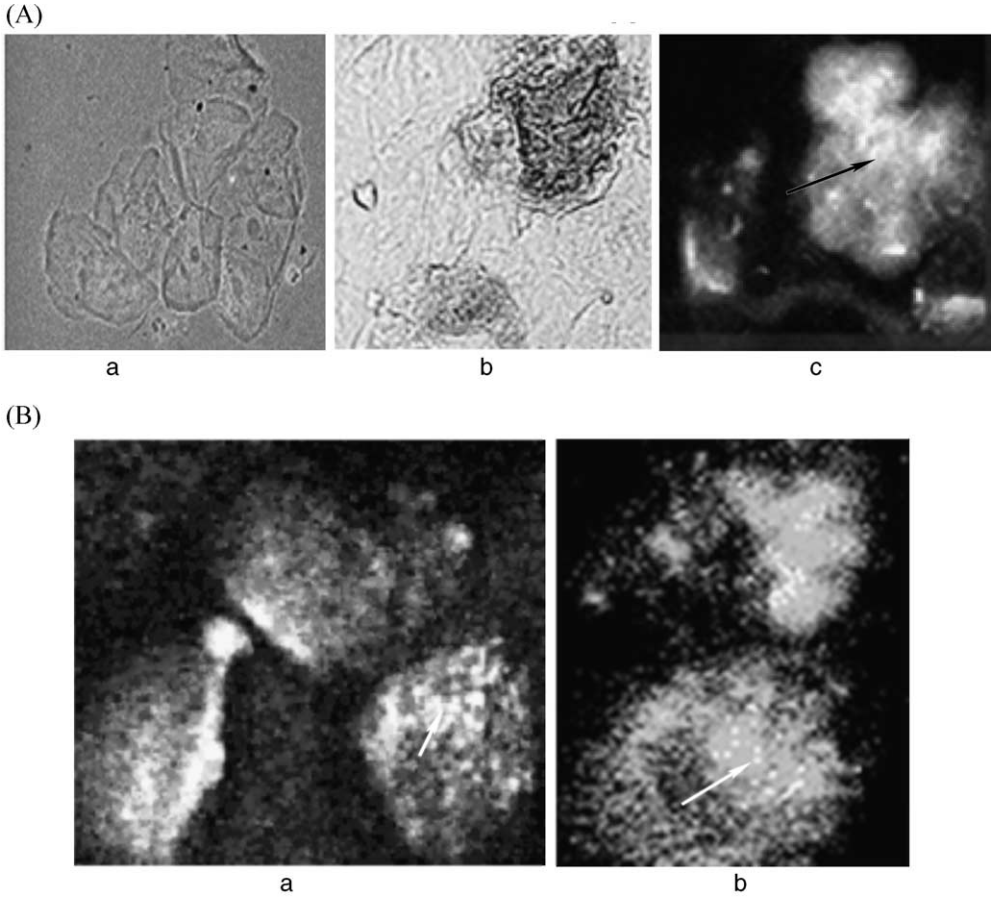

Figure 2 In this figure (A) dark field for non-smoker oral keratinocytes disclosed no visible detectable fluorescence for 8-OHdG DNA adduct (a,b,c). The level of darkfield was adjusted to demonstrate the presence of cells in the field. (a,c). Brightfield for oral keratinocytes from a smokers shows their presence. (b) Darkfield of the identical oral keratinocytes indicated the presence of 8-OHdG in the nucleus of the cells (see arrow) (200×) (c). Oral cytology produced oral keratinocytes exhibiting B[*a*]P-DNA adduct (see arrow), which is visible from the non-smoker (a) and the smoker (b).

Table 2 Summary of scatter-plot analysis showing B[*a*]P-DNA and 8-OHdG adduct formation (%) by DNA content

Quadrants[a]	I	II	III	IV
B[a]P-DNA and DNA content comparison				
Smoker #1	55.8	30.3	24.9	14.9
Smoker #2	45.0	28.5	25.5	5.5
Smoker #3	59.0	23.0	15.5	3.0
Non-smoker #1	20.0	30.8	0.4	1.4
Non-smoker #2	15.5	34.5	0.1	1.8
Non-smoker #3	12.5	20.0	0.5	2.0
8-OHdG and DNA content comparison				
Smoker #1	60.5	11.8	25.6	1.9
Smoker #2	65.0	10.0	22.0	2.0
Smoker #3	55.0	19.0	20.0	3.0
Non-smoker #1	3.6	45.3	0.3	50.7
Non-smoker #2	5.5	40.6	0.5	53.0
Non-smoker #3	5.0	50.9	0.9	42.8

[a] I=Low DNA content, low adduct level of expression; II=HighDNA content, low adduct level of expression; III=High DNA content, high adduct level of expression; IV=Low DNA content, high adduct level of expression.

Table 3 Flow cytometric determination of ploidy by smoking status

Subjects	Diploid (%)[a]	Aneuploid (%)
Smoker #1	60.3	39.7
Smoker #2	36.3	62.7
Smoker #3	53.9	47.1
Smoker #4	28.6	71.4
Smoker #5	14.7	85.3
Smoker #6	24.9	75.1
Non-smoker #1	83.4	16.6
Non-smoker #2	82.2	17.8
Non-smoker #3	81.5	18.5
Non-smoker #4	76.8	20.8
Non-smoker #5	52.8	26.1
Non-smoker #6	92.7	13.5

[a] G_2/G_1 fixed ratio, DNA content determined and the diploid and aneuploid results obtained from with the Expo-ADC software.

low DNA content levels is found in quadrant IV and smokers have 7.8±2.2% of oral cells while non-smokers have 1.7±0.3%. The result suggest smoker compared to non-smoker derived oral keratinocytes have higher DNA content and B[*a*]P-DNA adduct levels. Another example of the scatter quadrant plot analysis was conducted for 8-OHdG adduct. Smokers show a high DNA content and high adduct expression in 22.5±2.8% of the oral keratinocytes and in non-smokers 0.5±0.3%. In contrast, low DNA content and low adduct expression among smokers was 60.1±5.0% for keratinocytes and among non-smokers 4.7±0.9%. Oral keratinocytes from smokers with low levels of DNA content and high adduct formations were 2.3±0.6% and non-smokers 48.8±5.3%. These results showed smokers with high DNA content and high levels of expression for the oxidative adduct, 8-OHdG compared to non-smokers (Table 2). These results suggest that increased DNA content (aneuploidy) is not an absolute product of high levels of oxidative adduct formation. DNA adducts can appear without increases in DNA content.

DNA content, aneuploidy, cell cycle and apoptosis

In another assay the DNA content, was evaluated for ploidy, in relation to cell cycle and apoptosis and the results are shown in Table 3. A summary (mean±standard deviation) indicates that among smokers there is a notable increase in the percentage of cells, which are aneuploid (high DNA content). 63.5±17.3% of the keratinocytes in the smoker group compared to the non-smoker group 18.8±4.2% were aneuploid. A cell cycle analysis of these samples indicated an accumulation of cells in S (70.1±2.1) and G_2+Mitosis (16.9%±6.4) in smokers compared to non-smokers S (20.9±6.5) and G_{2+}Mitosis (11.4±16.2). G_1% was higher (63.1±19.1) among non-smokers than smokers (20.5±11.0) oral keratinocytes. Apoptosis as assessed by the hypodiploid curve showed an increase in the oral keratinocytes derived from smokers (31.2%±21.2) in comparison to the non-smoker group (2.6%±0.4) (Table 4). These results show, among smokers in comparison to non-smokers an increased aneuploidy, S phase (proliferation), and induction of apoptosis was noted.

Immunohistochemistry analysis of premalignant and malignant histopathology

Immunohistochemical markers were used to evaluate in tissue sections from smokers with premalignant and malignant lesions sites (Table 5). These indicators were also analyzed in malignant lesions compared to adjacent apparent normal sites. There was an enhanced level of proliferation as measured by PCNA, (cancer: 81.7±9.1%; dysplasia: 69.2±22.0%; normal: 5.1%±12.0); p53(cancer: 56.6±19.0%; dysplasia: 52.2±17.0%; normal: 1.3±1.5%); increases in apoptosis, TUNEL (cancer: 46.7±9.9%; dysplasia: 32.2±14.0%; normal: 2.5%±3.3) and inhibition of apoptosis, Bcl-2 (cancer:

Table 4 Flow cytometric determination of diploid cell cycle and apoptosis by smoking status

	Diploid cell cycle (%)[a]			Apoptosis (%)
	G_1	S	G_2+Mitosis	
Smoker #1	12.8	67.7	17.7	21.1
Smoker #2	11.5	70.9	22.9	55.7
Smoker #3	18.4	71.5	10.1	17.0
Smoker #4	24.8	59.4	4.8	14.9
Smoker #5	40.9	58.7	3.4	15.6
Smoker #6	14.8	66.5	18.7	24.5
Non-smoker #1	81.6	15.7	2.7	2.6
Non-smoker #2	79.5	18.9	1.5	2.2
Non-smoker #3	41.5	28.3	30.2	3.1
Non-smoker #4	79.6	18.9	1.5	4.5
Non-smoker #5	41.5	28.3	30.2	17.5
Non-smoker #6	58.3	26.5	15.2	7.0

[a] G2/G1 fixed ratio, Cell Cycle determined from DNA content results obtained from Laser Scanning Cytometry and digital processing using the Phoenix ''WinCyte'' software in the flow cytometer.

Table 5 Immunohistochemistry analysis of oral biopsy tissues

Immunohisto-chemical markers[a]	Normal (% of cells)	Dysplasia (% of cells)	Carcinoma (% of cells)
PCNA	5.1±12.0	69.2±22.0	81.7±9.14
P53	1.3±1.5	52.2±17.0	56.6±19.0
TUNEL	2.5±3.3	32.2±14.0	46.9±9.9
Bcl-2	2.2±1.6	18.2±6.3	43.3±8.1

[a] Mean level of intensity for a gated reference area divided by the total number of positive dark areas in the field. Object Areas: 364→ 3604.

43.3+8.1%; dysplasia: 18.2±6.3%; normal: 2.2±1.6%) in carcinoma containing tissues compared to normal appearing mucosa (Table 6). Oral keratinocytes from the above sites were evaluated using flow cytometry in an identical manner indicated above.

Laser microdissection harvesting of keratinocytes from tissue sections and analysis

Using laser microdissection of the tissue sections and flow cytometric analysis an increased DNA content (aneuploidy) in the dysplasia +carcinoma sites (8.1±0.5%) compared to the normal appearing sites (3.1±0.4%) (Table 7) was noted. These

Table 6 Flow cytometric analysis for DNA adducts in mucosa cells

Groups	DNA-adducts	
	B[*a*]P-DNA	8-OHdG
Untreated	2.1	3.0
B[*a*]P	67.7	88.2
B[*a*]P-diol epoxide	72.4	79.7

Table 7 Laser cytometry analysis of DNA index from oral cancer patients

Types of histopathology	% of cells with chromosome number (N) values			
	(1)	(2)	(3)	(4)
Normal	40.6	52.2	2.1	1.1
Dysplasia + carcinoma	69.7	20.2	3.8	4.3

Table 8 Laser cytometry analysis of proliferation markers from oral cancer patients

Histopathology	Level of marker expression		
	P53	Apo 2.7	Cytokeratin
Normal	10.6±8.2	27.0±9.6	33.5±3.8
Dysplasia+ Carcinoma	20.8±4.0	43.9±8.7	23.2±12.7

oral keratinocytes were also evaluated for cell cycle, changes, the dysplasia+carcinoma sites had more cells in G_1 (25.9±2.9%), and S (48.7±2.3%), and G_2 (24.1±5.8%) than the normal appearing sites G_1 (33.6±2.4%), S (30.0±1.6%) and G_2 (17.5+1.2%). The normal tissue is estimated to have more cells at rest. p53 expression was higher among premalignant and malignant oral keratinocytes than cells in normal sites (20.8±4.0% dysplasia, 10.6±8.2%, normal). This value is lower than the recorded immunohistochemical assessed level. The level of apoptosis (Apo 2.7) was also elevated (dysplasia+cancer: 43.9±8.7%, normal: 27.0±4.0%) while cytokeratin expression was depressed (dysplasia+cancer: 23.2±12.7%, normal: 33.5±3.8%) in dysplasia in comparison to the normal sites (Table 8). Among oral keratinocytes from histopathologic verified sites of dysplasia and oral cancer an increase in aneuploidy, S phase (proliferations) and apoptosis confirmed an association with oral cytology samples results from healthy smokers.

Table 9 Laser cytometric analysis of DNA content in mucosa cell line

Group	% of cells with chromosome number (N) values			
	(1)	(2)	(3)	(4)
Untreated	16.7	61.0	11.9	10.3
B[*a*]P	1.5	60.6	22.8	15.1
B[*a*]P-diol epoxide	0.9	8.3	79.6	11.1

Table 10 Flow cytometric analysis of ploidy in mucosa cell line

Groups	Diploid (%)	Aneuploid (%)
Untreated	87.6	3.4
B[*a*]P	72.9	27.1
B[*a*]P-diol epoxide	30.6	60.4

DNA adduct and content in vitro keratinocyte cell line exposed to B[*a*]P and BPDE

Laser scanning cytometry findings showed that the keratinocyte cell line treated with B[*a*]P or the oxidative reactive derivative BPDE increased the levels of adduct formation (Untreated: B[*a*]P-DNA adduct, 2.1%; 8-OHdG, 3.0%; B[*a*]P: B[*a*]P-DNA adduct, 67.7%; 8-OHdG; 88.2%, and BPDE: B[*a*]P-DNA adduct, 72.4%; 8-OHdG, 79.7%) (Table 6). High ($>$2N) DNA content and anueploidy were recognized (Untreated: 3.4%; B[*a*]P: 27.1%; BPDE: 60.4%) (Tables 9 and 10). Exposure to B[*a*]P or BPDE increased the percentage of cells in S phase (Untreated: 46.4%; B[*a*]P: 75.4% and BPDE: 60.6%). The percentage of cells in apoptosis was also recognized (Untreated: 14.8%; B[*a*]P: 24.4%, and BPDE: 29.4%) (Table 11). The results showed the exposure of keratinocytes to B[*a*]P or the oxidative derivative. BPDE increased DNA damage, adduct formation; DNA content, aneuploidy, increased proliferation, and the induction of apoptosis.

Discussion

A proof of principal for a flow cytometry approach to determine specific critical cell biologic changes in the oral mucosa of smokers before the presence of a lesion is identified in this study. First an identification of DNA damage, adduct lesion formation, using the current state of art, nuclear staining was undertaken. Later, DNA damage was also evaluated using flow cytometry. This method provided precision and consistency without possible investigator prejudice. Flow cytometric study from oral cytology samples were further substantiated by examining flow cytometric results obtained from clinically identified premalignant and oral cancer tissues. Flow cytometric evaluation of oral keratinocytes exposed to tobacco derived carcinogen, such as B[*a*]P provided a third approach validating the original oral cytology findings. The results confirmed exposure to smoke or direct contact to B[*a*]P or BPDE increased DNA damage, oxidative adduct formation, aneuploidy, cell proliferation and the induction of apoptosis among keratinocytes.

It has been well established that smoking increases significantly the risk for oral cancer.[39,40] To produce a malignant oral cancer cell requires the presence of a precursor non-malignant cell, which exhibits increased DNA damage, content, cell proliferation, and apoptosis. Among smokers the scatter-plot analysis showed a relatively high percentage of cells with low levels of DNA content but high levels of adduct formation. This finding suggests DNA adduct damage (e.g., bulky and oxidation lesions) can exist without the presence of aneuploidy and DNA damage, adduct formation precedes DNA content changes. The DNA damaged diploid cell a product of this precursor stage develops nuclear instability and malignant phenotypic changes (e.g., chromosomal instability, cytogenetic aberrations, mutations, and histone-chromatin epigenetic events).[41,42]

Findings from this study begin to establish links between smoking, ploidy changes identified in precursor non-malignant cells and oral malignant keratinocytes. In non-smokers, higher levels of adducts in oral cytology samples have been reported than for peripheral blood samples, possibly because the site, the tongue comes into direct contact with environmental or dietary PAHs from tobacco smoking.[40] Aneuploidy shown in this study and in previous studies precedes but segregates with malignant transformation.[43] For example, preneoplastic abnormal DNA content, aneuploidy, can be a product of DNA damage following exposure to tobacco-derived carcinogens (e.g., polonium, formaldehyde, nicotine and nitrosoamines etc.) and/or the life style of the smoker (e.g., diet, higher risk with use of recreational drugs, exposure to infectious agents such as human papilloma virus, increased stress, etc.). Additionally, in vitro and in animal tumors increased aneuploidy parallels and precedes karyotypic heterogeneous abnormalities.[23]

Cell studies have indicated that immortalization with HPV can result in increased cell proliferation

without the identification of abnormal DNA damage or content.[44] Suggesting increased proliferation presents before DNA adduct formation or DNA content. Oral keratinocytes exhibiting ploidy changes, from smokers have increased cell proliferation. Increased percentages of cells in S and G_2 phases of the cell cycle were noted in comparison to the cells harvested from non-smokers. To our knowledge, this is the first data that directly shows the presence of aneuploidy is found among smoker derived oral keratinocytes exhibiting a disrupted cell cycle but increased numbers of cells in S phase has been identified in tissue biopsies from smokers with premalignant and malignant oral lesions using flow cytometry.[30–32]

Data also indicated that increased DNA content, ploidy segregated with increased apoptosis. Apoptosis will produce enhanced selection of a unique population of oral keratinocytes at risk for further transformation and malignant phenotype. These cells have high proliferation, adduct formations and DNA repair resulting in faulty repaired but diploid cells (Fig. 3). Future studies are expected to show non-smokers have higher and more efficient DNA repair than smokers. Apoptosis in some keratinocytes and Bcl-2 inhibition of apoptosis in other cells from the identical tissue sections also indicated a complex selection for the survival of the transformed and proliferation cell clones of keratinocytes. It is hypothesized, smokers have more faulty repair sites and accelerated repair enzyme activity in their genome at any one moment increasing the persistence of nuclear damage and a higher DNA content, aneuploidy. In a recent study of mouth and buccal mucosa cells, the author concluded adduct formation can-not be used as individual markers for cancer risk because they are easily removed by repair processes in non-smokers. The level of effective DNA repair is therefore critical in assessing the risk for aneuploidy formation.[45]

Smoker oral keratinocytes also show several nuclear manifestations. These include binucleation, micronucleation, and increased nuclear–cytoplasmic ratio reversal. Some of these features were also identified in cells from premalignant and malignant tissue sections. The flow cytometric analysis of these cells indicated proliferation, which suggested a rapid expansion of the oral keratinocyte population from the stratum corium,

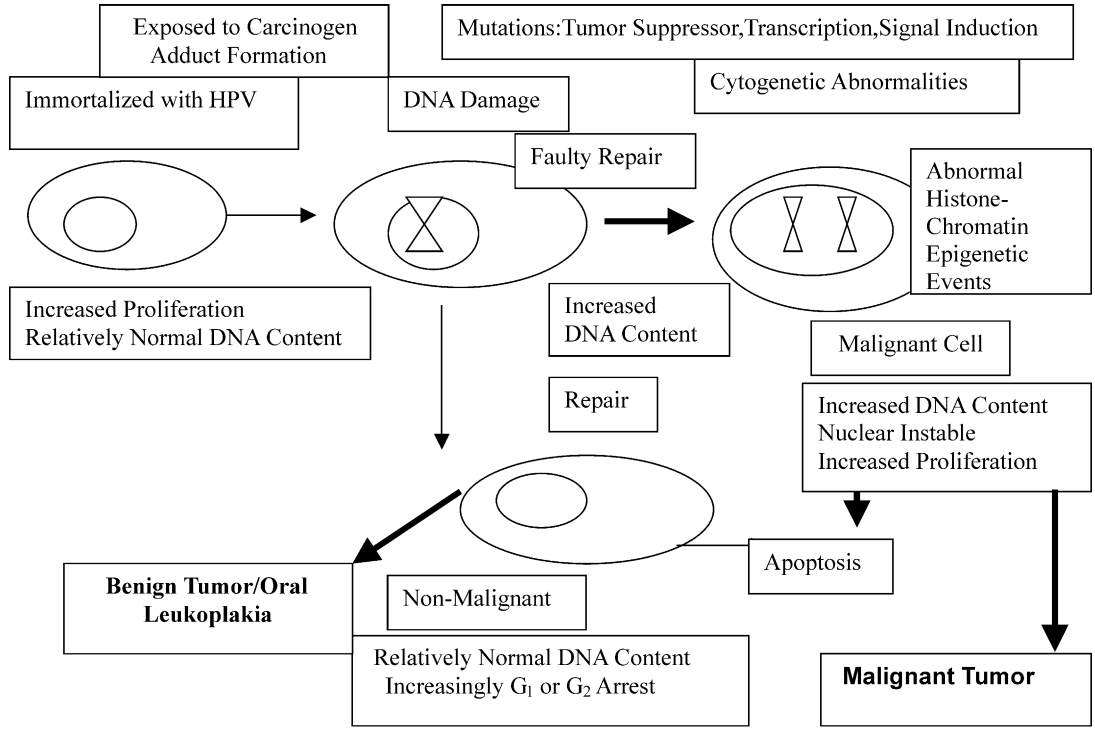

Figure 3 This scheme depicts the early cellular events resulting in malignant transformation in keratinocytes. Initially following infection with human papilloma virus (HPV, 16/18) the keratinocyte demonstrated increased proliferation and relatively normal DNA content. Oral cytology from smokers exhibited DNA adduct formation had increased DNA content, increased proliferation which induced apoptosis. A small population of these cells persist with faulty repair and with further damage a malignant cell is produced resulting in increased DNA content, nuclear instability, and increased proliferation. A larger portion of the non-malignant and malignant cells becomes apoptotic and is eliminated from the proliferating pool of cells.

stratum spinosum or basilis. The basilis layer is the most important region of the mucosa because the presence of stem cells. Damage to these cells results in malignant transformation and proliferation through clonal expansion enhancing the risk for oral carcinoma.[28,33]

A single aliquot of cells can provide an array of flow cytometric results potentially describing a early screening method for at risk individuals developing moderate to severe oral dysplasia or cancer.

These methods require further development before they can be used for any screening application. This includes determining the reliability of each measurement (e.g. the consistency of the findings in repeat samples), and interobserver reliability among flow cytometric facilities. Comparisons between smokers and nonsmokers need greater numbers in order to develop population standards. A need to determine a dose—response effect by cigarette amount and duration is evident. In particular, persons who quit smoking have genetic damage in the lungs that persists for decades.[46] Similar damage can occur in oral tissue identifying those former smokers or chewers that are at highest risk of oral cancer. The analysis of tissue from oral cancer patients needs to be repeated with greater attention to the patients smoking history. Although all patients had a smoking history, we did not have information on the smoking habits or the type of cigarette patients smoke. Likewise, further analysis will include determining karyotypic loss of heterozygosity for non-random cytogenetic markers in conjunction [47] with DNA adduct formation, repair enzyme activity, and markers for aneuploidy and cell growth.

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