Reduction in Oral Mucosa Micronuclei Frequency Following Alpha-Tocopherol Treatment of Oral Leukoplakia

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Abstract
Micronuclei frequency, a marker of genotoxicity, was studied within a trial of α-tocopherol for chemoprevention of oral leukoplakia. Oral swabs were obtained from two sites, the leukoplakia lesion and normal-appearing mucosa, at baseline and following 24 weeks of therapy with 400 international units of α-tocopherol twice daily. These specimens were analyzed for micronuclei frequency. The major risk factors for oral carcinogenesis in the group studied were cigarette smoking and alcohol consumption. α-Tocopherol therapy produced a significant reduction in micronuclei frequencies in specimens from both the visible lesions (P < 0.01) and the normal-appearing mucosa (P < 0.01). The micronuclei frequencies, both at baseline and following therapy, were greater in specimens taken from the lesion than in those from the normal-appearing mucosa. Although these results indicate that α-tocopherol has a beneficial effect in oral carcinogenesis, there was no significant clinical or histological response associated with the change in micronuclei frequency. Micronuclei frequency has not yet been validated as a biomarker for cancer incidence, and consequently, its utility as an intermediate end point for chemoprevention trials is not known. Determining clinical significance of micronuclei frequency patterns in oral carcinogenesis and chemoprevention will require further study.

Introduction
In chemoprevention trials, biomarkers may ultimately serve both to define high risk populations and as intermediate endpoints (1). Micronuclei, extranuclear fragments of DNA formed as the result of clastogen exposure, have been widely studied as a potential marker of cancer risk (2). Micronuclei frequency provides a quantifiable, although nonspecific, assessment of recent DNA injury. Stich et al. (3) have reported an important series of trials which have demonstrated that oral cavity micronuclei frequency is increased in populations of smokeless tobacco and betel nut users (who are at high risk of developing oral cancers) and appears to be greatest at the site with the most intense carcinogen exposure (e.g., where the quids are held) (4). A decrease in micronuclei frequency was observed following vitamin A and β-carotene treatment (5–7). Other studies in the oral cavity, bronchial mucosa, and esophagus have indicated that micronuclei frequency is not closely associated with clinical or histological change (8–11).

In an effort to further define the role of micronuclei as a biomarker for chemoprevention studies, the response of micronuclei frequency to treatment with α-tocopherol was incorporated into a United States oral leukoplakia chemoprevention study. We recently reported a study of the clinical and histological responses to α-tocopherol, a lipid phase antioxidant, in 43 patients with oral leukoplakia (12). The present paper describes the expression and modulation of oral micronuclei frequency associated with α-tocopherol therapy.

Materials and Methods
The clinical trial was performed through The University of Texas M. D. Anderson Cancer Center Clinical Community Oncology Program. Subjects with at least one two-dimensional measurable oral leukoplakia lesion were enrolled after informed consent was obtained. Participants received 400 international units of α-tocopherol, orally, twice daily, for 24 weeks. Biopsies of the leukoplakia lesions were obtained at baseline and at the completion of the therapy. Participants underwent clinical evaluation during follow-up visits at weeks 6, 12, and 24. The methods of assessing toxicity and both clinical and histological responses have been described previously (12).

Scrapings from the oral mucosa also were obtained prior to treatment and at the completion of the 24-week α-tocopherol therapy. They were obtained from the leukoplakia lesion and the normal-appearing buccal mucosa of the contralateral side. The scrapings from each site were smeared onto two microscope slides. The slides were immediately sprayed with a fixative (Adams Spray-Cyte, Parsippany, NJ) and allowed to air dry.

The slides were stained with Feulgen stain, which allows clear visualization of DNA, using the following procedure. The slides were first hydrolyzed in 1 N HCl at 56° for 10 min. The reaction was stopped by immersing the slides in chilled dH2O for 1 min. The slides were immersed in Feulgen stain in a dark refrigerator for 90 min and then washed in running tap water for 10 min. The slides were then dehydrated, counterstained with Fast green for 45 s, and mounted, using permount.

The slides were interpreted in a blinded fashion; the observer was unaware of the site of the specimen, the time the specimen was obtained, or other counts for the same participant. Micronuclei frequency was reported as the num-
Oral Mucosa Micronuclei after Alpha-Tocopherol

Table 1 Study participant characteristics (n = 24)

<table>
<thead>
<tr>
<th>Study location</th>
<th>United States</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female, 11 (46%); male, 13 (54%)</td>
</tr>
<tr>
<td>Age</td>
<td>29–83 years, median 58 years</td>
</tr>
<tr>
<td>Past tobacco use</td>
<td>19 (77%)</td>
</tr>
<tr>
<td>Present tobacco use*</td>
<td>12 (50%)</td>
</tr>
<tr>
<td>Past alcohol use</td>
<td>17 (71%)</td>
</tr>
<tr>
<td>Present alcohol use*</td>
<td>15 (63%)</td>
</tr>
</tbody>
</table>

* No changes in tobacco or alcohol use during the study were reported.

Results

Major characteristics of the 24 study subjects are listed in Table 1. Mean micronuclei frequencies were significantly lower in specimens taken from the normal-appearing mucosa than in those from the lesions (Table 2). Micronuclei frequencies declined after α-tocopherol treatment both in specimens from the lesions (n = 17, P < 0.01) and in those from the normal-appearing mucosa (n = 22, P < 0.01). The significant overall reduction in micronuclei frequency in the leukoplakia lesions is demonstrated in Fig. 1. Of the 17 patients with evaluable specimens from the leukoplakia lesion, 12 had a reduction in micronuclei frequency after treatment, 3 had no change, and 2 had an increase. Among the 22 participants with evaluable normal-appearing mucosa, 12 had 0 micronuclei per 1000 cells both before and after α-tocopherol treatment, 9 had a decrease in micronuclei frequency after treatment, and 1 had an increase in micronuclei. There was no statistical association between change in micronuclei frequency and clinical or histological response (Table 3). To assess interobserver agreement, 10 slides were independently reviewed by two observers. The Pearson correlation coefficient for these measurements was 0.89.

Discussion

Micronuclei frequency has been evaluated as a potential intermediate endpoint biomarker of carcinogenesis in both clinical and epidemiological studies (13). Using Feulgen stain, the test is not specific with regard to the extent or chromosomal location of the genetic injury but, as demonstrated in this study, assessment of micronuclei frequency has several important qualities that may be exploited to further develop this test as a biomarker of carcinogenesis and an intermediate endpoint for chemoprevention trials. The test is noninvasive and reproducible and provides an estimate of recent DNA injury. In addition, micronuclei frequency may be used to assess the impact of the chemopreventive agent directly within the tissue of interest.

It has been shown that micronuclei frequencies are increased in a site-specific fashion among individuals with known carcinogen exposure, such as within the oral cavity of tobacco users and betel nut chewers. Specimens obtained from different sites in a single individual reflect the degree of carcinogen exposure. In this study, micronuclei frequencies were higher in specimens obtained from the leukoplakia lesions than in those obtained from the normal-appearing contralateral mucosa. The dose-response relationship between carcinogen exposure and micronuclei frequency is a useful characteristic for a potential biomarker. Furthermore, increased micronuclei frequency in the grossly normal-appearing oral sites in these high risk individuals is consistent with the concept of field carcinogenesis.

Micronuclei frequency has been shown previously to decline following administration of chemopreventive agents. Oral leukoplakia chemoprevention studies in Asian smokeless tobacco and betel nut users have shown a decrease in micronuclei frequencies following administration of retinol and β-carotene. This first United States study has shown that micronuclei frequency is reduced in a population composed predominantly of smokers by the administration of α-tocopherol. The demonstration of a reduction in micronuclei frequency following a 24-week course of the agent further indicates that this test may have potential application in the screening of chemopreventive agents.

There has been wide variation in the reported micronuclei frequencies in oral leukoplakia studies. This reflects the marked differences in populations studied as well as differences in techniques. For example, betel nut chewers in Asia have micronuclei frequencies in the oral mucosa that are 10-fold higher than those in smokers from the United States. This variation underscores the need for both internal comparisons within a study, such as pretreatment and post-treatment measurements, and for caution in the interpretation of absolute micronuclei frequencies reported in different studies.

Our earlier report indicates that the chemopreventive agent α-tocopherol induced both clinical and histological responses in oral leukoplakia lesions. As reported here, α-tocopherol treatment led to reductions in micronuclei frequency in specimens obtained from both the lesions and the normal-appearing mucosa. There was, however, no short-term association between lesion response and the change in micronuclei frequency that might be useful as a marker of the biological effects of chemopreventive agents. The interpretation and timing of clinical, histological, and micronuclei responses over time and their correlation with cancer incidence will require larger and longer studies.

Recent studies have indicated that genetic changes such as amplification of epidermal growth factor receptor,
Table 2  Micronuclei frequencies at baseline and completion of α-tocopherol therapy

<table>
<thead>
<tr>
<th>Micronuclei count per 1000 cells</th>
<th>0</th>
<th>&gt;0-1</th>
<th>&gt;1-2</th>
<th>&gt;2</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa (n = 22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>13 (59%)</td>
<td>4 (18%)</td>
<td>3 (14%)</td>
<td>2 (9%)</td>
<td>0.70 ± 1.11</td>
</tr>
<tr>
<td>24 weeks</td>
<td>19 (86%)</td>
<td>2 (9%)</td>
<td>1 (5%)</td>
<td>0</td>
<td>0.14 ± 0.44</td>
</tr>
<tr>
<td>Leukoplakia lesion (n = 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2 (12%)</td>
<td>6 (35%)</td>
<td>5 (29%)</td>
<td>4 (24%)</td>
<td>1.94 ± 1.80</td>
</tr>
<tr>
<td>24 weeks</td>
<td>8 (47%)</td>
<td>6 (35%)</td>
<td>2 (12%)</td>
<td>1 (6%)</td>
<td>0.71 ± 0.90</td>
</tr>
</tbody>
</table>

Table 3  Changes in Micronuclei count by clinical and histological response to α-tocopherol treatment

<table>
<thead>
<tr>
<th>Micronuclei count</th>
<th>Not evaluable</th>
<th>Progressive disease</th>
<th>No change</th>
<th>Partial response</th>
<th>Complete response</th>
</tr>
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<tr>
<td>Clinical assessment</td>
<td></td>
<td></td>
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<tr>
<td>increased</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>unchanged</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>decreased</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
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<tr>
<td>Histological assessment</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>increased</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>unchanged</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>decreased</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

**bcl-1, int-2, polysony of chromosomes 7 and 17, and p53 mutation** are all associated with oral carcinogenesis (14–16). Double minute chromosomes containing the amplified oncogene MYC have been shown in cell lines to be incorporated into micronuclei, possibly as a mechanism of cell protection (17). These findings indicate that micronuclei may not simply reflect random DNA injury. The genetic composition of micronuclei must be studied to determine if they contain specific genes associated with oral carcinogenesis. Results of these studies could have a significant impact on the future use of micronuclei as a biomarker.

Biomarkers are being sought for chemoprevention trials which will both reflect cancer risk and have their expression modulated by the chemoprevention agent. The essential association is between changes in biomarker expression and cancer incidence. Biomarkers which can reliably predict the benefit of a chemopreventive agent before the development of invasive cancer will be extremely valuable in the development of intervention strategies. Micronuclei frequency, as with other proposed markers, has not yet been shown to be associated with cancer incidence. Micronuclei frequency has not been validated as an intermediate end point for chemoprevention trials. Consequently, the utility of this biomarker is not known.

There are difficulties which would complicate wider application of micronuclei as a biomarker. Although statistically significant reductions in micronuclei incidence have been observed with α-tocopherol treatment, the frequency of micronuclei per counted cell is very low. This raises concern about the potential for sampling error or the impact of poor techniques. Although automated procedures have been proposed, as employed in this study, assessment of micronuclei is a labor-intensive test. In addition, there is considerable variability in the micronuclei frequency reported for different patients. This variability indicates that changes in micronuclei frequency, rather than a fixed number of micronuclei, may be more useful in assessing response to a chemoprevention agent. A role for micronuclei frequency as a biomarker for chemoprevention trials will require demonstration that the small but statistically significant reduction of micronuclei incidence, observed with chemoprevention agents such as α-tocopherol has clinical relevance.

Micronuclei frequency in oral leukoplakia has been shown to increase at the site of the lesion and to decrease following the administration of α-tocopherol. If, as we assume, epithelial carcinogenesis is a multistep process driven by DNA damage and specific genetic events, it is a reasonable hypothesis that long-term suppression of DNA injury may have a favorable impact on cancer incidence. Future studies of α-tocopherol and micronuclei in oral leukoplakia will help determine whether this agent successfully prevents cancer and whether the reduction in micronuclei frequency, over time, predicts this benefit.

References
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