ORIGINAL ARTICLE

DNA damage in peripheral blood leukocytes in tobacco users

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ABSTRACT

Aim: To Quantify the DNA single-stranded breaks in the peripheral blood leukocytes (PBLs) of tobacco-habituated individuals with clinically normal mucosa and patients with oral carcinoma. **Objectives:** To evaluate DNA damage levels in PBLs of tobacco-habituated individuals with clinically normal mucosa and patients with oral carcinoma and compare with a control group of healthy volunteers. To evaluate the extent of DNA damage in PBLs using Single Cell Gel Electrophoresis (SCGE) in the above groups. **Materials and Methods**: Patients who were attending the outpatient department were enrolled in this study. A control group of 30 healthy volunteers included in Group I were selected from various age groups who are not tobacco users in any form. Thirty patients with tobacco habituation but with clinically normal mucosa were included in Group II, while 30 tobacco-habituated patients with oral squamous carcinoma were included in Group III. A biopsy was taken from the representative area and confirmed histologically. Intravenous blood samples were collected from all the groups for evaluation of the extent of DNA damage using ethidium bromide-stained slides under fluorescent microscope. The DNA tail length was calculated by subtracting the diameter from the total length. Twenty-five randomly selected cells per slide were analyzed and mean calculated. **Results**: The mean DNA damage levels in patients with tobacco habits were compared with that of the control group and the results were found to be statistically significant. The mean DNA damage level in PBLs between tobacco-habituated patients with normal mucosa and oral cancer patients was found to be statistically significant. The DNA damage in cancer patients was compared with the control group and the results were found to be statistically significant. **Conclusion**: DNA damage evaluation in PBLs by SCGE technique is a sensitive and reliable indicator of tobacco insult.

Key words: DNA damage, single cell gel electrophoresis (comet assay), tobacco users

INTRODUCTION

Single cell gel electrophoresis (SCGE) is a sensitive technique for measuring and analyzing DNA damage in eukaryotic cells.[1] It is also known as Comet assay,[2] which is a rapid, simple, visual and reproducible biochemical technique for measuring and analyzing DNA single-stranded breaks and/or alkali labile sites within individual cells.

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DNA damage was found to be significant in epithelial dysplasia and in carcinoma of the cervix using this technique.[3] Statistically significant results in DNA damage were found in the peripheral blood leukocytes with respect to the clinical staging and histopathological grading of oral squamous cell carcinoma.[4] DNA damage in the exfoliated buccal cells due to tobacco has been assessed.[5] The possible DNA damage in the peripheral blood cells of tobacco addicted individuals has not been studied. Hence, an attempt is made to ascertain and quantify the DNA single-stranded breaks in the peripheral blood leukocytes of tobacco-habituated individuals with clinically normal mucosa and with oral carcinoma.

MATERIALS AND METHODS

Patients attending the outpatient department were enrolled for this study. In Group I, control group of 30 healthy

volunteers were selected from various age groups who are not tobacco users in any form [Table 1]. Thirty patients with tobacco habituation but with clinically normal mucosa were included in Group II [Table 2] while 30 tobacco-habituated patients with oral squamous carcinoma were included in Group III [Table 3]. Patients on drug therapy and suspected heavy systemic illness were excluded from this study. The history and clinical details were recorded. A biopsy was taken from the representative area and confirmed histologically.

Intravenous blood samples collected from group II and III patients were evaluated for the extent of DNA damage and compared with Group I. The values obtained among different groups, i.e. Groups I, II and III, were mentioned in Tables 1-3 respectively.

The reagents and slides were prepared according to the method outlined by Singh *et al.*[1] under non-ultraviolet light and the DNA damage was quantified using ethidium bromide-stained slides using fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. Cells with DNA damage appeared as comets. Using an oculometer fitted to the eyepiece, the total image length (nuclear and migrating DNA) and the image (nuclear) diameter were measured. The DNA tail length was calculated by subtracting the diameter from the total length. Twenty-five randomly selected cells per slide were analyzed and the mean was calculated.

Statistical analysis was performed using Student's t test. A $P < 0.05$ was considered to be statistically significant.

RESULTS

The DNA damage levels in the leukocytes of tobacco users [Figure 1] were compared with those of the normal subjects [Figure 2]. The mean DNA damage levels in normal subjects, tobacco users with normal mucosa and tobacco users with oral cancer patients are tabulated. [Table 4] The mean DNA damage levels in patients with tobacco habituation were compared with the control group and the results were found to be statistically significant. [Table 5] The mean DNA damage level in the peripheral blood leukocytes between tobacco-habituated patients with normal

Table 1: Details of patients included in group I

Age	Sex	Mean DNA damage (µm)
35	Male	2.00
28	Male	2.00
45	Male	2.50
25	Female	1.75
50	Male	2.40
22	Male	1.75
50	Female	2.25
30	Male	1.80
75	Male	3.60
30	Female	2.20
36	Female	2.00
62	Male	2.20
57	Male	2.60
45	Female	2.25
48	Male	2.00
48	Female	1.80
57	Female	3.20
55	Female	1.60
26	Male	2.00
67	Male	1.80
27	Male	2.00
41	Male	2.40
25	Male	1.60
26	Male	2.05
24	Male	2.05
27	Male	2.25
28	Male	1.80
26	Female	1.85
32	Female	2.10
34	Male	2.05

DNA: Deoxyribonucleic acid

Table 2: Details of patients included in group II

Age	Sex	Mean DNA damage (µm)
60	Male	3.25
40	Male	2.50
67	Male	3.15
26	Male	2.50
30	Male	2.50
52	Male	2.75
50	Male	2.75
58	Male	2.50
22	Male	2.25
40	Male	2.25
45	Female	2.25
28	Male	2.25
44	Male	2.50
50	Female	2.50
35	Female	2.25
50	Male	3.20
45	Female	2.25
38	Female	2.40
40	Male	2.40
18	Male	2.60
30	Female	2.40
50	Male	3.00
40	Female	2.75
33	Male	2.60
35	Male	2.50
27	Male	2.50
50	Male	3.15
47	Male	3.15
48	Male	2.75
45	Male	2.75

DNA: Deoxyribonucleic acid

mucosa and oral cancer patients was found to be statistically significant. [Table 6] Further, the DNA damage in cancer patients was compared with that in the control group and the results were found to be statistically significant [Table 7].

Table 3: Details of patients included in group III

Age	Sex	Mean DNA damage (µm)
35	Female	30.20
55	Male	32.45
55	Male	26.80
58	Female	25.80
55	Female	26.60
36	Male	28.00
53	Male	20.20
45	Male	21.60
60	Male	28.20
50	Male	23.20
56	Male	19.80
65	Female	24.00
60	Male	20.40
58	Female	17.40
30	Male	23.60
75	Female	32.70
45	Female	25.00
60	Female	24.60
45	Male	31.60
40	Male	31.80
65	Male	24.40
60	Male	28.00
42	Female	26.75
45	Female	26.75
40	Male	24.00
60	Male	30.00
65	Male	25.00
35	Male	22.50
48	Male	25.75
50	Male	20.00

DNA: Deoxyribonucleic acid

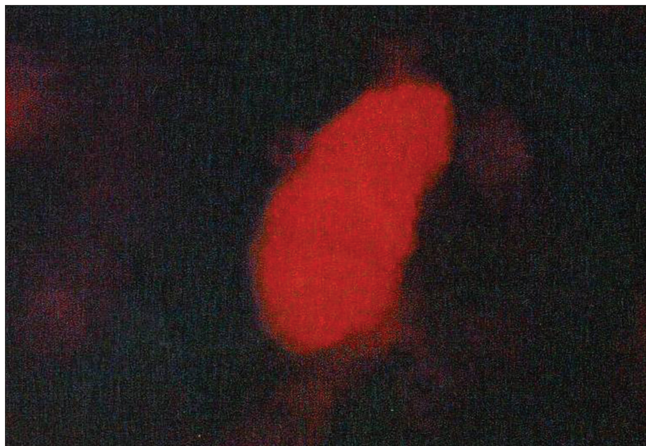

Figure 1: DNA damage in a tobacco user

DISCUSSION

DNA damage is central to cancer development. A variety of factors are responsible for DNA damage. Different carcinogens attack different sites on the DNA leading to the accumulation of cellular and DNA damage. They include single- and double-stranded breaks, DNA protein crosslinks, loss of bases from the DNA backbone and modified base residues.[6]

Tobacco smoking and smokeless tobacco have been accepted as important etiologic agents in oral cancer. Carcinogens, such as polyaromatic hydrocarbons nitrosamines and aromatic amines present in tobacco, upon deactivation in the liver to electrophilic intermediates, react with DNA to from covalently bound adducts.[7] The formation of specific DNA adducts and the resulting mutations can be viewed as being responsible for oncogene activation and inactivation of tumor suppressor genes, leading to cancer development.

The peripheral blood cells were known to exhibit changes in cancer of various organs. Even as early as 1959, Nieburgs[8] found abnormalities in the polymorphonuclear leukocytes (PMNL) and he named it as malignancy-associated changes (MAC). MAC could also be recognized in megakaryocytes, lymphocytes, plasma cells and erythroid elements from the bone marrow in patients with carcinoma.

Table 4: DNA damage levels in normal subjects, tobacco users with Normal oral mucosa and oral cancer patients

Group	No. of cases	Mean DNA damage (µm)
I		
Control	30	2.18
II		
Tobacco users with normal oral mucosa	30	2.5833
III		
Oral cancer patients	30	25.375

DNA: Deoxyribonucleic acid

Figure 2: DNA damage in a normal subject

Table 5: Comparison of DNA damage between Group I and Group II

Number of cases in different groups	Mean DNA damage (µm)	Standard deviation	'*t*' value	*P* value	Significance
30 (normal mucosa without habits)	2.18	0.4789	3.8489	<0.01	S
30 (tobacco users with normal mucosa)	2.5833	0.2984			

DNA: Deoxyribonucleic acid

Table 6: Comparison of DNA damage among Group II and III

Number of cases in different groups	Mean DNA damage (µm)	Standard deviation	'*t*' value	*P* value	Significance
30 (tobacco users with normal mucosa)	2.5833	0.2984	29.5826	<0.01	S
30 (tobacco users with OSCC)	25.375	4.1382			

DNA: Deoxyribonucleic acid, OSCC: Oral squamous cell carcinoma

Table 7: Comparison of DNA damage among Group I and Group III

Number of cases in different groups	Mean DNA damage (µm)	Standard deviation	'*t*' value	*P* value	Significance
30 (control group)	2.18	0.4789	30.0056	<0.01	S
30 (tobacco users)	25.375	4.1382			

DNA: Deoxyribonucleic acid

Chomet *et al*. (1996)[9] showed atypical nuclear changes in monocytes in routine peripheral blood smears of patients with malignant tumors. Significant increase in the frequencies of sister chromatid exchanges and chromosomal aberrations have been estimated in peripheral blood lymphocytes and the percentage of micronucleated cells in exfoliated cells of the buccal mucosa in Areca nut consumers.[10]

The comet assay was used to assess single-strand breaks in cervical carcinomas, oral neoplasms and in potentially malignant lesions of cervix and oral cavity. Using the comet assay, it has been shown that the patients with potentially malignant and cancerous lesions of cervix exhibited increased DNA damage levels in peripheral blood leukocytes (PBLs) when compared to normal individuals.[3] According to a pilot study conducted in PBLs of patients with oral squamous cell carcinoma by SCGE, a significant increase in DNA damage depending on the clinical staging and histopathological grading.[4] This result was attributed to SCGE assay, a sensitive technique to identify DNA damage in PBLs, even before morphological changes become apparent. As all the patients in the study group were tobacco users, the observed DNA damage could be attributed to tobacco habituation. These findings of various workers in recognizing changes in PBLs lend support to the concept of a systemic host response in malignancy.

Rogers *et al.* (1996),[5] using the comet assay, evaluated the DNA damage in exfoliated buccal mucosal cells of smokers and nonsmokers. The extent of DNA damage was found to be significantly increased in the smoker group than in a non-smoker group. It is quite probable that the leukocytes show a similar change in patients with tobacco habituation. To provide evidence to this assumption, the present study was carried out to evaluate DNA damage in PBLs of patients with tobacco habituation, using the SCGE technique.

The finding of statistically significant differences in DNA damage of PBLs between tobacco users with normal oral mucosa and oral cancer patients habituated to tobacco and higher mean values in cancer patients could be attributed to the continual insult of tobacco and its role in the progression to oral carcinoma. Further, evidence for this conclusion is available when control subjects without tobacco habituation were compared with oral cancer patients (mean damage value 25.375 µm, $P < 0.01$). The results of the observation were comparable to that of the study by Venkateswara Rao *et al.* (1997).[4]

CONCLUSIONS

The following conclusions were drawn from the study:

1. A statistically significant increase in DNA damage levels in peripheral blood leukocytes of tobacco-habituated patients with normal oral mucosa and in oral cancer patients suggests that the PBLs reflect the DNA damage in oral tissues
2. The finding of increased DNA damage in PBLs in tobacco-habituated patients with a clinically normal oral mucosa, much before the clinical alterations are evident, suggest that the DNA damage evaluation in PBLs is a sensitive indicator of tobacco insult
3. It can also be inferred that by evaluating DNA damage in PBLs by SCGE technique, potential malignancy, predisposition and cancer development may be predicted in susceptible patients, due to significant difference between the study group and the control group.

Though the DNA damage in oral epithelial cells due to tobacco smoking has been observed, the observation in PBLs appears to be unreported. A study involving a larger sample is required to corroborate these findings.

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