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# Effects of Protease Inhibitors on Levels of Proteolytic Activity in Normal and Premalignant Cells and Tissues

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**Abstract** Our studies utilizing different types of protease inhibitors as anticarcinogenic agents in *in vivo* and *in vitro* systems have recently been reviewed. These studies suggest that the protease inhibitors which prevent carcinogenesis affect processes in the early stages of carcinogenesis, although they can be effective at long time periods after carcinogen exposure in both *in vitro* and *in vivo* systems. While there is strong evidence that these protease inhibitors can affect both the initiation and promotion stages of carcinogenesis, they have no effect on already transformed cells. Our results have suggested that the first event in carcinogenesis is a high frequency epigenetic event and that a later event, presumably genetic, leads to the malignant state. Protease inhibitors appear capable of reversing the initiating event, presumably by stopping an ongoing cellular process begun by carcinogen exposure. The major lines of investigation on the mechanism of the protease inhibitor suppression of carcinogenesis relate to the ability of anticarcinogenic protease inhibitors to affect the expression of certain oncogenes, and the levels of certain types of proteolytic activities. The anticarcinogenic protease inhibitors have no observable effects on normal cells, but can reverse carcinogen-induced cellular changes for several different endpoints studied.

The most direct method of determining the mechanism of action of the anticarcinogenic protease inhibitors is to identify and characterize the proteases with which they interact. In the cells of the *in vivo* and *in vitro* systems in which protease inhibitors can prevent carcinogenesis, only a few proteases have been observed to interact with the anticarcinogenic protease inhibitors. Proteases have been identified by both substrate hydrolysis and affinity chromatography. Using substrate hydrolysis, we examined the ability of cell homogenates to cleave specific substrates and then determined the ability of various protease inhibitors to affect that hydrolyzing activity. Affinity chromatography can isolate specific proteases that directly interact with anticarcinogenic protease inhibitors. As examples, the Boc-Val-Pro-Arg-MCA hydrolyzing activity was identified by substrate hydrolysis, and a 43 kDa protease has been identified by affinity chromatography. The isolation and characterization of these proteases has been and will continue to be a subject of investigation in our laboratory.

Our studies on anticarcinogenic protease inhibitors have suggested that the Bowman-Birk Inhibitor (BBI) derived from soybeans is a particularly effective anticarcinogenic protease inhibitor. BBI has been studied both as a pure protease inhibitor, or purified BBI (PBBI), and as an extract of soybeans enriched in BBI, termed BBI concentrate (BBIC). PBBI and/or BBIC have been shown to suppress carcinogenesis in three different species (mice, rats and hamsters); in several organ systems/tissue types (colon, liver, lung, esophagus and cheek pouch [oral epithelium]); in cells of both epithelial and connective tissue origin; when given to animals by several different routes of administration (including the diet); leading to different types of cancer (*e.g.*, squamous cell carcinomas, adenocarcinomas, angiosarcomas, *etc.*), and

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induced by a wide variety of chemical and physical carcinogens [1]. We originally identified BBI as an anticarcinogenic agent in an *in vitro* transformation assay system. BBI, as BBIC, has recently risen to the human trial stage and has achieved Investigational New Drug status from the FDA. In human trials, elevated levels of proteolytic activities known to be affected by BBI serve as intermediate marker endpoints (IME) in the cells of tissues having premalignant characteristics or which are known to be at higher-than-normal risks of cancer development. In previous animal studies, BBI was capable of bringing such elevated levels of proteolytic activity back to normal levels in the normal-appearing areas of carcinogen-treated tissue. We have recently discovered that BBI/BBIC can increase the levels of our marker proteolytic activities in premalignant cells and tissues, which could be highly relevant to the mechanisms of action of the anticarcinogenic protease inhibitors. These findings are summarized here.


**Key words:** β-carotene, intermediate marker endpoints, protease inhibitor, soybeans

Extensive data illustrate the fact that certain protease inhibitors are highly anticarcinogenic, with the ability to prevent cancer development in a variety of *in vivo* and *in vitro* systems, as has recently been reviewed [1,2]. One particular anticarcinogenic protease inhibitor derived from soybeans, the Bowman-Birk Inhibitor (BBI), has risen to the human trial stage in the form of BBI Concentrate (BBIC). Potential intermediate marker endpoints (IME) that could be used in BBIC human cancer prevention trials have been discussed elsewhere [3–5]. The most promising of these IME have been the levels of certain types of proteolytic activities affected by BBI/BBIC in the cells of the *in vivo* and *in vitro* carcinogenesis systems in which BBI/BBIC have been shown to suppress carcinogenesis. Our studies on these IME will be discussed in this article.

Our data collected on these IME in cells, animals and humans have used particular synthetic substrates for which the proteolytic activity is known, from previous studies, to be affected by BBI/BBIC. Proteolytic activities in our studies of proteolytic activity levels are detected by the methods described by Manzone *et al.* [6]. The first carcinogenesis system studied in the laboratory in terms of the effects of BBI/BBIC on levels of proteolytic activities involved dimethylbenz-(*a*)anthacene (DMBA)-induced hamster oral carcinogenesis; in these studies we focused on the Boc-Val-Pro-Arg-MCA hydrolyzing activity [7]. Results from these studies are shown in Fig. 1. DMBA treatment of the hamster oral epithelium resulted in a high level of Boc-Val-Pro-Arg-MCA hydrolyzing activity in the normal-appearing areas of hamster cheek pouch epithelium. This activity persisted for long periods of time after carcinogen treatment; levels of proteolytic activity remained high until the end of the carcinogenesis assay period (months after DMBA exposure), as shown in Figure 1. DMBA treatment led to a 10-fold increase in protease levels over normal in normal-appearing areas of DMBA-treated hamster cheek pouch epithelium, but DMBA treatment coupled with BBI exposure resulted in normal levels of activity. This inhibition of proteolytic activity by BBI was correlated with a highly significant suppression of oral carcinogenesis [7], suggesting that the effects of BBI on the levels of proteolytic activity were related to its mechanism of action as an anticarcinogenic agent in the system.

Similar results for carcinogen treatment with or without BBI/BBIC have been observed in other systems as well. Our studies on dimethylhydrazine (DMH)-induced colon carcinogenesis with and without BBI will be briefly described here. In these studies, isolated epithelial cell populations gave approximately the same results as those obtained from colon mucosal biopsy specimens; the colon mucosal biopsy specimen results are given here. The major proteolytic activities studied in colon carcinogenesis assay systems are detected by three different substrates, substrate 1—Boc-Val-Pro-Arg, substrate 2—Boc-Val-Leu-Lys, and substrate 3—Glt-Gly-Arg. In studies using rats, the animals were untreated or treated twice weekly for three weeks with DMH (known to be at a carcinogenic level; the total DMH dose was 80 mg/kg), with or without 0.1% dietary BBIC. Animals were sacrificed 1 month after the last DMH injection. Protease activity was measured in colonic cells (colon mucosal biopsy samples); data for these studies are shown in Figure 2. These studies show highly significant elevations in levels of proteolytic activities in

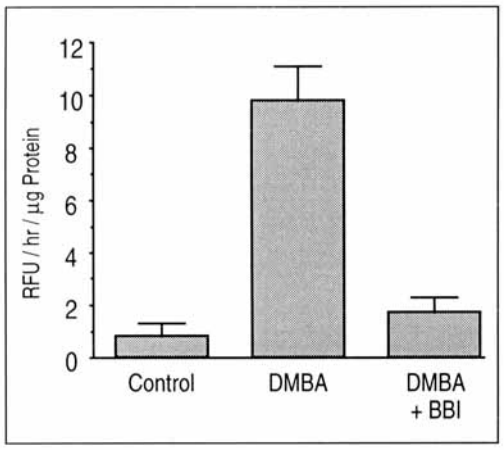


**Fig. 1.** Suppressing effect of BBIC on levels of the Boc-Val-Pro-Arg hydrolyzing activity in hamster oral epithelium treated with the carcinogen DMBA. DMBA leads to a persistent elevation of the Boc-Val-Pro-Arg hydrolyzing activity in these cells; this proteolytic activity remains elevated months after carcinogen exposure in this system, as described in detail elsewhere [7]. For these data collected at the times of animal sacrifices (at the end of the six month carcinogenesis assay period), the levels of proteolytic activity in the normal-appearing areas of DMBA-treated hamster cheek pouch epithelium were significantly higher than those observed in controls, while those areas treated with DMBA and BBIC were not significantly different from controls (when subjected to a t-test analysis).

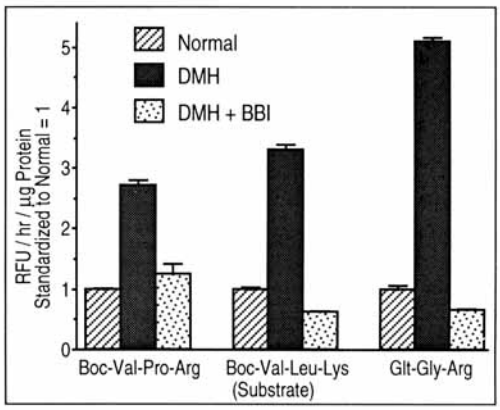


**Fig. 2.** Data showing reduction of proteolytic activities, as measured by three different substrates, in rats treated with DMH + BBI compared to those receiving DMH alone. In normal-appearing areas of colon mucosa, DMH treatment results in a significant elevation in levels of proteolytic activities compared to the levels observed in control animals, while BBI treatment of these DMH-exposed normal-appearing areas results in levels of proteolytic activities which are not significantly different from controls (when subjected to a t-test analysis).

biopsy samples one month after DMH treatments of animals fed a normal diet, whereas normal levels were restored in DMH-treated animals exposed to BBI. As part of the DMH/colon carcinogenesis studies, we determined the short-term effects of both DMH and BBIC on IME. Results from these studies are given in Figure 3, along with the experimental design. The results from these studies show that the effects of BBI on the IME are extremely rapid and can be observed within 48 hours after its administration to animals.

Results of studies in animals (Fig. 2) show that at a dose of DMH known to be carcinogenic (*i.e.*, known to result in colon cancer development in 50% of the exposed rodents), animals have significantly elevated levels of proteolytic activity one month after the last dose of DMH was administered. Under conditions in which DMH treatments lead to colon cancer in the animals, 0.1% dietary BBIC results in a highly significant

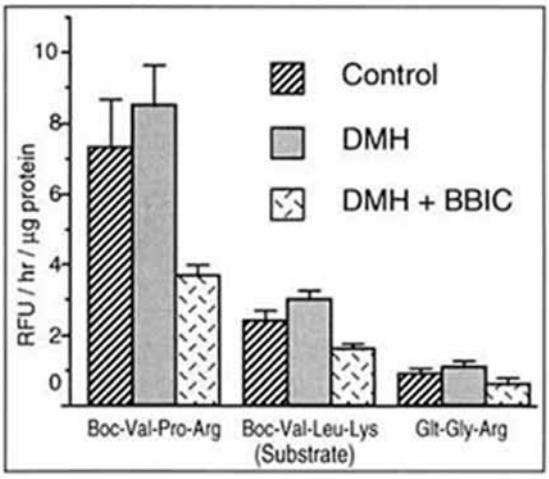


**Fig. 3.** Short-term effects of DMH and BBIC on the IME. Nine animals were treated as follows: 3 animals were controls (untreated), 3 animals were treated with a single dose of DMH (13.3 mg/kg), and 3 animals were treated with a single dose of DMH while dietary BBIC was being administered. All animals were sacrificed 48 hrs. after the single dose of DMH was given. Protease activity was measured in colon cells (colon mucosal biopsy samples) as described in Manzone *et al.* [6] and expressed as relative fluorescent units (RFU)/hr/mg protein.

reduction in the number of animals with colon cancer and/or the proportion of tumors/animal, as described in detail elsewhere [8–10]. This dose of dietary BBIC can completely prevent colon carcinogenesis when the dose of DMH is such that 50% (or fewer) of the animals are destined to get colon cancer [1,8–10], and can reduce the proportion of animals getting colon cancer by approximately 50% when the dose of DMH is such that 70–100% of the animals are destined to get colon cancer [1,4,8–11]. These data show that, under conditions in which dietary BBIC reduces the proportion of animals with colon cancer, it also decreases protease levels in the DMH-treated animals. BBIC treatment is capable of significantly reducing the DMH-elevated protease levels approximately to those observed in untreated control animals. The results of the experiment described above show that protease levels in the DMH + BBIC treatment group are significantly reduced when compared to the levels observed in the DMH treatment group as measured with the substrates listed above. The results presented here for DMH-treated rodents are comparable to those published previously for DMBA-treated hamsters in studies on oral carcinogenesis inhibition by BBIC, as described above.

Our studies on the BBI/BBIC suppression of oral carcinogenesis in humans have progressed to the trial stage. We plan to have the levels of certain proteolytic activities serve as IME in the BBI/BBIC human oral cancer prevention trials. In preparation for these human trials, studies have been done on human oral buccal mucosal cells to determine the levels of proteolytic activities in tissues at a higher than normal risk of developing cancer (oral leukoplakia or erythroplakia) as well as in normal buccal mucosal cells. Studies in the buccal mucosa of normal individuals have shown some variation, with smokers having particularly high levels of the proteolytic activities being studied. These IME data in people are shown in Figure 4. The differences observed in Figure 4 for normal subjects *vs.* smoking subjects, and normal subjects *vs.* patients with leukoplakia or erythroplakia, are significant for all substrates when tested by a t-test analysis. These studies are described in detail elsewhere [6]. It is of interest that the increased levels of Boc-Val-Pro-Arg-MCA hydrolyzing activity represent a persistent change in the human oral epithelial cells. We have observed that increased levels of this proteolytic activity remain significantly elevated even in a population of ex-smokers (Manzone *et al.*, unpublished data).

Most surprising in our studies on the IME in human buccal mucosa cells were extremely high levels of proteolytic activities serving as IME for some patients who had been taking daily doses of β-carotene for 3, 6, 9, or 12 months. The levels of proteolytic activity in some patients taking β-carotene for 3, 6, or 9 months are considerably higher than average values observed for either leukoplakia patients or normal individuals, as

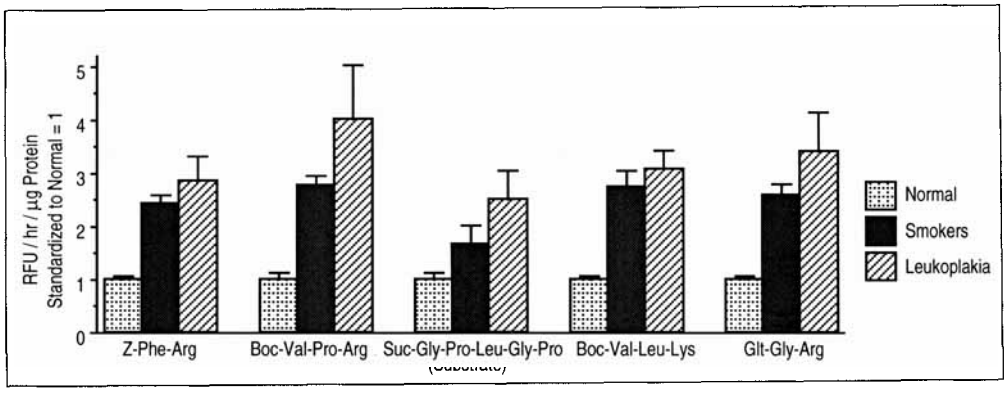


**Fig. 4.** Elevated levels of proteolytic activities, as measured by 5 different substrates, are observed in the buccal mucosal cells of patients with leukoplakia or smokers, as compared to the levels observed in normal individuals. For all 5 substrates, the levels of proteolytic activities in leukoplakia patients and smokers are significantly different from controls (when analyzed by a t-test).

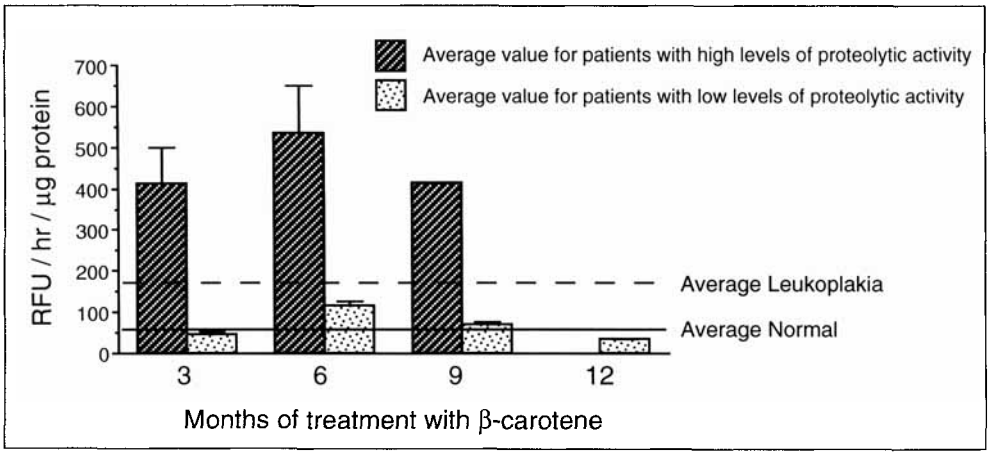


**Fig. 5.** Elevated levels of Boc-Val-Pro-Arg activity in the oral buccal mucosa cells of leukoplakia patients following treatment with 30 mg β-carotene /day for a period of 3, 6, 9, or 12 months. The average level of proteolytic activity in normal patients is indicated by a solid line; the average level of proteolytic activity for leukoplakia patients is indicated by a dashed line. In some patients, treatment with β-carotene for 3, 6, or 9 months resulted in near-normal levels of proteolytic activity, while others had greatly elevated levels at these same time points. We speculate that the increase in proteolytic activity in some patients may be due to induction of proteases involved in regression of the lesion.

shown in Figure 5. We have hypothesized that the highly significant increases in the levels of proteolytic activity observed in these patients are related to the ability of β-carotene to cause the regression/complete disappearance of the leukoplakia lesions in some patients with oral leuko plakia [6]; the regression of oral leukoplakia by β-carotene is discussed elsewhere [12].

The increase in levels of proteolytic activity in β-carotene-treated patients was the opposite effect from that expected from the results of our previous studies. The expectation in these studies was that oral cancer chemopreventive agents such as β-carotene would reduce elevated levels of proteolytic activity in patients with oral leukoplakia in parallel with a reduction in risk of oral carcinogenesis, as BBI is clearly capable of reducing carcinogen-elevated levels of proteolytic activity in DMBA-treated hamster oral buccal mucosa cells in parallel with its suppressing effect on oral carcinogenesis [7]. It is known that BBI/BBIC have suppressive effects on oral carcinogenesis [7,13] similar to those observed for β-carotene [14]. Thus, it is expected that they would have similar effects on the marker proteolytic activities related to oral carcinogenesis.

Our expectations were derived from the effects of cancer chemopreventive agents on carcinogen-treated normal-appearing tissues and cells, however. Oral leukoplakia represents a pre-malignant condition, as described elsewhere [6,12], and the effects of cancer preventive agents in these cells could be quite different from effects observed in normal cells. After studying several systems representing normal and premalignant variants of the parental cells, we found that BBI can have opposite effects in normal and premalignant cells of the same cell system. Our results using purified BBI in one of these cell systems, representing a keratinocyte cell system developed in Dr. Stuart Yuspa's laboratory, will be described here. BK-1 cells represent an immortalized, non-tumorigenic cell line [15], while 308 cells represent a partially transformed cell line capable of giving rise to hyperplastic, benign skin tumors in mice [16]. BBI-induced changes in levels of proteolytic activity in these two different cell lines are summarized in Figure 6. It can be observed in these cells that BBI has opposite effects in the two different lines, reducing the elevated levels of proteolytic activity in the irradiated normal cells (BK-1 cells) while leading to an increase in the levels of activities in irradiated partially transformed cells (308 cells).

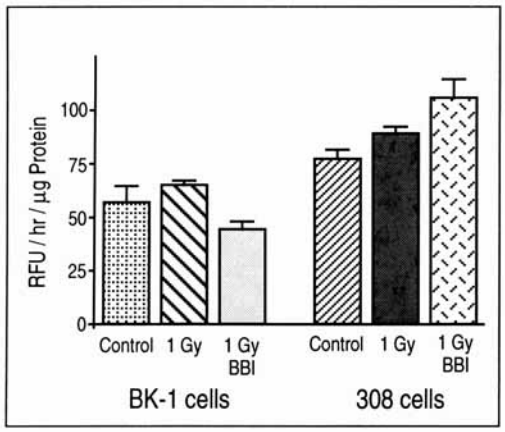


**Fig. 6.** Response of keratinocyte cell lines, representing immortalized (BK-1 cells) and partially transformed (308 cells) cells, with and without exposure to radiation (1 Gy), to incubation with BBI (2 µg/ml) for 9 days. BBI leads to a significant reduction in the level of proteolytic activity (*i.e.*, the Boc-Val-Pro-Arg-MCA hydrolyzing activity) in irradiated BK-1 cells and a significant increase in proteolytic activity in irradiated 308 cells compared to normal cells when analyzed by a t-test.

Our observations that protease inhibitor treatment can lead to increased levels of proteolytic activities in premalignant cells is unexpected, suggesting that BBI affects the levels of the proteolytic activities serving as IME in our studies through its effects on gene expression rather than a direct inhibition of proteolytic activity, as one might expect. BBI is known to be a powerful regulator of gene expression, as discussed elsewhere [1–5,17–22]. Through its effects on the expression of genes coding for proteolytic activities, it could result in either increases or decreases in the levels of proteolytic activities.

As part of the studies discussed here, we observed elevated levels of certain types of proteolytic activity in carcinogen-treated normal tissue, such as DMBA-treated hamster oral epithelium, and in premalignant tissues, such as oral leukoplakia in patients. It is conceivable that elevated levels of proteolytic activity in these premalignant, "initiated" cells are part of the body's defense mechanism to deal with damage produced by carcinogens. We have observed that β-carotene or BBI treatment increases already-elevated levels of proteolytic activity in the premalignant tissues. Perhaps this is part of the mammalian defense system against atypical tissue, and enzymes are mobilized in the attempt to return the tissue to a normal state. β-Carotene and BBI may be increasing the levels of these enzymes to make the natural defense system more effective in removing atypical tissue.

## ACKNOWLEDGMENTS

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