Electrophoretic Analysis of a Gastric Cancer-Associated Acid Proteinase Using a Highly Sensitive Detection System

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A highly sensitive detection system for acid proteinase separated on polyacrylamide gel was established. This system consisted of two-dimensional electrophoresis, combined with isoelectric focusing and polyacrylamide gel electrophoresis, and casein clotting (caseogram). Human urine, serum and gastric tissues obtained from normal individuals and gastric cancer patients were analyzed using this system. The previous electrophoretic method was not sufficiently sensitive to detect small amounts of pepsinogen (PG) C in normal urine. However, the new rapid and sensitive method clearly revealed its presence. In gastric tissue containing cancer cells, an additional proteinase, which was not present in normal tissue, was detected and named medium moving proteinase (MMP). MMP resembled PGs in alkaline stability rather than the non-PG proteinase, slow moving proteinase (SMP).

Keywords: pepsinogen; slow moving proteinase; caseogram; two-dimensional electrophoresis; gastric cancer; cancer-associated proteinase.

Human gastric mucosa contains several distinct types of aspartic proteinases that usually function under acid conditions. Samloff1) reported that eight bands (designated Pg 1 through Pg 7 and slow moving proteinase) having proteolytic activity were detected in mucosal extracts by agar gel electrophoresis. On biochemical and immunological grounds these have been divided into three groups.2)–6) Pg 1 through Pg 5 belong to pepsinogen group A (PGA, also called PG I; 43 kilodaltons). Pg 6 and 7 belong to pepsinogen group C (PGC, also called PG II; 38 kilodaltons). Slow moving proteinase (SMP), exhibiting slower anodal mobility than either PGA or PGC, may be classified as cathepsin E.7)8) PGA and PGC are present in not only gastric tissue but in serum5) and in the urine of premature infants9) or in patients with renal diseases.2) In normal urine, only PGA is detectable by electrophoresis.2) However, a recent immunoassay for PGA revealed the presence of a trace amount in normal urine.10,11)

Immunoassays for PGA or PGC have recently been used in the diagnosis of several diseases. Low serum PGA levels are found in patients with atrophic gastritis12)–14) or gastric cancer,15)–17) but a significant change of PGC levels is not found in these patients. Therefore, the serum PGA/PGC ratio is a potentially useful parameter for the diagnosis of gastric cancer and its precursor.18)–20) Not only this parameter (serum PGA/PGC ratio measured by immunoassay) but the visual elements of electrophoretic analysis of these proteinases in urine, serum, and gastric mucosa are also useful for second screening or for monitoring the course of the disease.

After agar or polyacrylamide gel electrophoresis, aspartic (acid) proteinases are usually detected by digestion of hemoglobin.1) However, Foltmann et al.21) reported that these proteinases could be detected more rapidly and sensitively by casein clotting, called a "caseogram." In this study we adapted this sensitive detection system to high resolution two-dimensional electrophoresis, and we report the application of this technique to the analysis of acid proteinases in several samples from normal controls and gastric cancer patients.

MATERIALS AND METHODS

Materials and Reagents Serum or early morning urine were collected from two healthy volunteers and two patients with gastric cancer, and stored at 20°C until use. The samples from the cancer patients were collected 2 d before surgery. Gastric tissues were extracted from four gastric cancer patients, and stored at 20°C until use. The tissue was divided into those with and those without cancer cells, and used as cancer tissue and normal tissue, respectively. The samples used in this experiment are summarized in Table I.

DEAE-Sephacel and Ampholines were purchased from Pharmacia LKB Biotechnology, Sweden. Pepsin A (from porcine stomach mucosa, 3000 units/mg) and Coomassie brilliant blue (CBB) R-250 were obtained from Sigma Chemical Co., U.S.A. Agarose (type S) and skim-milk powder were products of Nippon Gene Co., Japan and Snow Brand Milk Products Co., Japan, respectively. Bovine hemoglobin and all other chemicals were purchased from Wako Pure Chemical Industries Ltd., Japan.

Preparation of Samples Urine was concentrated from 400 µl to approximately 20 µl using an Ultrafree C3-TTK unit (Nippon Millipore Ltd., Japan). Serum (500 µl) was diluted with 1 ml of 25 mm sodium phosphate buffer (pH 7.0) then applied to a small column (1 × 1.5 cm) containing DEAE-Sephacel, equilibrated with the same buffer. The column was washed with the same buffer, and the materials that eluted between 0.15 and 0.5 M NaCl were collected. This fraction was concentrated to about 20 µl using an Ultrafree C3-TTK unit. The sample preparation from gastric tissue was performed according Samloff and Townes23) with slight modifications. Gastric tissue, 0.2 g, was homogenized with 1 ml of 0.1 M sodium phosphate buffer (pH 7.3) in a glass homogenizer with a Teflon pestle.
at 2000 rpm for 2 min. This step was carried out in an ice bath, and then the homogenate was centrifuged at 3900 × g for 5 min. The supernatant was diluted with this buffer and used as tissue extract.

**Enzyme Activity and Protein Concentration** Proteinase activity was measured by a turbidimetric milk-clotting assay as described by McPhie23 with slight modifications. The reaction proceeded at 30 °C in 2.5 ml of 0.264 M sodium acetate buffer (pH 5.3) containing 8 mM calcium chloride and 0.16% skim-milk. Sample or standard solutions (10 μl) were mixed, and the absorbance change at 510 nm was measured. Pepsin A solutions of five different known concentrations (0.25—4 units/ml) were used as the standards. Each standard solution was assayed three times, and a standard curve was obtained. The enzyme activity of each sample was determined with this standard curve.

The protein concentration was measured with a Protein assay kit II (Bio-Rad Laboratories, U.S.A.), using bovine serum albumin as the standard.

**Gel Electrophoresis** Ten to twenty microliter of samples were resolved by electrophoresis. Proteins were separated on continuous or discontinuous polyacrylamide gels (7 × 8 × 0.15 cm). The continuous system consisted of a separating gel (7.5% acrylamide-bisacrylamide C = 1.8%, in 50 mM Tris-acetate, pH 8.2) and 50 mM Tris-acetate electrode buffer, pH 8.2. The discontinuous system consisted of a stacking gel (3.75% acrylamide-bisacrylamide C = 2.7%, in 0.05 M Tris-HCl, pH 5.5), a separating gel (7.5% acrylamide-bisacrylamide C = 2.7%, in 0.14 M Tris-HCl, pH 7.5), and 8.25 mM Tris-30 mM veronal electrode buffer, pH 7.4. Electrophoresis proceeded at a constant voltage of 110 V for 1.5 h.

Two-dimensional electrophoresis included a combination of isoelectric focusing (IEF) and polyacrylamide gel electrophoresis (PAGE). The first dimension (IEF) gel consisted of 5% acrylamide-bisacrylamide (C = 3%), 2% Ampholines (pH 3.5—10/pH 3.5—5 = 1/4), and 100 mM 3-(N-morpholino propanesulfonic acid, which was added as a chemical spacer to spread the acidic region.23) The IEF gel was poured to a height of 10 cm in glass tube (1.5 mm i.d.). The anodic and cathodic reservoirs were filled with 0.01 M H3PO4 and 0.04 M NaOH, respectively. Electrophoresis was continued for 30 min at 150 V, then for 3 h at 300 V. IEF gel was equilibrated with 50 mM Tris-acetate buffer (pH 8.2) and placed on the second dimension (PAGE) gel in the continuous system described above.

**Detection of Acid Proteinases** Acid proteinases separated on polyacrylamide gel were activated by incubation in 0.1 M HCl for 15 min at room temperature. Activated proteinases were detected by hemoglobin digestion (hemoglobin zymogram) or casein clotting (caseogram). The hemoglobin zymogram was produced by the method of Defize et al.24 with slight modifications. The HCl-treated gel was immersed in 1% bovine hemoglobin in 0.1 M for 15 min at 37 °C. The gel was then removed from the hemoglobin and digested for a further 40 min at 37 °C in a humidified chamber. The gel was subsequently stained with 0.6% CBB R-250 in 9% acetic acid and 45%

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**Fig. 1. Detection of Acid Proteinases Separated by One- or Two-Dimensional Electrophoresis on Polyacrylamide Gels**

(A) Concentrated urine (NI-U, lane 1) and gastric tissue extracts (C2-nT, lane 2) were analyzed by standard one-dimensional PAGE and caseogram. PAGE was performed using the discontinuous system described under Materials and Methods. (B and C) The gastric tissue extracts (C2-nT) were analyzed by means of two-dimensional electrophoresis and a caseogram (B), or a hemoglobin zymogram (C). PAGE was performed using the continuous system.
methanol for 60 min, then destained in 9% acetic acid and 45% methanol. The activated proteinases hydrolyzed hemoglobin and produced unstained areas.

Caseograms were produced by the method of Foltmann et al. with slight modifications. The HCl-treated gel was incubated in 0.3 M sodium acetate buffer (pH 5.3), and 1% agarose gel containing 1% skim-milk in the same buffer was placed on top of the electrophoresis gel. Casein clotting was continued for 30—120 min at 37°C. The proteinase-containing zone produced clearly white precipitates. For permanent documentation the precipitates on the skim-milk gel were stained with 3% black ink (Pilot Corp., Japan) for 2 min. Non-precipitated proteins were rapidly eluted with water.

RESULTS

Detection of Acid Proteinases by Two-Dimensional Electrophoresis and Caseograms Acid proteinases in human urine and gastric tissue were analyzed by standard one-dimensional PAGE or two-dimensional electrophoresis (IEF + PAGE). As shown in Fig. 1A, PGA in urine (lane 1) and PGA, PGC and SMP in gastric tissue (lane 2) were clearly visible on the caseogram. However, these proteinases were better separated and more clearly visualized by a combination of two-dimensional electrophoresis and the caseogram (Fig. 1B). As shown in Fig. 1B, the isoelectric points of PGA, PGC and SMP were between 4 and 4.5. The bands or spots that appeared on caseogram gels diffuse fairly quickly. This is a downside of caseograms, but it was overcome by means of two-dimensional electrophoresis. Furthermore, the caseogram was superior in simplicity and sensitivity to the hemoglobin zymogram (Fig. 1C). For these reasons, we used this separation—detection system (two-dimensional electrophoresis and caseogram) for all further studies.

Acid Proteinases in Human Urine and Serum The electrophoretic profiles of acid proteinases in human urine and serum are shown in Fig. 2. In all of them (A—H), one PGC (indicated by arrowhead) and several PGA spots were detected; however, SMP was undetectable. Small amounts of PGC in normal urine have been difficult to detect by regular electrophoresis. However, it was clearly visualized using the new method (Fig. 2A and B).

The PGA/PGC ratio was determined by densitometry of each spot. The PGA/PGC ratio of normal urine was greater than that in the urine of cancer patients, because of the increase of PGC or decrease of PGA in the urine of the latter (Fig. 2C and D). Figure 2E—H show the profiles of acid proteinases in human serum (E and F, normal controls; G and H, cancer patients). In serum samples, the PGC spots were much more intense than those in urine samples. As shown in profiles G and H, the PAG spots in the serum of cancer patients were remarkably decreased.

Acid Proteinases in Human Gastric Tissues The

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![Electrophoretic Heterogeneity of Pepsinogens in Human Urine and Serum](image-url)

Urines (A—D) and serum (E—H) samples were analyzed by means of two-dimensional electrophoresis and a caseogram. (A) N1-U; (B) N2-U; (C) C3-U; (D) C4-U; (E) N3-S; (F) N4-S; (G) C3-S; (H) C4-S. The PGC spot is indicated by an arrowhead. The other spots are PGA isozymes. The absorbance of each spot was measured by densitometry using a dual-wavelength chromatoscanner (Shimadzu CS-900, Japan), and the PGA/PGC ratio was calculated. a) PGA spots were not detected in pattern H.
electrophoretic profiles of acid proteinases in human gastric tissues are shown in Fig. 3. A (C1-nT) and C (C4-nT) are the profiles of normal tissues; B (C1-cT) and D (C4-cT) are those of cancer tissues. As shown in Table I, normal and cancer tissues (C1-nT and C1-cT; C4-nT and C4-cT) were derived from same patients. As compared with the profiles of normal tissue (A and C), no significant change in the PGC spot was evident in cancer tissue samples. However, there was a remarkable decrease in the intensity of the PGA spots. Moreover, an additional proteinase spot (indicated by an arrow) appeared in B and D. This gastric cancer-associated proteinase was designated "medium moving proteinase (MMP)."

The classification of cancer tissue and the acid proteinases in normal and cancer tissues obtained from four patients (C1—C4) are summarized in Table II. As compared with normal tissues, there was a considerable decrease in the proteinase activity and the PGA/PGC ratio in cancer tissues as decreased previously. We detected MMP in all cancers are not any in normal tissues (Table II).

**Table I.** Origin of Human Urine, Serum and Gastric Tissue

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td></td>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>N1</td>
<td>35</td>
<td>M</td>
<td>N1-U</td>
</tr>
<tr>
<td>N2</td>
<td>24</td>
<td>M</td>
<td>N2-U</td>
</tr>
<tr>
<td>N3</td>
<td>24</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>N4</td>
<td>22</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>Cancer patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>61</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>88</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>48</td>
<td>M</td>
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</tr>
<tr>
<td>C4</td>
<td>67</td>
<td>M</td>
<td>C4-U</td>
</tr>
</tbody>
</table>

a) M, male; F, female. b) nT, normal tissue (without cancer cells); cT, cancer tissue.

Alkaline Stability of PGs, SMP and MMP Acid-treated PGA (pepsin A), PGC (pepsin C) and SMP have somewhat different alkaline stability. Athauda et al. reported that pepsin A was rapidly inactivated by incubation at pH 7.0, whereas pepsin C showed considerable stability at this pH. Moreover, Samloff et al. reported that unlike PGs, SMP retained proteolytic activity after sequential acidification and neutralization, and Muto et al. reported that acid-treated cathepsin D-like acid proteinase (probably SMP) was stable up to pH 9.5, but rapidly inactivated at higher pH values. Therefore, we examined the alkaline stability of the MMP in gastric cancer tissues.

Normal (C2-nT) and cancer (C1-nT) tissues were analyzed by caseograms (Fig. 4A and C), or by the additional modification at pH 8.6 (Fig. 4B and D). The
**TABLE II. Properties of Cancer Tissues and Electrophoretic Heterogeneity of Acid Proteinases**

<table>
<thead>
<tr>
<th>Gastric tissue</th>
<th>Phenotype of cancer tissue</th>
<th>Proteinase activity (units/mg protein)</th>
<th>Acid proteinases</th>
<th>PGA/PGC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class a) Stage</td>
<td>Histology b)</td>
<td>A(2)</td>
<td>C(2)</td>
</tr>
<tr>
<td>C1-αT</td>
<td>B-4</td>
<td>IV</td>
<td>sig</td>
<td>97.0</td>
</tr>
<tr>
<td>C1-εT</td>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>C2-αT</td>
<td>B-4</td>
<td>III</td>
<td>tub₁-tub₂</td>
<td>50.5</td>
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<tr>
<td>C2-εT</td>
<td></td>
<td></td>
<td></td>
<td>&lt;1.0</td>
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<tr>
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<td>II</td>
<td>por-sig</td>
<td>348.9</td>
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<tr>
<td>C3-εT</td>
<td></td>
<td></td>
<td></td>
<td>55.4</td>
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<tr>
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<td>B-3</td>
<td>III</td>
<td>tub₂</td>
<td>169.4</td>
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<tr>
<td>C4-εT</td>
<td></td>
<td></td>
<td></td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

a) Classification of Bormann type.  b) sig, signet-ring cell carcinoma; tub₁, well differentiated tubular adenocarcinoma; tub₂, moderately differentiated tubular adenocarcinoma; por, poorly differentiated adenocarcinoma.  c) A(2), two isozymes of PGA; A(4), four isozymes of PGA; C(2), two isozymes of PGC; SMP, slow moving proteinase; MMP, medium moving proteinase.

**Fig. 4. The Effect of Sequential Acidification and Neutralization on Acid Proteinases**

(A and B) C2-αT; (C and D) C1-εT. A and C, acid-treated proteinases on the gels were immediately detected by caseogram. B and D, samples were also incubated in 50 mK Tris-acetate (pH 8.6) for 30 min at room temperature. MMP (indicated by arrow) is present in C, not in D.

SMP spot did not change, whereas PGC remarkably decreased and PGA was completely eliminated in all profiles. The MMP spot (indicated by arrow in pattern C) was also eliminated by this procedure (Fig. 4D). This result indicates that MMP resembles PGs in alkaline stability rather than SMP.

**DISCUSSION**

We have described here, (i) the establishment of a highly sensitive detection system for acid proteinases separated on polyacrylamide gels, (ii) the application of this system to the analysis of human urine, serum and gastric tissue samples, and (iii) the detection of a gastric cancer-associated acid proteinase (designated MMP).
The previous electrophoretic method was not sufficiently sensitive to detect small amount of PGC in normal urine. However, this method clearly revealed its presence. The presence of PGC in normal urine has been shown by means of an enzyme-linked immunosorbent assay (ELISA). Kate et al. reported that the urinary excretion (mg/24 h) of PAG and PGC in normal individuals \((n = 21)\) were \(1.8 \pm 1.1\) and \(0.02 \pm 0.003\), respectively (PGAG/PGC = 30–170). Our PAG/PGC ratio for normal urine \((171.1\) for N1-U and 63.6 for N2-U) was consistent with their report.

The decrease of PAG/PGC ratio in urine and serum of gastric cancer patients was also observed by this method. Westerveld et al. determined the serum PAG and PGC levels by ELISA, and reported that the PAG/PGC ratio in normal controls \((n = 19)\) and gastric cancer patients \((n = 60)\) were \(3.12 \pm 1.20\) and \(1.29 \pm 1.05\), respectively. However, the PAG/PGC ratio in serum \((0.8 \pm 1.0\) for normal controls, \(0.1 \pm 0.1\) for cancer patients) we obtained was considerably lower than that they obtained. The PAG/PGC ratio was determined by densitometry of each spot in this study. Therefore, our data are based on the enzymatic activity of PGs toward casein. This may be one of the reasons for this difference. The assay systems of this method and ELISA are completely different. Therefore, it might be difficult to compare directly our data with those of ELISA. But the difference of both systems means that this method has the ability to supplement the ELISA screening for stomach cancer. Though further studies are needed, it is potential to get more useful information by not comparison but combination of both systems.

This method is essentially a detection system for acid proteinases. It remains to solve some problems in order to apply this method to assays for PGs. For example, the strong casein clotting changes gradually to the casein degradation. The linearity of densitometry is, therefore, relatively limited. If the very weak spot(s) is present in this system, it may be difficult to determine the exact PAG/PGC ratio by densitometry. To read the weak spot (for example, PGC in normal urine) by a densitometer, a long-clotting time is necessary. The long-clotting time causes the other spots to become larger, and then these strong white spots change gradually to clear spots. In that case, the exact PAG/PGC ratio may not be obtained by densitometry. However, it is possible to cut out the proteinase-containing zone from the electrophoresis gel after detection with a caseogram print and to measure each proteinase activity directly. This direct assay of the proteinase-containing gel pieces is in progress, and it will reveal how accurate the PAG/PGC ratio is by the densitometer reading. We have no data about loss of each proteinase in the step of sample preparation. This may be a problem if this method is applied to assays for the absolute value of PGs. However, we used this method for measuring the relative value of PAG and PGC. To confirm our PAG/PGC data, we plan to collect more data from many samples under the same conditions (e.g., sample preparation, electrophoresis, clotting time or temperature).

In gastric tissue containing cancer cells, a new acid proteinase was detected by this method and named MMP. The acid proteinases in human gastric mucosa are mainly PAG, PGC, and SMP, but it contains also the lysosomal acid proteinase, cathepsin D, as a minor component. Cathepsin D has been detected in normal and cancer tissues, and the isolectric points of human cathepsin D isoenzymes are 5.7, 6.0 and 6.5. However, MMP was only detectable in cancer tissue, and its isolectric point was 4.3–4.4. Therefore, MMP may be different from cathepsin D. Although further studies are necessary to clarify the structural and other properties of MMP, our findings suggest that MMP resembles PAG or PGC in enzymatic properties rather than SMP.

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