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Levels of Proteolytic Activities as Intermediate Marker Endpoints in Oral Carcinogenesis[1]

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Abstract

It is essential to identify intermediate marker endpoints of carcinogenesis for the evaluation of the effectiveness of cancer-chemopreventive agents. We have observed that levels of proteolytic activities (as detected by 4 different substrates) are increased 2–3-fold ($P < 0.003$) in oral buccal mucosa cells of smokers and patients with oral leukoplakia or erythroplakia as compared to a nonsmoking comparison group. In addition, proteolytic activity levels in the buccal cells were increased nearly 3-fold in patients with oral trauma ($P < 0.01$) or diabetes ($P < 0.02$), as well as pregnant women ($P < 0.04$). Excluding these subgroups of patients in epidemiological studies increases the differences in levels of proteolytic activities between both the nonsmoking comparison group and smokers and between the comparison group and patients with oral leukoplakia or erythroplakia. Evaluation of prerandomization levels of proteolytic activities of patients in cancer chemoprevention trials will increase the statistical power by allowing stratified randomization based on levels of proteolytic activities. The observed increases in levels of proteolytic activities in tissues at higher than normal risk of cancer development suggest that levels of proteolytic activities should be used as immediate marker endpoints in human cancer prevention trials using protease inhibitors as potential anticarcinogenic agents.

Introduction

Protease inhibitors are highly effective cancer-preventive agents, with the ability to prevent or suppress carcinogenesis completely in different assay systems *in vivo* and *in vitro* (1, 2). Treatment of cultured cells with certain protease inhibitors inhibits radiation- and carcinogen-induced transformation (2). In animal models, carcinogen treatment raises the levels of certain types of proteolytic activities in clinically normal-appearing tissue, and protease inhibitor treatment reduces these elevated levels to normal levels (*e.g.*, Ref. 3). The ability of protease inhibitors to suppress several stages of the carcinogenic process indicates that such compounds may be useful human cancer-chemopreventive agents. As with all candidate chemopreventive agents, IMEs[3] must be established before human trials can quantitate the effects of treatments. We hypothesize that premalignant human tissue might be analogous to carcinogen-treated tissue in animals and will have elevated levels of proteolytic activities that could serve as IMEs in human cancer prevention studies (4, 5). Human oral leukoplakia and erythroplakia are used frequently as premalignant lesions in cancer prevention studies, most notably those involving antioxidant vitamins (6). β-carotene has been proposed as a cancer-preventive agent (7), and both retinoids and carotenoids have been shown to cause regression of oral leukoplakia in some patients (8).

The first human trial in which protease inhibitors will be evaluated as cancer-preventive agents will involve the soybean-derived BBI as the anticarcinogenic protease inhibitor and oral leukoplakia/erythroplakia as the human tissue at higher than normal risk of cancer development. It has been observed previously that BBI is highly effective as a cancer-preventive agent in hamster cheek pouch carcinogenesis studies (1, 3, 9) in which leukoplakia represents a premalignant lesion (10). Exposure of hamster oral epithelium to the polycyclic hydrocarbon carcinogen 7–12-dimethylbenz(*a*)anthracene results in increased levels of certain types of proteolytic activities. Such increases can be reversed or reduced by treatment of the hamster oral epithelium with BBI. In parallel with the reduction in levels of proteolytic activities, BBI inhibits oral carcinogenesis (1, 3, 9). It is believed that the inhibition of proteolytic activity in carcinogen-treated tissue is related to the mechanism by which BBI suppresses carcinogenesis (1–5, 9, 11).

We assessed levels of proteolytic activity as possible IMEs in individuals at higher than normal risk of oral cancer development, *i.e.*, smokers and those with oral leukoplakia or erythroplakia. It is widely recognized that smokers have an elevated risk of oral cancer development and that tobacco is the most common cause of oral cancer (6). In the studies reported here, we used four different synthetic substrates to detect proteolytic activities in the buccal cell samples collected. We also report data from a prospective study involving 18 leukoplakia patients who were taking 30 mg/day β-carotene for periods of 3, 6, 9, or 12 months.

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[3] The abbreviations used are: IME, intermediate marker endpoint; BBI, Bowman Birk inhibitor; MCA, amino-methyl coumarin.

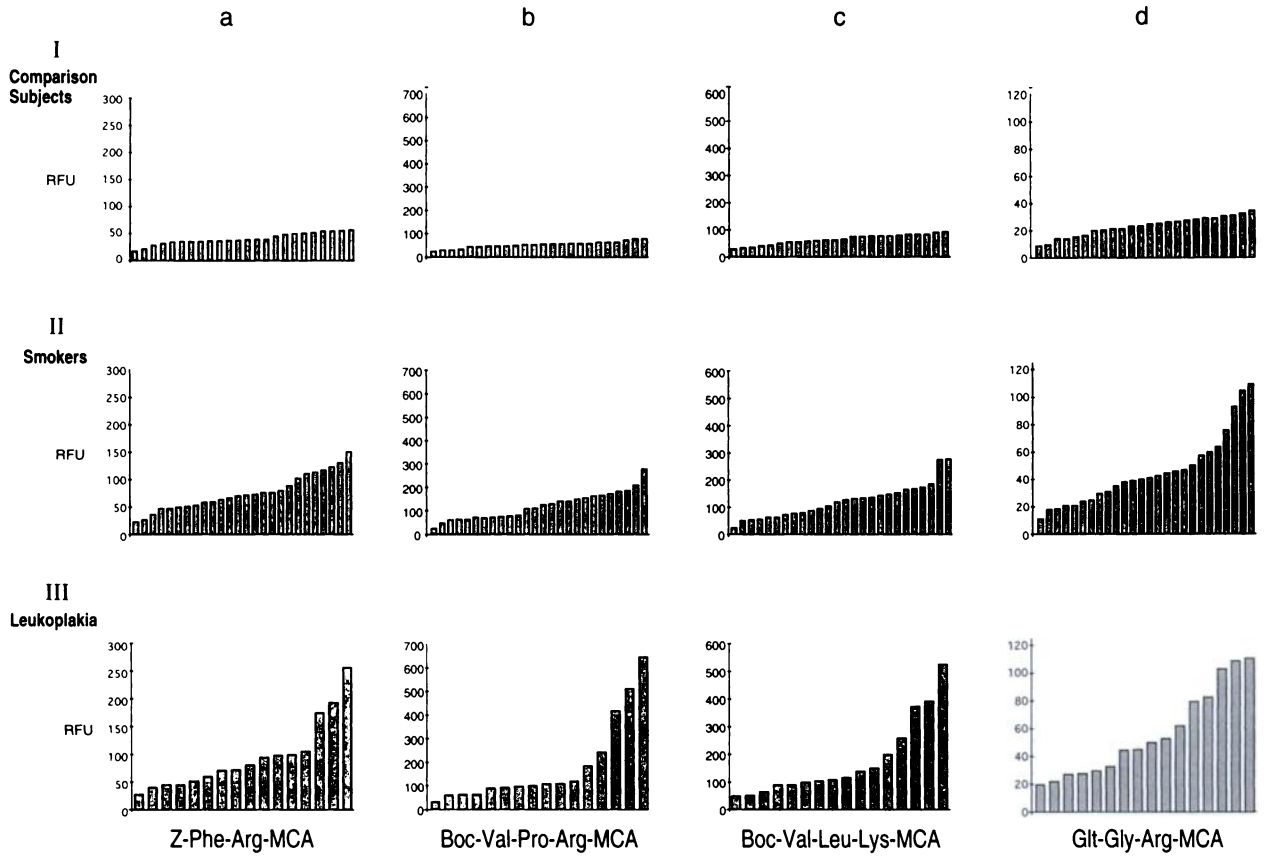

Fig. 1. Levels of proteolytic activities in the oral buccal mucosa cells. *I*, 26 comparison subjects; *II*, 41 smokers; and *III*, 20 patients with leukoplakia. Data are expressed as the relative fluorescent units (*RFU*) per μg protein. RFU are determined for each of four substrates [Z-Phe-Arg-MCA (*a*), Boc-Val-Pro-Arg-MCA (*b*), Boc-Val-Leu-Lys-MCA (*c*), and Glt-Gly-Arg-MCA (*d*)] based on the absorbance of the MCA reporter group relative to that of 10^{-7} M MCA standard. Data for each subject group are plotted on the same scale and show the consistent low level of protease activity for each substrate among comparison subjects (20 males and 6 females) of various ages. Patients with leukoplakia and smokers have much more variable levels of proteolytic activities in their buccal mucosa cells, and the levels may be much higher than that of comparison subjects.

Materials and Methods

The 113 participants in this study were selected as outpatients from dental clinics at the University of Pennsylvania and Veterans Administration Medical Centers in Philadelphia, PA. The study group was composed of 90 males and 23 females, ranging in age from 18 to 79 years. Patients were interviewed and grouped on the basis of medical histories (including history of smoking and drinking) and intraoral examination. The purpose of the study and sample collection procedure was explained, and all participants signed a consent form. The comparison population was selected as healthy, nonsmoking individuals with no history of heavy drinking and who on intraoral examination were deemed to be "within normal limits." Leukoplakia patients were selected after clinical examination and diagnosis. Smokers were selected as those with a history of smoking, but not heavy drinking, and who were regarded as within normal limits by intraoral examination. Although we recognize that ethanol is an important promoter of tobacco-induced oral cancer (6), we chose to limit the present study and exclude heavy drinkers. The subgroups of patients with oral trauma or diabetes and pregnant women were excluded from the comparison groups because the patients had elevated levels of proteolytic activities associated with these conditions. The validity of these exclusions was established by recruiting additional patients to each of these subgroups and testing that the mean levels of proteolytic activities of the subgroups and comparison group were significantly different. The levels of proteolytic activities in buccal mucosa were determined for an additional 18 patients from the University of California Medical Center (Irvine, CA) who were diagnosed as having leukoplakia and were administered β-carotene capsules (30 mg/day) for periods of 3, 6, 9, or 12 months.

Buccal cells were obtained by gently brushing the entire surface inside the mouth of each patient with a cytology brush and collecting the cells in sterile PBS. The brushing procedure was performed gently to avoid bleeding (bloody samples were discarded). The cells were pelleted by centrifugation at 5000 × *g* for 5 min at 4°C, flash frozen in liquid nitrogen, and stored at −80°C until analyzed.

Measurement of Protease Activity. Protease activity was analyzed as described previously (11). Briefly, buccal cell pellets were thawed on ice and homogenized in 600 μl ice-cold PBS by 10 strokes in a sterile glass-teflon homogenizer. The level of proteolytic activity was measured by incubating 50-μl aliquots of sample in 0.1 M Tris (pH 7.5)-5 mM $CaCl_2$, with each of the following synthetic substrates; Z-Phe-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Val-Leu-Lys-MCA, or Glt-Gly-Arg-MCA (Peninsula Laboratories, Inc., Belmont, CA). These particular

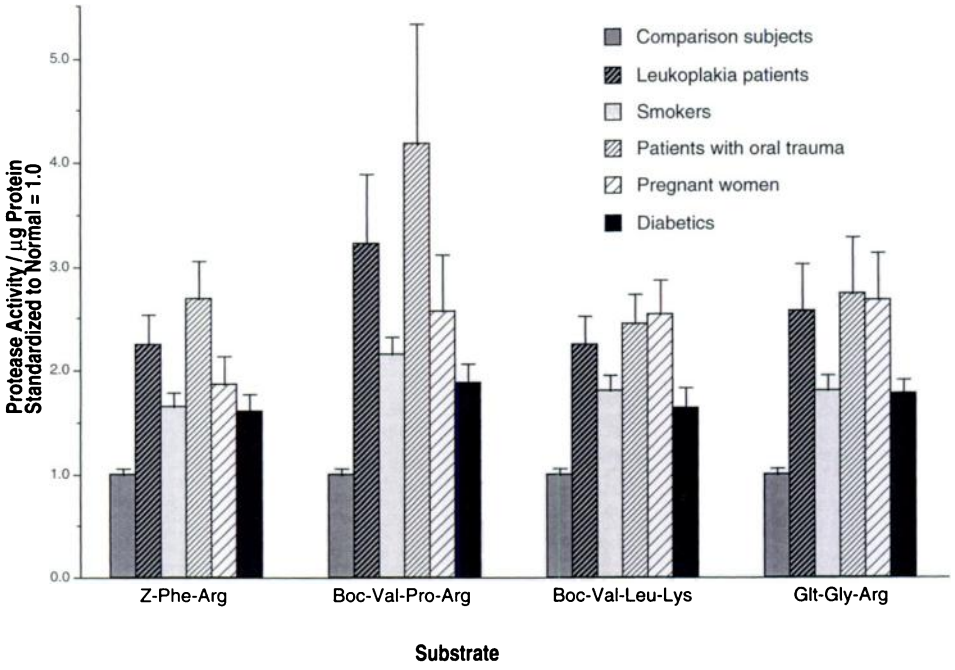

Fig. 2. Factors associated with increased levels of proteolytic activities in oral buccal mucosa cells. Data are standardized such that the level in comparison subjects for each substrate is equal to 1.0. The pattern of elevated levels of protease activity related to the various conditions is similar in each of the four substrates. All of the increased levels of proteolytic activity are statistically significant ($P < 0.02$). *Columns*, mean; *bars*, SE.

Table 1 *t* test of comparison subjects *versus* smokers and leukoplakia patients

Mean levels of proteolytic activity of four substrates in 26 comparison subjects compared to levels in 41 smokers and 20 patients with leukoplakia. In both cases, levels of proteolytic activity were significantly elevated over levels detected in comparison subjects when analyzed by a two-tailed Student's *t* test.

Substrate	Comparison subjects		Smokers			Leukoplakia		
	Mean	SD	Mean	SD	*P*	Mean	SD	*P*
Z-PHE-ARG-MCA	42.3	10.9	69.7	34.0	0.0001	94.7	56.7	0.0006
BOC-VAL-PRO-ARG-MCA	54.0	14.3	115.8	59.4	0.0001	173.9	164.6	0.0042
BOC-VAL-LEU-LYS-MCA	23.5	7.5	42.3	23.8	0.0001	52.6	29.4	0.0003
GLT-GLY-ARG-MCA	63.0	19.3	114.3	60.3	0.0001	162.0	129.5	0.0029
n	26		41			20		

substrates were chosen because they are known to be cleaved by proteases the level of activity of which are reduced in normal-appearing areas of BBI-treated hamster oral epithelium exposed to 7–12-dimethylbenz(*a*)anthracene (3). After a 2-h incubation at 37°C, the reaction was terminated by dilution with 1.8 ml distilled H_2O. Release of the fluorescent reporter group, amino-methyl coumarin, was determined spectrophotometrically at excitation and emission wavelengths of 380 and 460 nM, respectively, in a Perkin Elmer Cetus fluorescence spectrophotometer. Protein content was determined by the Bradford method (12) with the use of BSA as standard. Data were standardized on the basis of protein content and expressed as proteolytic activity/µg protein. The results were analyzed by a two-tailed Student's *t* test, and $P < 0.05$ was considered significant.

Results

The primary comparisons are between the levels of proteolytic activities in buccal mucosa of 26 healthy comparison subjects (20 males and 6 females), 20 patients with leukoplakia, and 41 smokers. In addition, 13 patients with oral trauma, 8 diabetics, and 5 pregnant women were evaluated to confirm the validity of the exclusion of individuals with these characteristics from the comparison group. Samples from an additional 18 patients with leukoplakia were evaluated following treatment with β-carotene for periods of 3, 6, 9, or 12 months. The range of proteolytic activities for each of the 4 substrates in the 26 comparison subjects was very narrow, with no significant differences between male and female patients or variation on the basis of age of the patient (Fig. 1; *panel I*). Two comparison subjects and one pregnant female were reevaluated at later intervals of 2–19 months, and overall results varied by <10%. Smokers and patients with leukoplakia had much wider distributions of levels of proteolytic activities in their buccal cells (Fig. 1; *panels II* and *III*). Significantly higher levels of proteolytic activities for each of the 4 substrates were observed in smokers ($P < 0.0001$) and patients with oral leukoplakia ($P < 0.005$) when compared to the levels in the 26 comparison subjects (Fig. 2; Table 1).

The upper limit of normal proteolytic activity for each substrate was defined by ranking all the subjects from the leukoplakia, smoking, and comparison groups from lowest to highest and assuming that the lowest 26 values, the number of subjects in the comparison group, represented the range of normal or low values. This approach allows subjects in each of the three groups an equal chance to be in either the normal or high

Table 2 Relative odds of above median levels of proteolytic activities for comparison subjects *versus* smokers and nontreated leukoplakia patients

Number of subjects with levels of proteolytic activities above the median (high) and below the median (low) levels determined for subjects in the comparison group. The OR[a] and *P*-value of the OR is presented for each substrate in the subgroups of smokers and patients with leukoplakia.

Substrate	Total		Comparison subjects		Smokers				Leukoplakia			
	High	Low	High	Low	High	Low	OR	*P* value	High	Low	OR	*P* value
Z-PHE-ARG-MCA	61	26	11	15	31	10	4.2	0.006	19	1	25.9	0.003
BOC-VAL-PRO-ARG-MCA	61	26	8	18	34	7	10.9	0.0001	19	1	42.7	0.000
BOC-VAL-LEU-LYS-MCA	61	26	12	14	31	10	3.6	0.014	18	2	10.5	0.005
GLT-GLY-ARG-MCA	61	26	12	14	32	9	4.1	0.007	17	3	6.6	0.011

[a] OR, odds ratio.

Table 3 *t* test of comparison subjects *versus* patients with oral trauma, diabetics, and pregnant women

Mean levels of proteolytic activity of four substrates in 26 comparison subjects compared to levels in 13 patients with oral trauma, 8 diabetics, and 5 pregnant women. In all cases, levels of proteolytic activities were significantly elevated over levels detected in the comparison subjects when analyzed by a two-tailed Student's *t* test.

Substrate	Comparison subjects		Oral trauma			Diabetics			Pregnant women		
	Mean	SD	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Z-PHE-ARG-MCA	42.3	10.9	113.6	55.7	0.0006	67.6	20.0	0.0090	78.9	25.5	0.0343
BOC-VAL-PRO-ARG-MCA	54.0	14.3	226.3	221.7	0.0016	101.2	27.8	0.0016	138.6	66.1	0.0101
BOC-VAL-LEU-LYS-MCA	23.5	7.5	57.3	25.7	0.0005	38.3	13.1	0.0158	59.6	17.5	0.0106
GLT-GLY-ARG-MCA	63.0	19.3	172.5	125.8	0.0089	111.6	25.0	0.0006	168.8	64.0	0.0215
n	26		13			8			5		

category under the null hypothesis. Smokers were significantly more likely to have elevated levels of proteolytic activities than were nonsmokers; the odds ratio of smokers having elevated proteolytic activities ranged from 3.6 (for the Boc-Val-Leu-Lys substrate) to 10.9 (for the Boc-Val-Pro-Arg substrate), as shown in Table 2. The statistical significance and strength of the association was even stronger when patients with leukoplakia were compared to the comparison group. The odds ratio of having elevated levels of proteolytic activities ranged from 6.6 (for the Glt-Gly-Arg substrate) to 42.7 (for the Boc-Val-Pro-Arg substrate).

We also observed significantly elevated levels of proteolytic activities in several other conditions: 13 cases of oral trauma ($P < 0.01$), 8 diabetics ($P < 0.02$), and 5 pregnant women ($P < 0.04$), as shown in Fig. 2 and Table 3. These subgroups were examined in additional detail because individuals in the initial survey with these characteristics had high levels of proteolytic activities. The confirmation of the high levels of activities in these subgroups of patients allowed their exclusion from the final comparison and patient groups.

In the prospective intervention trial with β-carotene, the levels of proteolytic activities in the buccal cells of the 18 individuals treated with β-carotene appear to vary by length of time on treatment; the results of measurements from individual patients are shown in Fig. 3*a*. To examine the early effects of treatment, the patients with 3- or 6-month treatment with β-carotene were combined; later effects were examined by combining patients with 9 or 12 months of treatment. Comparison of the cumulative distributions of the levels of proteolytic activities suggests a trend in which the early effects of treatment result in higher levels of proteolytic activities and the later effects result in lower levels of proteolytic activities when compared to the distribution of values for nontreated leukoplakia patients (Fig. 3*b*). However, the small number of samples available for analysis had inadequate statistical power to test for significant differences between these treatment and the comparison groups.

Discussion

The levels of proteolytic activities in the buccal mucosa cells of normal volunteers had a narrow distribution. Neither gender nor age of the patients affected the levels of activities (Fig. 1, *panel I*). The variability of levels of proteolytic activities for patients with leukoplakia was higher than in the comparison group, although some patients have levels of proteolytic activities that are comparable to those of comparison subjects (Fig. 1, *panel II*).

The overlapping distribution of patients in the comparison group with those in the leukoplakia group suggests that there is need to further clarify the clinical diagnoses of leukoplakia. It is conceivable that patients with clinical oral leukoplakia and elevated levels of proteolytic activities are at the highest risk of developing oral cancer. Clearly, not all clinical leukoplakia lesions are premalignant; estimates of the rate at which leukoplakia gives rise to cancer have varied from 3–17.5% (13–16). The highest estimate, 17.5%, was reported in a study of 257 patients followed for a mean period of 7.2 years (13). We speculate that the differences we observed in levels of proteolytic activities may allow us to distinguish between those lesions that have a high malignant potential and those that are less significant. Similarly, the high levels of proteolytic activities in the buccal mucosa cells of some smokers may predict which individuals are at the highest risk for oral cancer development. The overlapping distribution of some smokers with the comparison group requires additional investigation to try to identify the factors that result in elevated levels of proteolytic activity. Additional study is also needed to determine whether a dose-response relationship exists between smoking intensity and elevated levels of proteolytic activities.

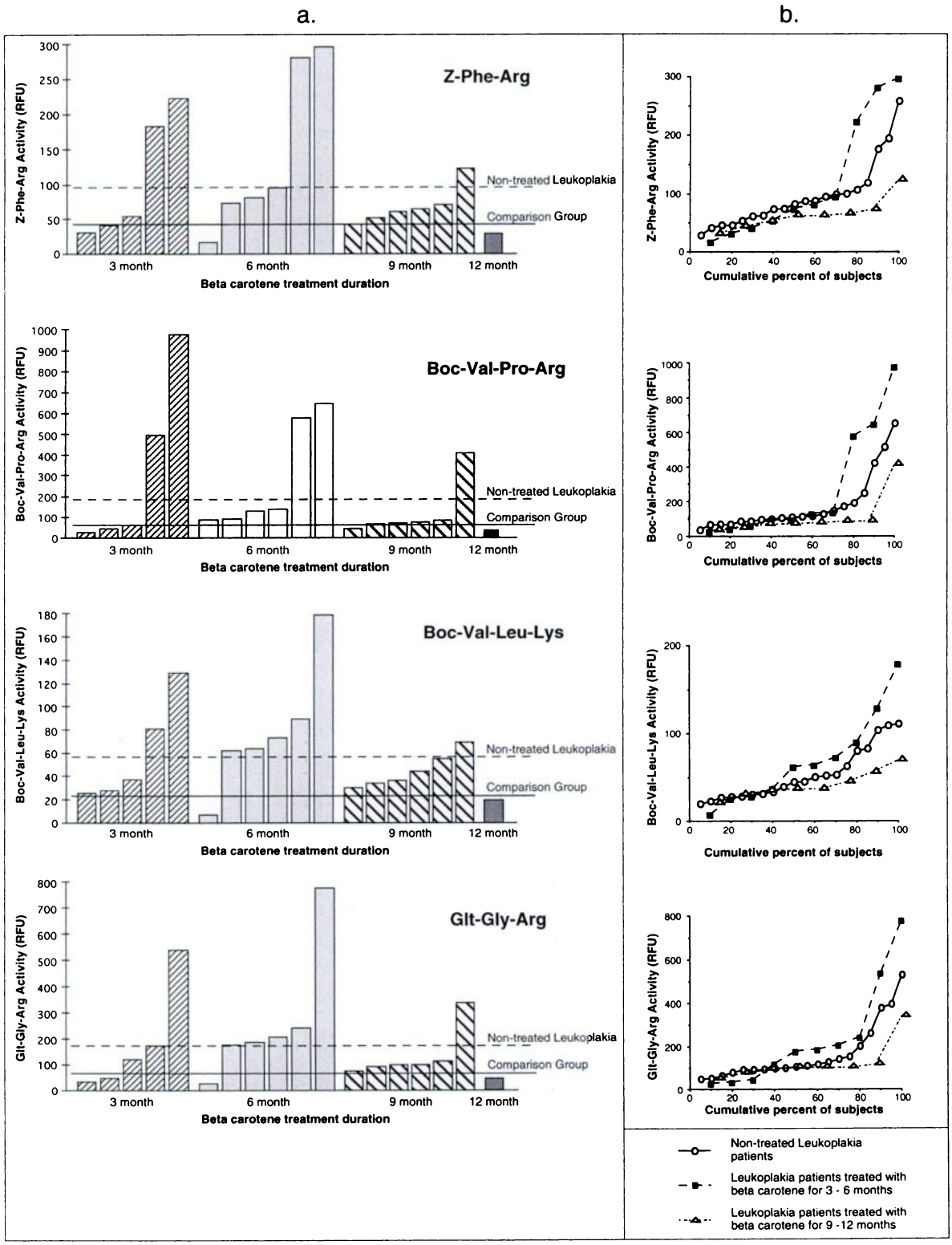

Fig. 3. *a*, levels of proteolytic activities in the oral buccal mucosa cells of 18 leukoplakia patients after treatment with 30 mg/day β-carotene for a period of 3 months (5 patients), 6 months (6 patients), 9 months (6 patients), or 12 months (1 patient). ————, average level of proteolytic activity in comparison subjects for each substrate; - - - - -, average level of proteolytic activity for nontreated leukoplakia patients. High levels of proteolytic activities were observed in 2 of the 5 patients after 3 months treatment with β-carotene, 5 of the 6 patients after 6 months treatment, and in only 1 of 6 patients who had 9 months treatment. *b*, cumulative distributions of levels of proteolytic activities in the oral buccal mucosa cells of nontreated leukoplakia patients and leukoplakia patients treated with 30 mg/day β-carotene for periods of 3–6 and 9–12 months. Each group of subjects is represented by a different line with each point on the line representing the level of proteolytic activities for the given substrate for an individual subject. The points are arranged on the basis of their percentile in the distribution. *RFU*, relative fluorescent units.

From our results, it appears that conditions other than clinical leukoplakia can also lead to a significant elevation in levels of proteolytic activities in buccal cells. Clinical hyperkeratosis or trauma in the mouth caused by dental appliances or habitual cheek biting can result in increased levels of proteolytic activities. The increased levels of proteolytic activities observed in certain conditions (Fig. 2) are consistent with the findings of Holly *et al.* (17) of increases in circulating proteases in the sera of pregnant women, patients with acute injury, diabetics, and patients with various types of cancer. These proteases are capable of altering insulin-like growth factor-binding proteins and, thereby, may serve to regulate the bioavailability of insulin-like growth factor. The elevation in levels of proteolytic activities observed in diabetics may have significance; it is known that diabetics have elevated rates of some forms of cancer (18–20). It is interesting that two activities elevated in diabetics were detected by cleavage of the Boc-Val-Leu-Lys and Glt-Gly-Arg substrates. These substrates are known to be cleaved by plasmin and plasminogen activator, proteases involved in thrombolysis (Fig. 2). It has been speculated recently that impairment of the plasminogen-plasmin system may contribute to angiopathic complications in diabetics (21).

A surprising observation in our results, which requires confirmation, was the high levels of proteolytic activities in some patients with oral leukoplakia who had been taking β-carotene for 3 or 6 months (Fig. 3). β-carotene and BBI have similar suppressive effects on oral carcinogenesis in hamsters (3, 9, 22–24), and β-carotene treatment can result in complete regression of oral tumors in hamsters (22, 24) and oral leukoplakia in some patients (25). Approximately 8% of patients with oral leukoplakia respond to β-carotene treatment with complete regression of lesions, whereas a higher percentage of patients exhibit a partial response to β-carotene therapy (*e.g.*, Ref. 25; reviewed in Ref. 8). Thus, the expectation in this study was that β-carotene would reduce the levels of proteolytic activities in the buccal mucosa cells of patients with oral leukoplakia. The increases in levels of proteolytic activities observed in our study follow a similar time course to the delayed response of leukoplakia to β-carotene treatment reported by Toma *et al.* (26). We speculate that the induction of proteolytic activities by β-carotene could be involved in the destruction of tissue necessary for the complete or partial regression of oral leukoplakia. The differences in responses of these patients may reflect variation in their responsiveness to β-carotene treatment.

Our findings suggest that levels of proteolytic activities may serve as IMEs for the evaluation of cancer chemopreventive agents such as BBI. We have observed that many individuals with a higher than normal risk of oral cancer development (associated with chronic smoking habits or their diagnosis of leukoplakia or erythroplakia) have significantly elevated levels of certain proteolytic activities that are known to be affected by BBI. It is now appropriate to evaluate BBI in Phase II and III cancer prevention trials. It would also be extremely useful to further investigate the epidemiology of levels of proteolytic activities in other organs, such as the breast, colon, rectum, and prostate, and the ability of BBI to reduce the levels of proteolytic activities in individuals with high levels of proteolytic activities before randomization. Some individuals, such as pregnant women, diabetics, or those with oral trauma, may have higher levels of proteolytic activities that are independent of a history of smoking or diagnosis of oral leukoplakia; these patients can be excluded easily from prevention trials by baseline examinations. Similarly, patients with oral leukoplakia and normal levels of proteolytic activities should be either excluded from prevention trials using protease inhibitors or randomized into a separate strata to both increase the statistical power of the trial and to maintain balance for this important factor in the randomization. The design of such trials will need to incorporate measurements of oral mucosa levels of proteolytic activities at baseline and at each clinical evaluation to evaluate further the usefulness of oral mucosa proteolytic activity levels as IMEs. The results presented here suggest that the levels of proteolytic activities in buccal mucosa cells could be useful as IMEs and can be determined easily during each patient visit in a prevention trial and that trials of BBI, and perhaps other cancer-chemopreventive agents, should have a high priority for evaluation in Phase I and II cancer prevention trials.

Acknowledgments

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H Manzone, P C Billings, W N Cummings, et al.

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