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Original

Reduction of micronuclei in oral lichen planus supplemented with beta-carotene

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Abstract: An elevated frequency of micronucleated exfoliated cells (MEC) in atrophic and erosive oral lichen planus (OLP) has been reported. To evaluate the effects of supplemental beta-carotene (BC) on MEC frequency in OLP lesions, we performed an open trial in 20 patients with atrophic and/or erosive OLP. Each patient received 15 mg of BC four times daily for 3 months. The frequency of MEC in both lesions and adjacent normal mucosa in each patient was evaluated and compared before and after supplementation. Serum levels of BC and retinol were also determined. After BC supplementation, all patients had higher levels of serum BC and retinol. The MEC frequency in OLP lesions was significantly reduced ($P < 0.01$). No significant changes were seen in the MEC frequency in adjacent normal mucosa. BC supplementation thus significantly reduces MEC frequency in atrophic and erosive OLP. (J. Oral Sci. 50, 461-467, 2008)

Keywords: oral lichen planus; beta-carotene supplementation; micronucleated exfoliated cells.

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Introduction

Oral lichen planus (OLP) is a common chronic inflammatory mucocutaneous disease. The pathogenesis of OLP is complex, representing T cell-mediated cytotoxicity (1). Various forms of OLP are seen clinically; reticular, papular, plaquelike, atrophic, erosive, and bullous lesions, and these can occur separately or simultaneously (2). Atrophic and erosive forms usually cause symptoms of pain and discomfort (3). Malignant transformation of OLP has also been reported, thus suggesting that OLP is a premalignant condition (4). Recent studies in histologically confirmed OLP indicate that patients with atrophic and erosive OLP have an increased risk of developing oral squamous cell carcinoma (5,6).

Micronuclei, cytoplasmic fragments of DNA, have been reported as markers for high cancer risk, as they arise in response to carcinogens. They can be detected in exfoliated cells and are used as an indicator of recent DNA injury within oral mucosa (7;8). Micronucleated exfoliated cells (MEC) are typically elevated in oral premalignant lesions (9). MEC frequency in atrophic and erosive OLP has been found to be elevated in comparison to adjacent normal mucosa (10). About 60% of oral mucosal dysplasia, including oral leukoplakia and OLP, responds to beta-carotene (BC) and cis-retinoic acid treatment (11). Remission and inhibition of new oral leukoplakia were confirmed in patients receiving 90 mg of BC twice weekly for three months (12). A significant reduction in micronuclei in mucosal cells also occurred in this group of patients. Conversion from oral leukoplakia to OLP or lichenoid

changes in patients receiving antioxidant supplements, including BC, was reported to be an actual transformation (13). Although the explanation remains uncertain, this phenomenon indicates a relationship between OLP and oral leukoplakia.

The aim of this open trial was to determine the effects of supplemental BC on MEC frequency in atrophic and erosive OLP, as the effects of BC on MEC in OLP have never been studied.

Materials and Methods

Patients

Patients from the Oral Medicine Clinic, Faculty of Dentistry, Mahidol University, Bangkok, Thailand, were enrolled in the study according to the following inclusion criteria:

1. Presence of painful atrophic and/or erosive OLP;
2. Histological confirmation of OLP according to WHO criteria (14); and
3. Wash-out period of 2 or 4 weeks for patients receiving topical or systemic medications, respectively, for OLP.

Patients were excluded from the study if they:

1. had been taking any drugs known to induce lichenoid reactions;
2. had oral lichenoid lesions due to restorative materials;
3. were pregnant or lactating.

The study method was approved by the Institutional Review Board of Mahidol University. All patients received information on the study and signed consent forms. Patients were interviewed for thorough medical history, dietary intake and habits (smoking, betel quid chewing, etc.). Each patient received 15 mg of BC four times daily for three months. BC (Betacar-15, Medicrafts) was supplied by Mega Product (Thailand) Ltd. Liver function test (liver enzymes) was assayed at baseline and on the last day of supplementation.

Assessment of OLP

At the beginning of the study, and in the second week, and the first, second and third months of the study, OLP lesions were assessed by one of the authors (W.B.). Type, site, distribution and severity of OLP were recorded. The most severe lesion at the beginning in each patient was selected as a representative. The criteria for severity scoring was as follows (15):

Score 5 = white striae with erosive area more than 1 cm^2
Score 4 = white striae with erosive area less than 1 cm^2
Score 3 = white striae with atrophic area more than 1 cm^2
Score 2 = white striae with atrophic area less than 1 cm^2
Score 1 = mild white striae, no erythematous area
Score 0 = no lesion, normal mucosa.

At each visit, the selected lesions were given a severity score and photographed.

Sampling of exfoliated cells and quantitation of MEC

OLP patients were asked to rinse their mouths with water in order to remove food particles. Exfoliated cells were swabbed from the selected lesions and normal-appearing mucosa about 2 cm from the lesions. Cells were transferred onto a pre-cleaned microscopic slide, allowed to air-dry, and fixed in 85% methanol. Cytoplasmic assessment was performed (C.A.) in a blinded manner. Smear preparations and screening for MEC were performed as described previously (10). Briefly, 500 cells were screened for nuclear anomalies, including micronuclei, karyorrhexis, pyknosis and chromatid clumping. The frequency of MEC was recorded.

BC and retinol assay

Serum levels for BC and retinol were assayed at baseline and on the last day of supplementation. Blood samples were obtained from an antecubital vein using vacutainer tubes without anticoagulant. Serum was collected by centrifuging clotted blood for 10 min at 1,000 × *g*. Supernatant fluid was placed in brown polystyrene tubes for protection from light, and was stored at -80°C. Samples to be assayed were thawed and a 200-µl aliquot was added to 50 µl of alpha-tocopherol acetate (internal standard) and 150 µl methanol in foil-wrapped tubes. Samples were mixed using a vortex for 30 s. After adding 460 µl of hexane, samples were mixed for one min, making sure that the bottom layer was thoroughly extracted. After centrifugation for 10 min at 3000 × *g*, 40 µl of the solution was injected into a high-performance liquid chromatography (HPLC) system. Solvents were freshly prepared by degassed sonication for 30 min prior to use. The analysis is part of the simultaneous method for BC and retinol determination reported previously (16).

HPLC instrumentation (LDC/Milton Roy Company, Rochester, NY, USA) consisted of a model CM 4000 multiple solvent-delivery system, a 100 autoinjector, a solvent reservoir bottle, a 50-µl injector valve, a multi-wavelength detector at 454 nm for BC and 325 nm for retinol at a flow rate of 1.5 ml/min, and a CI-10B integrator. The analytical stainless steel column was a Phenomenex C-18 ODS2 (25 × 4.6 mm) paired with a matching guard column (pre-column).

Results

Patient characteristics

Twenty patients (1 male and 19 females) with atrophic and/or erosive OLP participated in and completed the study. The average age of the patients was 48.1 ± 7.9 years (range, 31-61 years). OLP lesions were most commonly found on the gingiva (55%), followed by mucobuccal fold (35%), buccal mucosa (25%), tongue (5%) and palate (5%). The mean duration of lesions was three years (range, two months to twelve years). The patients were otherwise healthy. They had no medical problems and were not taking any medication. None of them reported betel quid chewing or tobacco use.

OLP assessment

Representative areas of the lesions were at gingiva (55%), mucobuccal fold (20%), buccal mucosa (15%), tongue (5%) and palate (5%). Patients, classified by severity scores according to the selected lesion before and after BC supplementation, are shown in Table 1. At the beginning, the mean score for the lesions was 2.6 ± 0.7, whereas at the end of the study, it was 1.8 ± 0.8. After treatment, most patients still had atrophic and erosive forms of OLP.

Micronucleated exfoliated mucosal cells

A representative microscopic view of MEC found in OLP is shown in Fig. 1. The frequency of MEC in OLP lesions with different severity scores is shown in Table 1. Mean MEC frequency in the lesions and in adjacent normal mucosa before and after BC supplementation are shown in Table 2. Using the Mann-Whitney rank sum test, it was found that at the beginning, the frequency of MEC was significantly higher for specimens obtained from lesions than those from normal-appearing mucosa ($P < 0.001$). Following the three-month BC supplementation, the frequency of MEC in the lesions declined significantly ($P < 0.001$), but not that in normal mucosa ($P > 0.05$), as analyzed by Mann-Whitney rank sum test. At the end of the study, MEC frequency in the lesions was significantly different from that in normal mucosa ($P < 0.001$), as analyzed by *t*-test. The change in MEC frequency for each participant during the course of the study is shown in Fig. 2. There was no relationship between MEC frequency and severity score, either at the beginning of or after supplementation.

Table 1 Frequency of MEC in OLP patients with different severity scores before and after BC supplementation

Severity score of OLP	Before treatment		After treatment	
	No. of subjects	Percent MEC Average (range)	No. of subjects	Percent MEC Average (range)
0	0	-	2	0.98 (0.8-1.16)
1	0	-	2	0.90 (0.8-1.0)
2	10	4.35 (1.94-6.4)	15	0.81 (0.4-1.2)
3	8	3.08 (2.0-4.59)	0	-
4	2	4.89 (3.89-5.88)	1	1.13 (1.13)

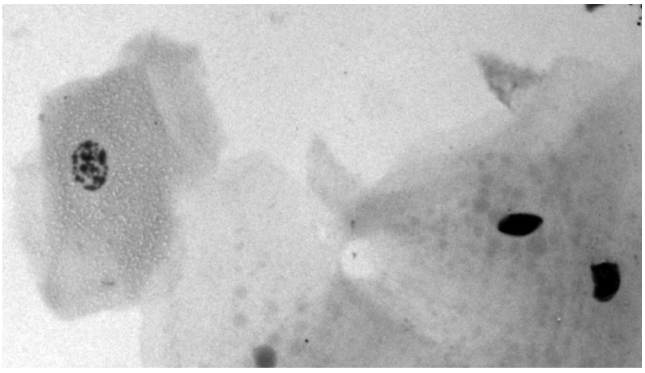

Fig. 1 Micronucleated exfoliated cells (MEC) from buccal mucosa. One karyorrhexic nucleus (left) and two pyknotic nuclei (right) are shown. Original magnification: ×400

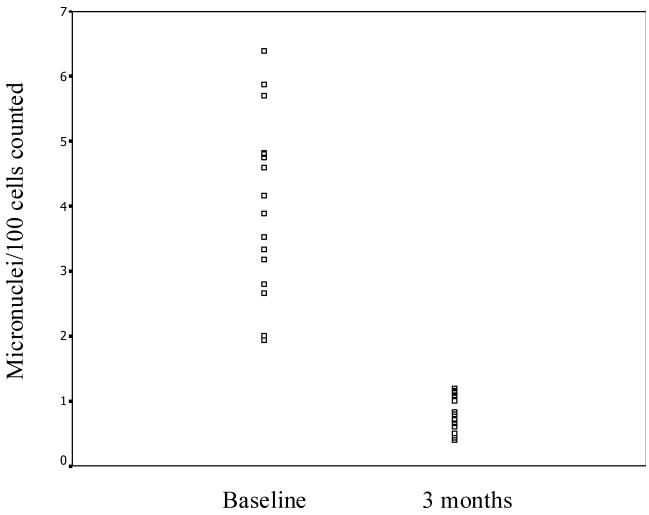

Fig. 2 Frequency of MEC from OLP lesions before and after 3 months of BC supplementation.

Table 2 Frequency of MEC in OLP lesions and normal-appearing mucosa before and after BC supplementation

Location	Percent MEC Mean ± SD (range)	
	Before treatment	After treatment
OLP lesion	3.89 ± 1.32 (1.94-6.4)	0.85 ± 0.25* (0.4-1.2)
Normal-appearing mucosa	0.41 ± 0.42 (0-1.78)	0.30 ± 0.26 (0-1.0)

*$P < 0.001$

Table 3 Serum levels of BC and retinol at baseline and after three-month BC supplementation

		Range (μg/dl)	Mean ± SD (μg/dl)
BC	Baseline serum	1.75 - 76.05	35.25 ± 20.51
	3-month serum	50.82 - 1,146.07	483.36 ± 384.95
Retinol	Baseline serum	15.36 - 61.35	29.75 ± 13.60
	3-month serum	24.50 - 82.63	38.09 ± 14.99

BC and retinol assay

Baseline and three-month serum BC and retinol are shown in Table 3. In pretherapy, 13 patients had serum BC levels lower than normal values. Three patients had low serum retinol. One patient had lower than normal serum retinol and BC values. After treatment, all patients had serum BC levels at or above normal values (50-300 μg/dl) (17), and had normal serum retinol (20.1-80.2 μg/dl) (18) (Table 3). No significant changes in serum retinol were seen after BC supplementation. Mild and reversible yellowing of the palms occurred in 12 patients. Of these, two patients also had a yellowish soft palate. Before and after treatment, liver enzymes were within normal limits in all patients.

Discussion

We evaluated the effects of three months of supplemental BC in patients with atrophic and/or erosive OLP. BC has been reported to exhibit strong antioxidant and immunoregulating properties with no toxicity (19-21). The BC dosage of 60 mg daily (15 mg, four times) was in the therapeutic range. The dosage was the same as in a recent study (17), but differed from that in a previous study (12). Increased BC levels at or above normal values were observed in all patients. This was comparable to the results of Silverman et al. (17). It has been shown that a proportional increase in tissue BC levels corresponds to increased serum BC levels (13,17). We found that supplementation of BC did not significantly alter the concentration of retinol. This finding was in agreement with other reports (17,22).

The treatment of oral mucosal dysplasia such as OLP with BC has been studied (11). A response rate of 44% for overall dysplastic lesions was reported. Although the pathogenesis of OLP remains unknown, it is clear that once the immune system has been activated, LP is principally cell-mediated. Lymphocytes are recruited into the mucosa by the upregulation of adhesion molecule expression and damage to the keratinocytes while the basement membrane is mediated predominantly by cytotoxic T-lymphocytes (23). Changes in the clinical features of OLP after 3-month supplementation of BC were observed. Without any topical medication for OLP, the changes could possibly stem from either the nature of the lesions themselves (1) or the immunoregulating properties of BC (19-21). Further investigation with a placebo group would clarify this issue.

Micronuclei are formed mainly from chromatids or chromosome fragments, which remain excluded from the main cell nucleus following mitosis. They indicate an increased probability of a reshuffling of gene sequences, which can lead to the activation of various oncogenes following their transposition or amplification (24). The second source of micronuclei is from aberrant chromosomes. This phenomenon leads to aneuploidy, a common feature of many human carcinomas (25). The frequency of MEC in normal mucosa (0.41%) in this group of patients was similar to previous reports (0.5%) (24,25). The frequency of MEC in OLP lesions (3.89%) was found to be similar to that sampled from areas of leukoplakia (3.5%) (26). None of our patients had plaque-type OLP, which is difficult to distinguish from oral leukoplakia. Furthermore, the OLP lesions showed no epithelial atypia or dysplasia and were confirmed by immunofluorescence techniques before inclusion in the study. A significant increase in the frequency of MEC in atrophic OLP and erosive OLP as compared to the adjacent normal mucosa has recently been reported (10).

A decrease in MEC frequency after BC supplementation in patients with oral leukoplakia has been confirmed (12).

With no placebo arm for comparison in this study, MEC frequency was compared before and after supplementation within the same location and also between OLP lesions and normal-appearing mucosa (about 2 cm from the lesions). The latter comparison was determined because the MEC frequency in atrophic and erosive OLP has been found to be elevated when compared with adjacent normal mucosa (10). After BC supplementation, the concentration of BC in buccal mucosal cells would increase along with serum BC levels (27). BC acts as an antioxidant (28;29), inhibiting free radical production. It has been demonstrated that carotenoids can prevent mutagenesis, genotoxic effects or malignant transformation (30). These biomechanisms seemed to be associated with a significant decrease in MEC frequency in OLP lesions. The findings showed that the antioxidant rather than immunoregulating properties of BC affected most lesions (16/20), as they remained atrophic and erosive. A statistically significant reduction in MEC frequency was not observed in normal mucosa, probably because the initial frequency of MEC was lower at the normal sites. Nevertheless, a significant difference in MEC frequency was found between the lesions and the adjacent normal mucosa after supplementation. Relapse of the lesions to high MEC frequency after discontinuation of BC supplementation may have occurred; this study did not examine MEC frequency after BC was discontinued.

Our findings show the benefits of BC supplementation on decreasing MEC frequency in Thai patients with OLP. We agree with Nagao et al. (31) on the uncertain benefits of BC on OLP treatment, as most patients experienced no improvement in lesions. Nonetheless, our results imply that diets rich in antioxidants might be useful in decreasing MEC frequency in OLP.

Most (14/20) of our atrophic and/or erosive OLP patients had low serum BC at the start of treatment. The relationship between atrophic and erosive OLP and BC deficiency should be investigated further. We were unable to compare the effects of BC supplementation on clinical changes in OLP in patients with normal or low serum BC, as the number of patients in each group was too low.

Micronuclei can serve as biomarkers for increased cancer risk, as they have been reported to arise in response to DNA-damaging agents (32). The advantage of using micronuclei is that the frequency can be measured repeatedly by non-invasive methods. Changes in MEC frequency are detectable within two months after a chemopreventive regime (24). Furthermore, the results can be compared with the frequency of MEC in normal mucosa in individual patients. Using this technique, high-risk lesions can be detected early and proper treatment can be initiated promptly (33). One concern on the use of micronuclei as a biomarker is their non-specificity. However, current evidence suggests that accumulated genetic damage is important in carcinogenesis. Micronuclei can be used to assess DNA damage and have been found to associate with the amplification of specific oncogenes (34). Exfoliative cytology performed for micronuclei detection can also be used to assess genetic/molecular changes in biopsy specimens from the same sites (35).

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