Relationship between Protease Activity and neu Oncogene Expression in Patients with Oral Leukoplakia Treated with the Bowman Birk Inhibitor

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Abstract

The protease catalyzing the hydrolysis of the tripeptide fluorescence substrate, butoxycarbonyl-valine-proline-arginine-(7-amino-4-methylcoumarin) (Boc-Val-Pro-Arg-MCA) and the neu oncogenic protein are potentially useful biomarkers for human cancer prevention studies. In the present study, we standardized a specific substrate hydrolysis method for measuring this protease activity in human oral mucosal cells and characterized the relationship between neu oncogene expression and protease activity in patients enrolled in an oral cancer prevention trial using Bowman Birk Inhibitor Concentrate (BBIC) as the cancer preventive agent. The results demonstrate that changes in the protease activity in oral mucosal cells after BBIC treatment correlated with the changes in the neu protein levels in oral mucosal cells (r = 0.726, P < 0.001) and serum (r = 0.675, P < 0.001), suggesting that the Boc-Val-Pro-Arg-MCA hydrolyzing activity can be as useful as neu oncogene expression as a cancer biomarker. In the 25 patients enrolled in the study, the level of neu protein in oral mucosal cells correlated with the serum neu protein concentration in the patients before BBIC treatment (r = 0.645, P < 0.001). However, such a correlation was not observed after the BBIC treatment, suggesting that BBIC may inhibit serine protease(s) involved in the cleavage of neu protein on the cell surface, thereby preventing the release of the extracellular domain of neu protein into the circulation. By inhibiting the cleavage of neu protein on the cell surface, BBIC could prevent malignant and premalignant cells expressing high levels of neu protein from escaping host immunological surveillance control.

Introduction

It has been suggested that premalignant human tissues have elevated levels of proteolytic activities that can be used as biomarkers for human cancer prevention studies (1–3). The protease that catalyzes the hydrolysis of the tripeptide fluorescence substrate, Boc-Val-Pro-Arg-MCA, is particularly relevant to human cancer prevention studies because the level of this protease activity was found to be elevated in several types of human tissues at higher than normal risks of cancer development (reviewed in Ref. 3). In mouse C3H10T1/2 cells, the Boc-Val-Pro-Arg-MCA hydrolysis activity has been attributed to a M(sub r) 70,000 neutral serine endopeptidase that is inhibitable by several antitumorogenic serine protease inhibitors, such as the soybean-derived BBI, chymostatin, and antipain (1). Because the abilities of these antitumorogenic protease inhibitors to inhibit this protease correlate with their abilities to suppress malignant cell transformation (1), this protease is likely to be involved in the carcinogenic process and, therefore, may be a good candidate biomarker for human cancer prevention studies.

In addition to the Boc-Val-Pro-Arg-MCA hydrolysis activity, there are numerous other biomarkers that are potentially useful for human cancer prevention studies. One such biomarker is the neu oncogene (4–11), which codes for a M(sub r) 185,000 protein commonly referred to as p185 (12). The neu oncogene was independently discovered by several groups (12–14) and has also been referred to as HER-2 (13) and c-erb-B-2 (14). The protein encoded by the neu oncogene has three domains, at the COOH-terminal is a cytoplasmic domain consisting of 580 amino acid residues, at the NH2-terminal is an extracellular domain composed of 632 amino acid residues, and between is a hydrophobic transmembrane anchor region consisting of 22 amino acids (13–17). The neu protein in serum is derived from the extracellular domain of the neu protein, which is released from the cell surface by proteolytic cleavage (18). The elevated level of neu protein on tumor cells has been shown to correlate with the elevated concentration of neu protein in serum (19, 20). Elevated levels of neu protein on tumor cells and/or in serum have been detected in patients with various cancers, including cancers of the breast, ovary, prostate, stomach, pancreas, colon, liver, and lung (8, 21, 22). In some cases, elevated serum neu protein concentrations were detected prior to the
clinical diagnosis of cancers, suggesting that the levels of neu protein can be a useful biomarker for human cancer studies (8).

The present study was undertaken to standardize a specific substrate hydrolysis method for measurement of the Boc-Val-Pro-Arg-MCA hydrolysis activity in human oral mucosal cells and to elucidate the relationship between protease activity and neu protein levels in patients with oral leukoplakia who were enrolled in a cancer prevention trial using BBIC as a cancer preventive agent. The results of the present study demonstrate that the Boc-Val-Pro-Arg-MCA hydrolyzing activity in oral mucosal cells can be reliably measured by a specific substrate hydrolysis method, and that changes in protease activity in oral mucosal cells and changes in levels of neu protein in oral mucosal cells and serum samples are correlated with one another in these patients after BBIC treatment.

Materials and Methods

Preparation of Human Oral Mucosal Cell Homogenates and Serum Samples. Human oral mucosal cells and serum samples used in the present study were collected from 25 patients with oral leukoplakia who were enrolled in a clinical trial using the soybean-derived BBIC as the cancer preventive agent. The patients were recruited from Orange County and Los Angeles, California. After the purpose and procedures were explained and an informed consent form was signed by each patient, the patients were given BBIC in the form of a mouthwash, which was swallowed at daily doses of 200 to 1000 chymotrypsin inhibition units for 1 month. Blood and oral mucosal cell samples were collected from the patients before and after the 1 month BBIC treatment period. The blood samples were allowed to clot and centrifuged at 1500 rpm for 10 min to separate the blood clot from the serum. The serum samples were saved and stored at −20°C before the neu protein assay. To collect oral mucosal cells, the patients were instructed to drink and hold ~15 ml of sterile physiological saline in the mouth and gently brush the entire surface inside of the mouth with a cytology brush. The cells brushed into the saline solution were collected into a 50-ml conical tube and centrifuged at 1000 g at 20°C before the neu protein assay.

Preparation of Other Cell and Tissue Samples for Protease Activity. The other samples included in the protease assay were normal human breast tissue, cultured MCF10 human breast epithelial cells and MCF7 human breast cancer cells, and normal rat liver tissue. The normal human breast tissue was obtained from an unidentified woman who underwent a breast reduction procedure at the Hospital of the University of Pennsylvania. MCF10 cells were cultured in a 1:1 mixture of DMEM and Ham’s F12 medium supplemented with 5% horse serum (Sigma Chemical Co.), 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 500 ng/ml hydrocortisone. MCF7 cells were cultured in DMEM supplemented with 10% fetal bovine serum. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The cells were harvested with cell scrapers at confluence, washed with PBS, and centrifuged at 5000 rpm for 5 min to precipitate the cells. After the centrifugation, the supernatant was decanted, and the cell pellet was stored frozen at −70°C until assayed.

Measurement of Protease Activity. The protease activity assay procedure used was adapted from the specific substrate hydrolysis method described previously by Billings et al. (1). Briefly, each oral mucosal cell pellet was thawed and homogenized on ice in 600 µl of ice-chilled PBS (pH 7.0). The protein concentrations in the cell homogenates were determined by a colorimetric method (23) using BSA as a standard. To select appropriate ranges for the sample protein concentration and the Boc-Val-Pro-Arg-MCA substrate concentration for the protease activity assay, a pooled oral mucosal cell homogenate containing 0.08–40 µg of protein was mixed with 2 ml of reaction buffer containing 20 mM Tris (pH 7.0), 0.5 mM CaCl₂, and 1.6–12.8 µM Boc-Val-Pro-Arg-MCA substrate (Peninsula Laboratories, Inc., Belmont, CA) and incubated at room temperature for 15–120 min. At the end of the incubation period, the concentration of the AMC fluororescent reporter group released from the substrate was determined with a Perkin-Elmer Corp. fluorescence spectrophotometer at excitation and emission wavelengths of 380 and 460 nm, respectively. The spectrophotometer was standardized such that the relative fluorescence unit for 10⁻⁷ M AMC was 700. The protease activity was expressed as number of μM of substrate hydrolyzed per hour per μg of sample protein (μM/h/μg).

The protease activity in the oral mucosal cells collected from individual patients was determined by the same method as described above, except that the sample protein concentration in the reaction buffer was limited to no more than 2.5 µg/ml, the Boc-Val-Pro-Arg-MCA substrate concentration in the reaction buffer was adjusted to 3.2 μM, and the incubation time was shortened to 60 min. The protease activity in the normal human breast tissue, MCF10 cells, and MCF7 cells, as well as normal rat liver tissue were also determined by the same method, except that the sample protein concentration was raised to ~20–80 µg/ml because of the relatively low protease activity in these cell and tissue samples.

Measurement of neu Protein. The levels of neu protein in the oral mucosal cell homogenates and in serum samples were quantitated with a dual-antibody ELISA kit (Caltibiochem, San Diego, CA) that detects the extracellular domain of the neu protein. To perform the assay, the oral mucosal cell homogenates and serum samples prepared as described above were diluted 1:50 (~200 µg protein/ml) and 1:80, respectively, in the sample diluant and applied in duplicate at 100 µl/well onto polystyrene microwells precoated with a capture antibody against the neu protein. The neu protein assay was performed according to the manufacturer’s instructions. To compensate for interassay variations of neu protein measurement, 10 selected oral mucosal cell samples were included in each run of the neu protein assay as internal standards. The raw data of neu measurement in different runs of the neu protein assay were normalized using conversion factors calculated based on the readings of the internal standards in each run of the neu protein assay.

Statistical Analysis. The protease activity in the homogenates prepared from the oral mucosal cells of the patients with oral leukoplakia were analyzed by regression analysis using the incubation time, the substrate concentration or the sample protein concentration as the independent variables and the amount of substrate hydrolyzed as the dependent variable. The levels and the changes in levels of protease activity and neu protein in the oral mucosal cell homogenates and neu protein in serum samples were also analyzed by regression analysis. The patients treated with BBIC at various doses were pooled into a single group for the purpose of statistical analysis, which was per-
The purposes of this investigation were to standardize a substrate hydrolysis method for the measurement of the protease activity involved in Boc-Val-Pro-Arg-MCA substrate hydrolysis and to study the relationships among the oral mucosal cell protease activity, the oral mucosal cell neu protein level, and the serum neu protein concentration in patients with oral leukoplakia. The validity of the substrate hydrolysis method for measuring protease activity is based on the condition that the substrate concentration does not change substantially during the reaction period. This condition is satisfied when the substrate concentration does not vary substantially with the change in the substrate concentration in the reaction buffer, regardless of the small SD in relation to the mean (Table 1). When the sample protein concentration in the reaction buffer exceeded 2.5 μM, the substrate concentration became a limiting factor, and the relationship between the sample protein concentration and the amount of substrate hydrolyzed was no longer linear. As a result, the specific protease activity could not be determined meaningfully by this method when the sample protein concentration exceeded 2.5 μg/mL because its calculated value decreased substantially as the sample protein concentration increased (Table 1). On the basis of these results, the upper limit of sample protein concentration for use in these studies was set at 2.5 μg/mL. To study the relationship between protease activity and neu oncoprotein expression, the protease activity and neu protein level in the oral mucosal cell homogenates and the neu protein concentration in serum were determined in 25 patients with oral leukoplakia enrolled in a oral cancer prevention trial using Boc-Val-Pro-Arg-MCA substrate hydrolysis activity in human oral mucosal cells.

<table>
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<tr>
<th>Sample protein (μg/ml)</th>
<th>Substrate hydrolysis rate (μM/h)</th>
<th>Protease activity (μM/h/μg)</th>
<th>Mean ± SD</th>
<th>SD/mean (%)</th>
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* The Boc-Val-Pro-Arg-MCA substrate concentration in the reaction buffer was 3.2 μM.
BBIC as the cancer preventive agent. Prior to the BBIC treatment, the levels of neu protein in oral mucosal cells and in serum correlated significantly with each other in the patients (Fig. 5), but the protease activity level in oral mucosal cells did not correlate with either the oral mucosal cell neu protein level ($P > 0.10$) or the serum neu protein concentration ($P > 0.25$). After the BBIC treatment, both increases and decreases were observed in the oral mucosal cell neu protein levels, serum neu protein concentrations, and oral mucosal cell protease activity in the patients. The change in the oral mucosal cell neu protein level correlated with the changes in the serum neu protein concentration (Fig. 6A) and the oral mucosal cell protease activity (Fig. 6B) in the patients. The change in the serum neu protein concentration also correlated with the change in the oral mucosal cell protease activity in these patients (Fig. 6C). The levels of neu protein in oral mucosal cells and in serum, which correlated with each other prior to the BBIC treatment, were no longer correlated with each other after the 1-month BBIC treatment period ($P > 0.15$). The levels of neu protein in oral mucosal cells and in serum, which correlated with each other prior to the BBIC treatment, were no longer correlated with each other after the 1-month BBIC treatment period ($P > 0.15$).
protein and protease activity in the oral mucosal cells, which did not correlate with each other prior to the BBIC treatment, were significantly correlated with each other at the end of the BBIC treatment (Fig. 7; \( P < 0.001 \)).

**Discussion**

It has been suggested that the protease activity involved in hydrolysis of the Boc-Val-Pro-Arg-MCA substrate can be a useful biomarker for cancer prevention studies because its activity is elevated in various cells and tissues at higher than normal risk of cancer development (1–3) and can be inhibited by several serine protease inhibitors known to be capable of inhibiting malignant transformation in vitro and cancer development in animal model systems (1, 3). The Boc-Val-Pro-Arg-MCA hydrolyzing activity was used as one of the biomarkers in a Phase IIa clinical human oral cancer prevention study using BBIC as the cancer preventive agent. Because the level of this protease activity in human oral mucosal cells was orders of magnitude higher than in other tissues, such as rat liver and human breast tissues, the assay conditions suitable for measuring levels of this protease activity in other tissues are not appropriate for use in human oral mucosal cells because of the premature reaction plateau caused by the high level of protease activity. This problem can be overcome by reducing the reaction time, increasing the substrate concentration, and/or decreasing the sample protein concentration in the reaction buffer. In the present study, we modified the procedure used previously and were able to quantitate accurately the levels of Boc-Val-Pro-Arg-MCA hydrolyzing activity in human oral mucosal cell samples by reducing the reaction time from 120 to 60 min, increasing the substrate concentration from 0.8 to 3.2 \( \mu \text{M} \), and limiting the sample protein concentration in the reaction buffer to no more than 2.5 \( \mu \text{g/ml} \).

The relationship between Boc-Val-Pro-Arg-MCA hydrolyzing activity and neu protein levels was analyzed in patients with oral leukoplakia enrolled in the Phase IIa BBIC oral cancer prevention trial. It was found that the neu protein levels in oral mucosal cells correlated significantly with the serum neu protein concentration before BBIC treatment (Fig. 5). The relationship between the oral mucosal cell neu protein level and the serum neu protein concentration was determined by a linear regression analysis. The neu protein measurement was carried out in duplicate.

**Fig. 5.** Correlation between the oral mucosal cell neu protein level and the serum neu protein concentration before BBIC treatment. The neu protein levels in oral mucosal cells and in serum were determined by the ELISA method in 25 patients with oral leukoplakia before BBIC treatment. The relationship between the oral mucosal cell neu protein level and the serum neu protein concentration was analyzed by a linear regression analysis. The neu protein measurement was carried out in duplicate.

**Fig. 6.** Correlation between the changes in levels of oral mucosal cell neu protein, serum neu protein concentration, and oral mucosal cell protease activity after BBIC treatment. The neu protein levels in oral mucosal cells and in serum and the Boc-Val-Pro-Arg-MCA hydrolysis activity in oral mucosal cells were determined in 25 patients with oral leukoplakia before and after the 1-month treatment with BBIC. The relationship between changes in the levels of neu protein and protease activity after BBIC treatment were analyzed by a linear regression analysis. The neu protein measurement and protease assay were carried out in duplicate.
Protease, Bowman Birk Inhibitor, and neu Oncogenic Protein

The neu protein was detected in a majority of oral mucosal cells and serum samples in this study, neu protein expression has also been reported in oral mucosa in various benign or malignant lesions, such as hyperplasia, dysplasia, and squamous cell carcinoma (25). However, these results do not necessarily suggest that the neu oncogene is overexpressed in most patients with oral leukoplakia because the neu protein is also expressed in normal tissues to various degrees (26, 27). The established cutoff values for neu protein expression in normal human subjects are 3.2 HNU per μg of protein in endometrial cells (28) and 1761 or 2050 HNU/ml in serum samples (29, 30). On the basis of the cutoff values of 3.2 and 1761 HNU/ml for neu protein in oral mucosal cells and serum, respectively; it was estimated that neu protein was overexpressed in 33–46% of the patients with oral leukoplakia in the present study. This is similar to the percentages of neu overexpression reported in other preneoplastic lesions (28, 31) as well as in patients with various malignant diseases (5, 8, 21, 22, 32, 33). It should be noted that due to differences in methodologies used for neu protein measurement by different groups, the percentages of neu overexpression documented in various published reports are not always comparable.

Although the levels of neu protein on oral mucosal cells and in serum significantly correlated with each other before BBIC treatment, such a correlation was not observed after BBIC treatment. The mechanism for the lack of a correlation between the levels of neu protein on oral mucosal cells and in serum after BBIC treatment is not clear. A possible explanation is that BBI inhibits the protease(s) involved in the proteolytic cleavage of the neu protein on the cell surface, thereby preventing the release of the extracellular domain of the neu protein into the circulation. It is conceivable that the extracellular domain of the neu protein is routinely cleaved and released into the circulation, which is the biological basis for the correlation between the levels of neu protein on the cell surface and in serum. Inhibition of the protease(s) by BBI would prevent the cleavage and release of the extracellular domain of the neu protein, thereby breaking the link between the neu protein level on the cell surface and the serum neu protein concentration. The release of the extracellular domain of the neu protein from the cell surface can be inhibited completely by combined treatment with the serine protease inhibitor leupeptin and EDTA (18), suggesting an involvement of a serine protease in the proteolytic cleavage of the neu protein. Because BBI is a potent serine protease inhibitor that inhibits both trypsin-like and chymotrypsin-like serine proteases, such as trypsin (34), chymotrypsin (34), cathepsin G (35, 36), elastase (35), and chymase (37), it is likely that BBI inhibits the protease(s) involved in the proteolytic cleavage of the neu protein. BBI and leupeptin are both effective anticarcinogenic protease inhibitors capable of suppressing the malignant transformation of cells, as reviewed previously (38). BBI may also affect other proteolytic events that are related to cancer development. For example, BBI is known to inhibit the activation of EGFR after radiation exposure, possibly by stabilizing specific tyrosine-phosphatases that interfere with EGFR activation (39). BBI also inhibits the proteolytic processing of gastrin-releasing peptide, a growth factor derived from small cell lung carcinoma cells (40). It remains to be determined whether the abilities of BBI to inhibit the cleavage of the extracellular domain of the neu protein, the activation of EGFR as well as the proteolytic processing of gastrin-releasing peptide are functionally related to its abilities to suppress malignant transformation.

The neu protein is a tumor-associated antigen that is considered to be a promising target for antigen-specific cancer immunotherapy (41, 42). The overexpression of neu antigen on the surface of cancer and premalignant cells could render the cells vulnerable to attack by the host immune surveillance mechanisms. Immune responses directed against neu antigen have been observed in patients with cancers that express high
levels of neu protein on the cancer cells (42). The immune responses against neu antigen are mediated by both antibodies and CTLs (42, 43). It has been demonstrated that antibodies directed against neu antigen can reverse the malignant phenotype of cells transformed by neu oncogene (44), and that CTLs specific for neu antigen can recognize and lyse neu oncogene-expressing cancer cells in an HLA-A2-restricted fashion (43).

In a recently finished Phase II clinical trial, i.v. administration of a humanized monoclonal antibody against neu antigen resulted in an objective clinical response in patients with advanced metastatic breast cancers that overexpress neu antigen (45). Cancer and premalignant cells that produce high levels of neu protein may escape host immunological surveillance control by shedding off the neu antigen from the cell surface, and thereby avoid being detected and targeted by antibodies and T lymphocytes against neu antigen. The neu antigen cleaved from the cell surface and released into the circulation may bind to anti-neu antibodies and T-lymphocytes and thereby prevent them from homing onto the neu-expressing cells. The neutralization of the anti-neu antibodies and T-lymphocytes could further weaken the host immune response against cancer and premalignant cells that express high levels of neu antigen on the cell surface. It has been demonstrated that the soluble neu antigen released from human breast cancer cells can neutralize the biological activities of anti-neu antibodies and abolish the antibody-mediated growth inhibition and antibody-dependent cellular cytotoxicity in the cancer cells (46). By inhibiting the proteolytic cleavage of neu protein on the cell surface, BBI may prevent the neu-expressing cancer and premalignant cells from escaping the host immunological surveillance control through these mechanisms and subject them to destruction by the host immune mechanisms and/or immunotherapeutic agents targeted to the neu antigen. In the Phase IIa BBIC oral cancer prevention trial in patients with oral leukoplaikia, a dose-dependent reduction in the size of the oral leukoplaikia lesions was observed after a 1-month treatment with BBIC (47). On the basis of the results discussed here, we hypothesize that one of the mechanisms by which the treatment with BBIC leads to the reduction in the size of oral leukoplaikia lesions is by inhibiting the proteolytic cleavage of neu protein at the cell surface, which, in turn, results in the destruction of the leukoplaikia lesions by the host immune system.

References
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