Anti-Cancer Drugs, 2, pp. 581–589

In vitro effects of β-carotene on human oral keratinocytes from precancerous lesions and squamous carcinoma

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Human keratinocytes, obtained from bioptic specimens of healthy and preneoplastic oral mucosa, and from human cell lines from oral cavity tumors (KB and SCC-25) were treated with β-carotene (10 μM). The colony forming efficiency (CFE), the proliferation rate and the frequency of micronucleated cells were measured in these cultures. CFE was significantly reduced ($p < 0.05$) by β-carotene treatment in cells from healthy mucosa and in KB cells. Decreases ($p > 0.05$; NS) were also observed in cells from pathological mucosa and in SCC-25 cells. Cell proliferation rate was not substantially affected by β-carotene in all cultures. Finally, a decreased frequency of micronucleated cells was found in treated cultures, but significant reductions ($p < 0.05$) were only observed in cultures from oral mucosa (healthy and pathological) as well as in KB cell cultures. Our results indicate that β-carotene is able to reduce the clonogenic activity (CFE), even if it does not seem to influence cell proliferation, and that it has a protective effect against genotoxic damage.

Key words: β-Carotene, cancer chemoprevention, cell cultures, oral premalignancy.

Introduction

Squamous cell carcinoma represents about 90% of malignant lesions of the oral cavity. The tumor may arise from normal mucosa but it may also start from 'oral leukoplakia', a clinically detectable white lesion. The relationship between leukoplakia and cancer has been reported by several authors.[1–5] In some cases oral leukoplakia is the local clinical occurrence of clinically undetectable precancerous illness of the whole oral mucosa (field cancerization).[6,7]

Over the last decade, agents inducing variations of cell differentiation have played a considerable role in chemoprevention of head and neck cancer. Retinoids, natural and synthetic analogs of Vitamin A (retinol), have been used to reverse oral leukoplakia[6,8] and to reduce the rate of new occurrences (second primary tumors) in patients without evidence of disease after local treatment with surgery and/or radiotherapy for squamous cell carcinoma of head and neck.[9]

During the last years, carotenoids and, particularly, β-carotene have attracted considerable interest as potential chemoprevention agents. β-Carotene is present in many foods as a Vitamin A precursor (provitamin). Epidemiologic studies have shown a correlation between high intake of carotenoids in the diet and low risk of cancer.[10,11] Even if recent data show that β-carotene is not protective for basal and squamous cell cancer of the skin,[12] there are several pieces of data that confirm the significant role of β-carotene in the prevention of head and neck cancer. It is known that low levels of β-carotene are present in exfoliated mucosa cells of subjects at elevated risk for oral cancer.[13] β-Carotene levels increase in cells of oral mucosa after oral administration of this provitamin.[14,15] Furthermore, β-carotene administration decreases the frequency of cells with micronuclei that are a

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sign of genotoxic damage.[16] Differently from retinoids, oral administration of β-carotene does not have any toxic effects and can therefore be taken for a long time in chemoprevention clinical trials with a good compliance of the treatment. Recent clinical data have demonstrated the efficacy of β-carotene in reversing oral leukoplakias.[17–20]

Clinical results of leukoplakia treatments, with several differentiating agents (retinoids, carotenoids etc.), are not consistent. Furthermore, it is not possible to forecast the clinical response in single patients. This information could be used in order to assess the treatment benefit (in consideration of side and toxic effects) that could derive by the long time of administration which is always required in chemoprevention therapy.

In the present study, in support of clinical application, we set up an *in vitro* system which allows us to investigate the action of differentiating substances on keratinocyte cultures obtained from preneoplastic oral mucosa.

In a previous report we have shown that, according to Rheinwald and Green,[21] keratinocytes of healthy oral mucosa can be cultured *in vitro* and that they are able to reconstitute a stratified epithelium suitable for transplantation.[22] In the same way, we obtained and cultured human keratinocytes starting from bioptic specimens of patients with oral white lesions. Moreover, we tested in culture the β-carotene in order to assess its *in vitro* influence on the colony forming efficiency (CFE), the proliferation rate and the frequency of micronucleated cells. Finally, we accomplished a similar evaluation on two human cell lines derived from oral squamous carcinoma (KB and SCC-25).

Materials and methods

Patients

Patients affected by clinically diagnosed oral leukoplakia were eligible for this study. All patients underwent an incisional small biopsy (2–4 mm^2) inside the lesion. A fragment obtained by longitudinal incision of the bioptic specimen was employed for histopathological diagnosis. Only hyperplastic and dysplastic lesions were considered. Only voluntary patients underwent another biopsy in a controlateral site of clinically healthy oral mucosa. After diagnosis, patients were introduced in a treatment plan comprehending either β-carotene or 13-*cis*-retinoid acid, orally administered. Final clinical results are still to be evaluated.

Keratinocyte primary cultures

Before the biopsy the oral cavity was carefully disinfected in order to reduce the rate of culture contamination. As reported above, keratinocytes of the lesion and healthy mucosa were obtained and cultivated according to the method previously described.[21] After several washes with phosphate buffered saline (PBS), bioptic specimens were minced and treated with a solution of trypsin (0.05%) and EDTA (0.01%) at 37°C to produce a suspension of single cells. Throughout the digestion (about 2 h) aliquots of cells were repeatedly collected (every 30 min). The overall number of cells obtained from every biopsy specimen ranged between 0.5 and 5×10^5. Cells were seeded ($1.5–3 \times 10^4$ cells/cm^2) on a feeder layer of lethally irradiated 3T3 J2 cells and cultured in Dulbecco's modified Eagle's (DMEM) and Ham's F12 media (2:1 mixture) supplemented with fetal calf serum (FCS) (10%), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 mg/ml), cholera toxin (0.1 nM), triiodo-thyronine (20 pM), penicillin (50 IU/ml) and streptomycin (50 μg/ml). After 2 days epidermal growth factor (10 ng/ml) was also added to the culture medium. Subconfluent cultures were trypsinized as above and isolated cells were seeded in secondary culture at a lower density than primary culture ($0.6–2 \times 10^4$ cells/cm^2).

Cell line growth conditions

Two human cell lines (supplied by American Type Culture Collection, Rockville Pike, MD) were used: KB (oral epidermoid carcinoma) and SCC-25 (squamous cell carcinoma of tongue). KB cells were cultured in DMEM. SCC-25 cells were cultured in DMEM and Ham's F12 media (1:1 mixture) containing hydrocortisone (0.4 mg/ml). Growth media were supplemented with FCS (10%), penicillin (50 IU/ml) and streptomycin (50 μg/ml).

β-Carotene treatment

β-Carotene (Sigma), dissolved in *n*-hexane, was added in culture medium to a final concentration of 10 μM, starting from the plating day. *n*-Hexane alone was added at equal volume to the control medium, not exceeding 1% v/v.

In order to destroy endogenous retinol, which is normally present in the serum, FCS was exposed

overnight to UV light[23] before the addition to the culture medium in all the experiments to investigate the effect of β-carotene on cultures.

CFE evaluation

Single cells isolated from subconfluent secondary cultures of patients were seeded in duplicate at very low density (0.4–1.2 × 10^2 cells/cm^2) on lethally irradiated supporting 3T3 cells. Cells were allowed to grow for 12 days. Cultures were then fixed with 3.7% formaldehyde. Colonies were stained with 1% rhodamyne and counted. Likewise, the CFE of KB and SCC-25 cells were obtained using the appropriate culture medium.

The CFE was expressed as ratio of colonies obtained versus cells plated × 100.

Cell growth evaluation

Keratinocytes derived from oral biopsy: Cells isolated from subconfluent secondary culture were seeded in duplicate at a density of 0.5 × 10^5 onto 35 cm^2 plastic dishes (6-well Multiwell, Falcon, Becton-Dickinson) and cultured as described above. Until confluence was reached, fresh medium was replaced every 2 days and cells were counted every 24–48 h on a hemocytometer (Neubauer improved).

Cell lines: KB (1.5 × 10^5 cells/dish) and SCC-25 cells (1 × 10^5 cells/dish) were seeded in triplicate onto 35 cm^2 plastic dishes without a 3T3 cell feeder layer. Medium change and cell count were accomplished as described above. On the same days as cell counting, cell kinetic evaluation was achieved by the labelling index (LI) of bromodeoxyuridine (BrdUrd) pulses. The procedure involves the incorporation of BrdUrd (Sigma) at a final concentration of 10 μM for 30 min by cell cultures, labeling the BrdUrd with APAAP immuno-alkaline phosphatase technique using a monoclonal antibody anti-BrdUrd (DAKO-BrdUrd, Dakopatts).[3]

The BrdUrd LI, expressing the percentage of cells in S phase, was calculated as the ratio of labelled cells versus total cells × 100.

Micronucleated cell assay

The frequency of micronucleated cells was scored on cellular suspensions obtained by trypsinization of confluent secondary cultures. To do this the procedure described by Stich *et al.*[16] was used with the following modifications: the smear preparation of the fixed cells (3:1, methanol:acetic acid) was stained with 3% Giemsa solution. Approximately 3 × 10^3 cells were examined from each cell sample in order to determine significant differences between β-carotene treated and untreated cells.

Table 1. Characteristics of patients and CFE of cells derived from oral biopsy

Patient No.	Sex/age	Tobacco/alcohol	CFE	
			Normal	Pathological
1	M/36	no/no	—[b]	—[b]
2	M/38	yes/yes	—[a]	16.9
3	M/59	yes/yes	1.8	0.6
4	F/62	yes/no	18.1	—[b]
5	F/57	yes/yes	13.3	—[c]
6	F/74	no/no	31.7	5.8
7	M/77	yes/no	16.8	—[b]
8	M/67	yes/yes	16.0	22.0
9	M/35	no/no	22.5	21.6
10	M/16	no/no	45.4	23.8
11	M/53	yes/yes	11.1	—[c]
12	M/68	yes/no	5.7	—[b]
13	F/70	no/yes	20.2	11.0[c]
14	F/61	yes/yes	28.3	23.1
15	M/66	no/no	21.1	35.5
16	F/45	yes/yes	—[c]	26.0

[a] Biopsy not taken.
[b] Abortive colonies.
[c] Contamination.

Results

We studied a total of 16 patients. Characteristics of patients are presented in the Table 1.

Seventeen biopsies were withdrawn from oral pathological sites. One patient (no. 13) underwent two biopsies from different oral lesions. Histologic analysis of the specimens was performed: one biopsy (no. 12) was diagnosed as mild–moderate dysplasia, all the others as hyperplastic lesions.

We obtained cultured epithelium starting from 10 out of 17 pathological mucosa biopsies (58.8%) and 13 out of 15 biopsies of clinically healthy mucosa (86.6%).

CFE

The CFE of different cell populations is presented in Table 1. The CFE of cells from healthy mucosa was more elevated than that found for the pathological counterparts. Table 2 shows that CFE was reduced by β-carotene treatment in culture from both healthy and pathological mucosa, but a significant decrease ($p = 0.02$) was only observed in keratinocytes from healthy mucosa, while the difference was not significant ($p = 0.2$) in keratinocytes from pathological mucosa.

Table 2. Influence of β-carotene treatment on CFE of cells derived from oral biopsy[a]

Patient no.	Cells from normal mucosa*		Cells from pathological mucosa**	
	β−	β+	β−	β+
2	—	—	8.7	6
3	0.9	0.3	—[b]	—[b]
4	12.1	12	—	—
5	9.1	8.2	—	—
6	28	25.9	1.2	0.8
7	11.8	9	—	—
8	8.4	5.3	1.3	0.9
9	10.9	8.4	22	17.7
10	15.7	10.3	9.7	9.7
11	4.9	3.4	—	—
12	3.6	3	—	—
13	11.5	6	4.5	1.3
14	28.3	21.7	26.2	4.6
15	13.8	17.8	25.1	31.6
16	—	—	16.9	14.4

[a] β−, untreated cultures; β+, treated culture.
[b] Abortive colonies.
Difference between CFE of treated and untreated cells: *$p = 0.02$; **$p = 0.2$ (NS).

Table 3. Influence of β-carotene treatment on CFE of KB and SCC-25 cell lines[a]

Cell line	β−	β+	p
KB	13.1	6.2	0.02
SCC-25	36.1	31.3	0.3 (NS)

[a] β−, untreated cells; β+, treated cells.

A similar assessment accomplished on the CFE of KB and SCC-25 cell is reported in Table 3. As shown, a significant decrease was observed in KB cells ($p = 0.02$) after treatment, while no significant decrease ($p = 0.3$) was observed in SCC-25 cells.

Cell growth evalutation

Keratinocytes from oral biopsies: The cytokinetic evaluation of keratinocytes originated from oral biopsies was achieved using only cell counting, because of the presence in culture of irradiated 3T3 cells troubled both BrdUrd and [^{3}H]thimidine cellular incorporation, especially during the first days of culture.

As shown in Figure 1, the β-carotene treatment did not produce substantial modifications in the growth rates of cell populations tested. The evaluation was accomplished on healthy mucosa cells (*upper panels*) and oral lesion cells (*lower panels*). In both cell populations, the doubling time, measured during the exponential growth phase, was not substantially modified by β-carotene treatment.

Cell lines: Growth curves of KB (a) and SCC-25 (b) cells are shown in Figure 2. No significant modification was observed after β-carotene treatment.

Cell kinetic evaluations, performed by BrdUrd LI (Figure 3), seem to confirm that β-carotene does not substantially influence cell proliferation rates.

As can be seen in Figures 2 and 3, data relative to KB cells, obtained by comparison between treated and untreated cells, is more uniform than data for SCC-25 cells.

Micronucleated cells assay

The micronuclei assay was applied to confluent secondary cultures of both pathological and healthy mucosa (10 and 13 patients, respectively). For each

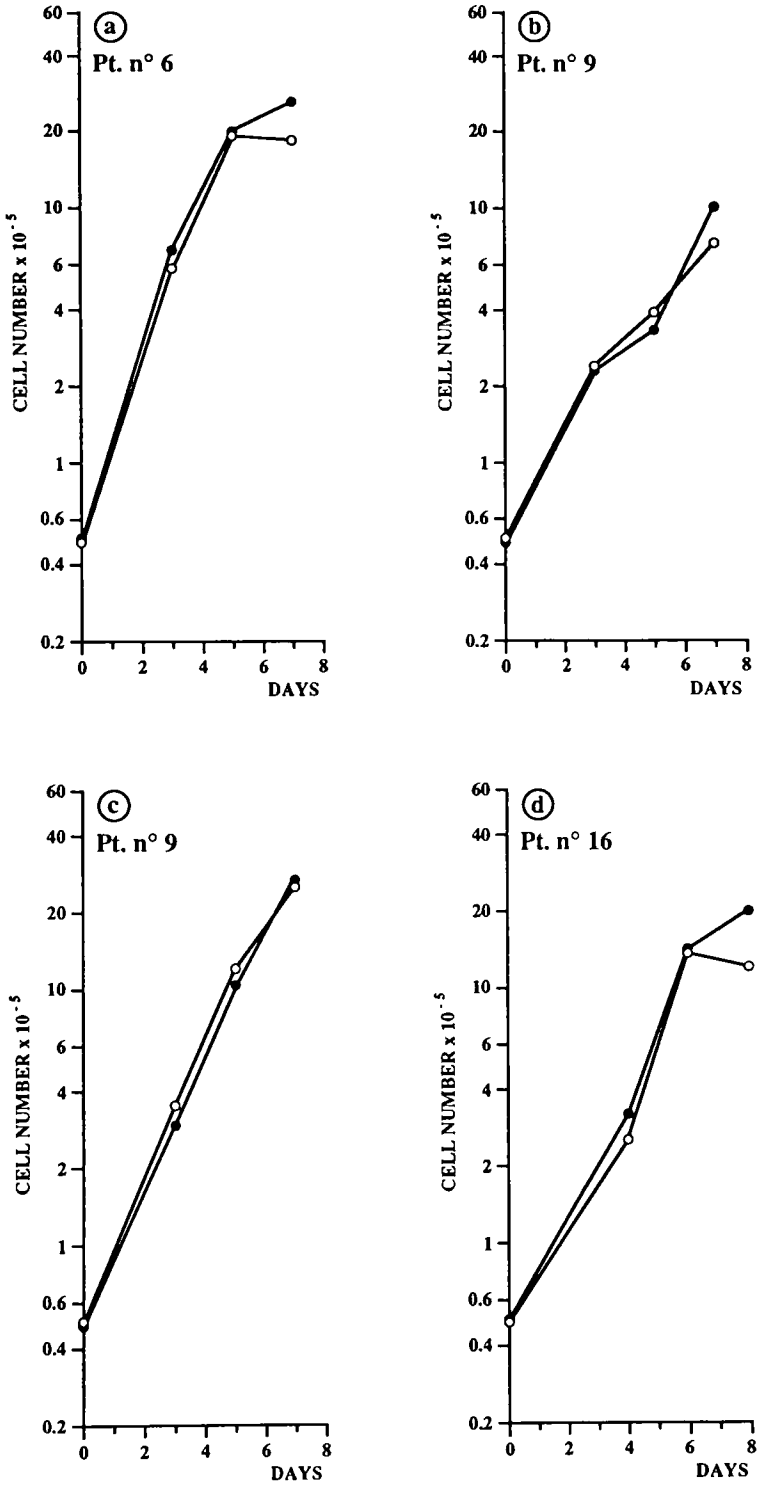

Figure 1. Growth curves of keratinocytes from oral biopsies of different patients in the absence (●) or in the presence (○) of β-carotene (10 μM). *Upper panels*: cells from healthy mucosa. *Lower panels*: cells from pathological mucosa. Experiments were performed in duplicate and the values presented are the mean of three different experiments. Doubling time (in hours) of the cell populations (untreated versus treated) during the exponential growth phase: a = (19 versus 21); b = (32 versus 32); c = (26 versus 27); d = (23 versus 21).

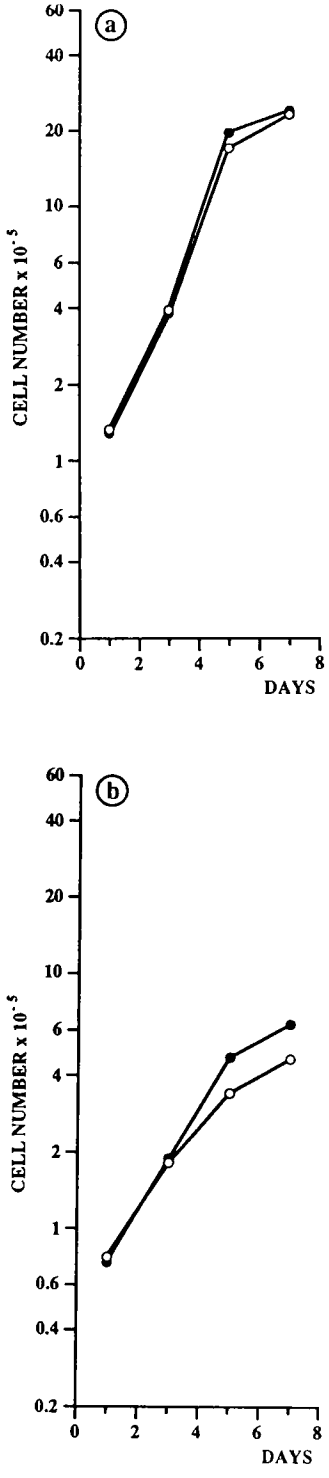

Figure 2. Growth curves of (a) KB and (b) SCC-25 cells in the absence (●) or in the presence (○) of β-carotene (10 μM). Experiments were performed in triplicate and the values presented are the mean of three different experiments. Doubling time (in hours) of the cell populations (untreated versus treated) during the exponential growth phase: a = (20 versus 22); b = (36 versus 39).

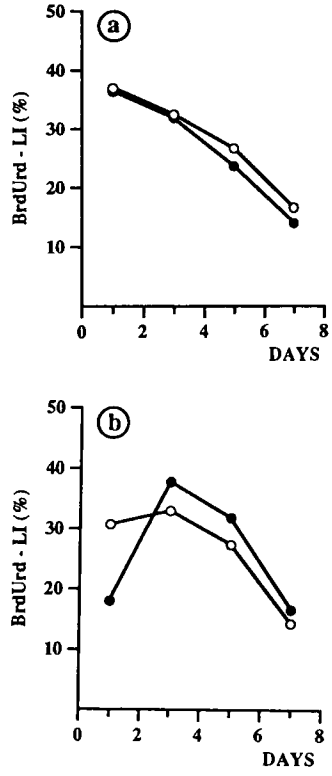

Figure 3. BrdUrd incorporation (labelling index) of (a) KB and (b) SCC-25 cells in the absence (●) or in the presence (○) of β-carotene (10 μM). Experiments were performed in triplicate and the values presented (percentage of labelled cells) are the mean of three different experiments.

subject studied the frequency of micronuclei was scored on both treated and untreated cells.

Results are reported in Table 4. Cellular suspension obtained from pathological mucosa presented a greater number of micronucleated cells than that obtained from healthy mucosa. Furthermore, in all cases examined, a highly statistically significant reduction (healthy cells $p = 0.0003$; pathological cells $p = 0.0001$) of micronucleated cells was found in treated cultures.

The test was also applied to cultures of KB and SCC-25 cells (Table 5). After treatment with β-carotene, the reduction of micronucleated cells was significant only in KB cells ($p = 0.04$).

In SCC-25 cells, a reduction in the percentage of micronuclei was observed in all samples examined, but did not reach a significant value ($p = 0.07$).

Discussion

Several sets of clinical data have demonstrated that β-carotene is effective as a chemopreventive agent in oral cancer.

Table 4. Influence of β-carotene treatment on micronucleated cell frequency of cultures derived from oral biopsy[a]

Patient no.	Cells from normal mucosa*					Cells from pathological mucosa**				
	Analyzed cells	β−		β+		Analyzed cells	β−		β+	
		N	‰	N	‰		N	‰	N	‰
2	—	—	—	—	—	3002	43	14.32	21	6.99
3	3005	15	4.99	4	1.33	3020	57	18.87	27	8.94
4	3004	21	6.99	17	5.66	—	—	—	—	—
5	3100	33	10.64	29	9.35	—	—	—	—	—
6	3003	6	2	2	0.67	3010	49	16.28	25	8.31
7	3001	24	8	11	3.66	—	—	—	—	—
8	3000	9	3	6	2	3002	39	12.99	31	10.33
9	3040	11	3.62	9	2.96	3024	51	16.86	33	10.91
10	3050	41	13.44	27	8.85	3015	42	13.93	29	9.96
11	3018	27	8.95	20	6.63	—	—	—	—	—
12	3010	19	6.31	11	3.65	—	—	—	—	—
13	3004	8	2.66	6	2	3050	54	17.70	30	9.84
14	3002	7	2.33	3	1	3034	69	22.74	34	11.21
15	3007	29	9.64	11	3.65	3021	47	15.56	29	9.60
16	—	—	—	—	—	3050	51	16.72	30	9.83

[a] β−, untreated cultures; β+, treated culture.
Difference between the mononucleated cells frequency of β+ and β−: *$p = 0.0003$; **$p = 0.0001$.

Table 5. Influence of β-carotene treatment on micronucleated cell frequency of KB and SCC-25 cell line cultures[a]

KB cells					SCC-25**				
Analyzed cells	β−		β+		Analyzed cells	β−		β+	
	N	‰	N	‰		N	‰	N	‰
3050	60	19.67	4	1.31	1558	37	23.75	7	4.49
3002	22	7.33	5	1.66	3010	11	3.65	7	2.32
3000	20	6.66	7	2.33	3100	56	18.06	24	7.74
4000	23	5.75	11	2.75	3077	44	14.30	18	5.85
2002	14	6.99	12	5.99					

[a] β−, Untreated culture; β+, treated culture.
Difference between CFE of treated and untreated cells: *$p = 0.04$; **$p = 0.07$ (NS).

We tested β-carotene on keratinocyte cultures obtained from oral mucosa of patients affected by oral precancerous lesions. In particular, we studied some biologic effects that may support the *in vivo* chemopreventive action such as the deviations of CFE, proliferation rate and presence of micronuclei. Analogous aspects were also assessed on human cell lines of oral cavity tumors.

Altogether, cells derived from oral lesion biopsies showed (1) a lower CFE and (2) a higher frequency of micronucleated cells than those from healthy mucosa biopsies. However, the number of studied patients is, up to now, too limited in order to reach a definitive biological conclusion. For this reason we stressed, principally, the influence of the β-carotene on each separate cell type.

The presence of *n*-hexane, used for β-carotene dissolution, and UV light, employed to destroy retinol of the serum, account for the global reduction of CFE, reported in Table 2, with respect

to CFE obtained in normal culture conditions and reported in Table 1.

Our data concerning the influence of β-carotene on both CFE and growth rates of cultured keratinocytes is apparently contradictory. In particular, β-carotene, at a concentration of 10 μM, in our culture conditions, does not seem to influence cell proliferation, but it is able to reduce clonogenic activity which is correlated to the fraction of cells in active proliferation (the only ones competent to form colonies).

In order to clarify this aspect, it must be considered that keratinocytes can release soluble growth factors, such as TGF-α, able to produce autocrine and paracrine cell stimulation.[24] Therefore, when cells are plated at heavy or standard concentrations, TGF-α or related molecules could mask the influence of β-carotene on cell differentiation. Culture conditions characterized by a very low density of cellular plating (that are employed to evaluate CFE) could be more sensitive to the influence of β-carotene.

In our system, β-carotene seems to be effective in reducing the frequency of micronucleated cells in both healthy and pathological mucosa cell cultures. These data confirm the results of *in vivo* studies which demonstrated a remission of oral leukoplakias and a reduction of micronuclei in oral mucosal cells of patients treated with β-carotene.[18,19]

A highly significant difference in the percentage of micronucleated exfoliated cells of oral mucosa between smokers and non-smokers individuals was recently reported.[25] Nevertheless, the effect of smoking and/or alcohol on the induction of micronuclei is unresolved. In our study, no significant correlation was found between patient characteristics and the frequency of micronuclei, but our sample of patients was small and did not comprise strong smokers or strong consumers of alcohol.

We believe that the experimental model employed is an effective system for oral premalignancy studies. We have demonstrated that it is possible to easily obtain keratinocyte cultures starting from a small biopsy of oral lesions. Cultured cells can be employed to determine biomarker expressions (e.g. micronuclei) and to test *in vitro* several drugs. Consequently, the system could be used as a predictive test of clinical responses by single drugs or association ones.

In the near future we will undertake a prospective study in order to evaluate the effect of *in vitro* and *in vivo* treatment with β-carotene.

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(Received 17 October 1991; accepted 30 October 1991)