Comparison of Proteinase Activities in Squamous Cell Carcinoma, Basal Cell Epithelioma, and Seborrheic Keratosis

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The correlation between proteinase activities and invasive and metastatic potentials was investigated by comparing three different kinds of tumors. Extracts from tumor homogenate of 11 squamous cell carcinoma (SCC), 5 basal cell epithelioma (BCE), and 8 seborrheic keratosis (SK) were prepared in order to examine the activity of acid phosphatase and proteinases such as cathepsin B and D, type I and IV collagenase, and plasminogen activator (PA). There was no difference observed between acid phosphatase and cathepsin D activities among the three tumors. Cathepsin B and PA activities were slightly elevated in SCC. Type I collagenase activity of SCC was 9-fold higher than that of SK (p < 0.01), and type IV collagenase was 3-fold higher per tissue DNA (p < 0.05). Type I and IV collagenase of BCE were elevated per tissue protein but not elevated per tissue DNA. Correlation was found between the level of cell differentiation in SCC and the activities of cathepsin B, PA, and type I collagenase. Poorly differentiated SCC exhibited a tendency to have higher proteinase activities. Proteinases that showed high activities in malignant tumor homogenate may be related to the degradation of the surrounding cell matrix in addition to intracellular metabolism. Type I and IV collagenase, in cooperation with cathepsin B and PA, might play a major role in invading the dermal stroma and basement membrane. J Invest Dermatol 90:869–872, 1988

Malignant tumor cells derived from keratinocytes penetrate the basement membrane to proliferate in dermal interstitial stroma and finally metastasize to distant organs. Epithelial basement membrane, dermal interstitial tissues, and endothelial basement membrane of blood vessels, which are composed primarily of collagens, elastin, glycoproteins, and proteoglycans, may work as physical and biochemical barriers. Considering the interaction of tumor cells and the extracellular matrix, it has been hypothesized that the existence of matrix-degrading enzymes from tumor cells may be required for their invasiveness. Recently, correlation between proteinase production and tumor cells’ invasive and metastatic potential has been discussed [1,2]. Elevated activities of plasminogen activator (PA) [3,5], cathepsin B [6–8], and type IV collagenase [9–11] were observed in highly metastatic tumor cells grown in vitro. The importance of these proteinases, including type I collagenase, was also reported in extracts of tumor homogenate [12–18], and their immunocytochemical localization was investigated [19,20]. However, simultaneous observations on several proteinases including type IV collagenase have not been demonstrated in the keratinocyte-derived tumor homogenates.

In this study, in an attempt to demonstrate the correlation between degrading enzyme activities and invasive and metastatic potentials, we assayed acid phosphatase activity and several proteinase activities such as cathepsin B and D, type I and IV collagenase, and PA in the tumor homogenates of squamous cell carcinoma, basal cell epithelioma, and seborrheic keratosis.

MATERIALS AND METHODS

Sample Sources Specimens were obtained at surgery from 11 cases of primary squamous cell carcinoma (SCC) of skin, from 5 cases of basal cell epithelioma (BCE), and from 8 cases of acanthotic seborrheic keratosis (SK). All recognizable surrounding normal tissue was removed macroscopically and rinsed with saline to remove blood. Samples were stored at −70°C until enzyme assay.

Histological Examination Two pieces of tissue were sliced from the margin of each tumor sample and fixed with 10% formalin solution. Sections 5 μm thick were prepared by hematoxylin-eosin stain. Samples that contained tumor lesions of more than 70% of the total section microscopically were used for enzyme assay. Tumor cell density was determined by counting the tumor cells in a square field at a magnification of 400X. The values were shown as the average counts of three fields. Microscopically, 11 cases of SCC were divided into three groups according to the grade of cell differentiation [21]. Grade I consisted of cells of which more than 75% were differentiated; in grade II, more than 50%; and in grade III, more than 25%. Correlation between cell density or maturity and enzyme activities was examined.
Preparation of Extracts from Tumor Homogenate  Tumor samples were roughly minced by scissors and rinsed with 0.05 M Tris-HCl buffer (pH 7.4), containing 0.15 M NaCl and 0.1% Triton X-100. Homogenization was carried out in 10 volumes (w/v) of the same buffer with Polytron (Kinematica, maximum intensity for 30 s three times) and subsequently with a sonifier (Branson, intensity 3 for 5 x four times). The homogenate was centrifuged at 5000 g for 30 min, and supernatants were used for enzyme assays.

Protein and DNA Contents  Protein contents in each sample were measured by Lowry’s method [22] with bovine serum albumin (Sigma) as a standard. Absorbance value at 750 nm was measured with a Hitachi spectrophotometer (model 100-40). DNA contents were measured by Kissane’s method [23] with calf thymus DNA (Sigma) as a standard. Pretreated 30-μl samples were reacted with 100 μl of 40% 3,5-diaminobenzoic acid dihydrochloride solution at 60°C for 30 min. Fluorescent intensity of the supernate was measured by a Hitachi fluorescence spectrophotometer (model 850) at 400 nm excitation and 492 nm emission.

Enzyme Assays

Acid Phosphatase  Acid phosphatase activity was assayed with 4.5 mM 4-nitrophenyl phosphate (Merck) in 0.1 M sodium acetate buffer (pH 4.5) [24]. The assay was started by adding 50 μl of sample to 1.45 ml of substrate solution. Incubation was carried out at 37°C for 1 h and stopped by the addition of 1.0 ml of 1 N NaOH. Absorbance was measured at 405 nm by a spectrophotometer. Enzyme assay was shown as OD 405 nm/h per milligram of protein or per microgram of DNA.

Cathepsin D  Cathepsin D activity was assayed with hemoglobin as a substrate [25]. Hemoglobin (Sigma H 2625) was denatured with 0.06 N HCl solution, and the final pH of the solution was adjusted to pH 3.5 by the addition of 1 N NaOH. One-tenth milliliter of sample was reacted with 0.5 ml of 2% hemoglobin solution and 0.4 ml of 135 mM sodium acetate buffer (pH 3.5) at 37°C for 3 h. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid (TCA) solution, and TCA-soluble peptide was measured after centrifugation by Lowry’s method [22]. The reaction tube to which the sample was added after the addition of TCA solution was used as a control. Enzyme activity was shown as mg/h/μl of milligram protein or per microgram DNA. In order to confirm that the hydrolysis of hemoglobin was due to cathepsin D, 10 μl of 1 mg/ml pepstatin (Peptide Institute Inc., Japan) solution was added before commencement of the assay.

Cathepsin B  Cathepsin B activity was assayed with a synthetic fluorogenic substrate, carboxbenzoxyl-arginyl-arginine 4-methoxy-β-naphthylamide (Bachem) [26]. Two milliliters of reaction mixture, containing 0.3 ml of sample solution and 1.7 ml of 40 mM citric acid–sodium phosphate buffer (pH 6.2), contained 0.2 mM substrate and 0.9 mM diithiothreitol (Sigma). Incubation was carried out at 37°C for 2 h, and the reaction was terminated by the addition of 0.5 ml of 1 N HCl. The liberated 4-methoxy-β-naphthylamide was measured by a fluorescence spectrophotometer at 292 nm excitation and 410 nm emission. Enzyme activity was shown as fluorescence intensity (FI) at 410 nm/h/mg protein or μg DNA.

Plasminogen Activator (PA)  Plasminogen activator activity was assayed with a synthetic fluorogenic substrate, N-butyloxy carbonyl-valiyl-leucyl-lysin 4-methylene coumarin-7-amine (Peptide Institute Inc.) [27]. Substrate solution consisted of 0.25 mM substrate in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% Tween 80. Plasminogen (Sigma) solution was prepared at 0.25 unit/ml in the same buffer. A 50-μl sample was reacted with 100 μl of substrate solution in the presence and absence of 20 μl of plasminogen solution at 37°C for 2 h. After the addition of 0.6 ml of cold water, the reaction tube was kept in ice water until analysis. The liberated 7-amino-4-methylethyl coumarin was measured by a fluorescence spectrophotometer at 380 nm excitation and 460 nm emission. Plasmin activity, which was already included in the sample without the addition of plasminogen, was subtracted from the total enzyme activity. PA activity was shown as fluorescence intensity (FI) at 460 nm/h per mg protein or per μg DNA. Linearity of PA assay was confirmed by urokinase as a standard.

Type I Collagenase  Type I collagenase activity was measured according to the method of Nagai et al [28,29]. Fluorescein isothiocyanate (FITC)-labeled bovine type I collagen in 0.1 M acetic acid (1 mg/ml) was purchased from Collagen Research Foundation, Japan. The substrate was diluted with an equal volume of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.4 M NaCl. Fifty microliters of each sample, which was expected to include latent collagenase, was activated by the addition of 20 μl of 1 mg/ml trypsin (Sigma T 8642) in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. Incubation was carried out at 35°C for 10 min, and the reaction was terminated by the addition of 30 μl of 3 mg/ml soybean trypsin inhibitor in the same buffer. The above-mentioned 100 μl of pretreated sample was reacted with 100 μl of substrate solution (50 μg collagen) at exactly 35°C for 16 h. The reaction was terminated by the addition of 10 μl of 80 mM 1,10-phenanthroline (Sigma) in 50% ethanol solution. After the addition of 200 μl of 0.05 M Tris-HCl buffer containing 0.2 M NaCl, the reaction tube was kept at 35°C for another hour. Finally, the reaction tube was kept in ice water for several minutes and strongly agitated with 280 μl of ethanol and 120 μl of 0.17 M Tris-HCl buffer (pH 9.5) containing 0.67 M NaCl. After centrifugation at 1500 × g for 10 min, the fluorescence intensity of the sample (FL) in the supernate was measured at 495 nm excitation and 520 nm emission. The same volume of heat-denatured FITC-labeled type I collagen (80°C for 5 min) was provided as 100% fluorescence intensity (FL0). The reaction mixture to which sample was added after the termination of incubation was provided as blank (FL0). Enzyme activity was shown as (FL0 – FL)/ (FL0 – Fl) × 50 μg/h per mg protein or per μg DNA. In order to confirm that the hydrolysis of type I collagen was due to collagenase, 1 μl of 20 mM ethylendiaminetetraacetic acid (EDTA, Sigma) solution was added before commencement of the assay.

Type IV Collagenase  Type IV collagenase activity was assayed with the same method as that of type I collagenase. FITC-labeled type IV collagen was prepared by the authors according to the method of Nagai et al [28]. Bovine lens type IV collagen (Nitta Gelatin Corp., Japan) was conjugated with FITC (Sigma) under alkaline conditions. FITC-labeled type IV collagen in 0.1 M acetic acid (1 mg/ml) was prepared through salting out and DEAE-cellulose chromatography.

RESULTS  Tumor cell contents in a square field at a magnification of 400× are shown in Table I. Cell densities showed a tendency to parallel tissue DNA. The BCE group showed high cell density compared with other tumors. On the other hand, the grade 1 SCC group showed low cell density because of the presence of large cells with horn cysts. Enzyme activities were compared according to the amount per milligram of protein or microgram of DNA. Each value was expressed by mean ± standard error. All data were analyzed by a

<table>
<thead>
<tr>
<th>Material</th>
<th>No. of Samples</th>
<th>Cell Density</th>
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<tbody>
<tr>
<td>Seborheic keratosis</td>
<td>8</td>
<td>220 ± 22</td>
</tr>
<tr>
<td>Basal cell epithelioma</td>
<td>5</td>
<td>296 ± 35</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>11</td>
<td>118 ± 16</td>
</tr>
<tr>
<td>Grade I</td>
<td>3</td>
<td>89 ± 20</td>
</tr>
<tr>
<td>Grade II</td>
<td>4</td>
<td>154 ± 35</td>
</tr>
<tr>
<td>Grade III</td>
<td>4</td>
<td>103 ± 18</td>
</tr>
</tbody>
</table>

* Tumor cell density was determined by counting the tumor cells in a square field at a magnification of 400×. The values were shown as the average amounts of three fields.
Figure 1. Acid phosphatase, cathepsin B and D, PA, and type I and IV collagenase activities in skin tumors. Enzyme activities were compared per mg protein or per μg DNA. Bars, standard error. Asterisks indicate that values are significantly different from that of SK (* p < 0.1, ** p < 0.05, *** p < 0.01, **** p < 0.001).

Student's t test. Figure 1 shows a comparison of enzyme activities among SCC, BCE, and SK. As BCE had a high DNA content per milligram of protein, enzyme activity based on tissue DNA exhibited a lower value than that based on tissue protein. Acid phosphatase activity did not significantly differ among the three tumors, although a little lower activity was observed in SCC. Hemoglobin hydrolyzing activity was more than 90% inhibited by the addition of pepstatin, which suggested that the enzyme activity was due to cathepsin D (not shown in figure). A similarly high cathepsin D activity was observed in all three tumors. Cathepsin B activity of BCE was not high based on tissue DNA, though it showed high activity per milligram of protein. Cathepsin B activity of SCC was slightly elevated over that of SK. The highest PA activity was evident in SCC. However, the statistical difference was minimal (p < 0.1), due to the large variation in the activity in each sample. Type I and IV collagenase activities in SCC showed 50–90% FITC release compared with heat-denatured positive control, and their activities were completely inhibited by the addition of EDTA (not shown in figure). These data suggested that the hydrolysis of collagens was due to collagenase. Type I collagenase activity of SCC was 9-fold higher than that of SK per tissue DNA (p < 0.01). Type IV collagenase activity of SCC was 3-fold higher than that of SK (p < 0.05). Type I and IV collagenase activities of BCE were elevated per tissue protein (p < 0.01, p < 0.1, respectively) but were not elevated per tissue DNA. Figure 2 shows cathepsin B, PA, and type I collagenase activities dependent on the grade of cell differentiation in SCC. Poorly differentiated cell group (grade III) exhibited a tendency to have higher proteinase activities, especially in cathepsin B (p < 0.05) and type I collagenase (p < 0.05).

DISCUSSION

Tumors derived from keratinocyte, SCC, BCE, and SK have different clinical characteristics based on invasive and metastatic potentials. A concurrent comparison of the enzyme activities in various kinds of tumors was thought to be beneficial to demonstrate the biochemical aspects of malignant tumors. When extracts from surgically excised tumors were used as samples, we had the advantage of knowing conditions similar to those in vivo. However, we should take into account the effects of contaminating cell matrix, fibroblasts, and infiltrating cells, even though surrounding normal tissue and blood were removed as much as possible. There does remain, however, the possibility that contaminating proteinases and natural inhibitors affected the results. Nevertheless, research work using cultured clonal cells also has limitations concerning any speculation related to the in vivo condition, as cultured cells do not necessarily maintain the original characteristics [30]. Recent research revealed interactions between cultured malignant cells and normal fibroblasts [31,32]. We felt that with extracts from tumor homogenates it was possible to know the balance of enzyme activities in tumors as they are found in vivo. As the volume of the tumor specimens, especially that of BCE, was restricted, optimal extraction conditions for all the enzymes were not necessarily obtained. The data from SK is valid for a benign tumor, not for normal tissues. Confirmatory data from normal thin epidermis were not obtained by exactly the same extraction methods (not shown in figure).

Of the enzymes assayed, the activities of acid phosphatase and cathepsin D were found not to vary among the three tumors. This result suggests that there is no significant difference in the lysosomal functions of the tumors. Sloan et al [6,7] also observed this phenomenon and then found increased cathepsin B activity in the plasma membrane fraction corresponding to the metastatic potentials of B16 melanoma. In our study, the enzyme activities of both cathepsin B and PA were elevated in SCC, but not significantly. There is a possibility that the existence of natural inhibitors, low salt extraction for PA, and high pH extraction for cathepsin B minimized the activity of each enzyme. Collagenase assay was carried out with FITC-labeled collagen [28,29], which was believed to have as high a sensitivity as C-labeled collagen. Activity of type I collagenase, the degrading enzyme of stromal type I collagen, was significantly elevated in SCC, which coincided with a previous report by Hashimoto et al [15]. Our study proved further that the activity of type IV collagenase is significantly increased in SCC. Analyzed type I and IV collagenase activities of BCE were more highly elevated than that of SK per tissue protein but were not.

Figure 2. Cathepsin B, PA, and type I collagenase activities dependent on the grade of cell differentiation in SCC. Enzyme activities were compared per mg protein or per μg DNA. Bars, standard error. Asterisks indicate that values are significantly different from that of grade I (*** p < 0.05).
elevated per tissue DNA. Considering that cell density showed a tendency to parallel tissue DNA, it seemed reasonable to compare enzyme activities by tissue DNA. We failed to clarify the clinical characteristics of BCE, which invades the surrounding cell matrix but rarely metastasizes, because we initially expected a high type I collagenase activity and a low type IV collagenase activity. The reason for the type IV collagenase activity in each tumor being about one-tenth of that of type I collagenase might be the membrane-bound localization of type IV collagenase [1]. It is important to note that a mild correlation was observed between the grade of cell differentiation and enzyme activities in cathepsin B, PA, and type I collagenase, though Abecasis et al. [17] failed to find this correlation in breast carcinomas. The elevated intracellular proteinases might be related to tumor cell invasion as well as to intracellular metabolism. In SCC, type I and IV collagenases may assume major responsibility for invasiveness and metastasis, and cathepsin B and PA may assist their activities. However, it is uncertain whether cathepsin B and plasmin directly degrade the cell matrix or indirectly degrade it by activating latent collagenases [1]. A fairly large variation in enzyme levels in each tumor may reflect the heterogeneity of tumor cells, as was pointed out by Fidler [30], but the effect of the contaminating proteinases and natural inhibitors must also be considered to be possibly relevant. A further study is now being performed with more cases, especially those of BCE.

REFERENCES
