Cathepsins D and E in normal, metaplastic, dysplastic, and carcinomatous gastric tissue: an immunohistochemical study

T Saku, H Sakai, N Tsuda, H Okabe, Y Kato, K Yamamoto

Abstract

Immunohistochemical distributions of cathepsins D and E were determined in normal mucosa, metaplastic, dysplastic, and cancerous lesions of the human stomach. Cathepsins D and E were localised in the foveolar epithelium and parietal cells of the normal gastric mucosa, but their intracytoplasmic distributions were different – cathepsin E distribution was even and diffuse in the cytoplasm while cathepsin D was found in coarse intracytoplasmic granules. Chronic inflammation and ulcer did not influence the distribution of these enzymes. No positive staining was obtained in the incomplete type of intestinal metaplasia, dysplasia, and well differentiated adenocarcinoma. Tumour cells of signet ring cell carcinoma and poorly differentiated adenocarcinoma cells, however, gave strong and diffuse stainings for cathepsins D and E in the cytoplasm. The results suggest that the distribution of cathepsins D and E is related to each specialised function of the foveolar epithelium and the parietal cells, and that their disappearance is associated with development of well differentiated adenocarcinoma from intestinal metaplasia.

Human gastric mucosa contains two different types of aspartic proteinase. One is a secretory type consisting of enzymes that function in extracellular spaces – for example, pepsin and gastricsin. The other is a non-secretory type consisting of enzymes that function primarily within the cell (cathepsin D and cathepsin E). Little is known about the functions of the intracellular aspartic proteinases, compared with those of the secretory enzymes. In particular, there has been no information about their clinical importance in gastric mucosal abnormalities.

Cathepsins D and E are the main intracellular aspartic proteinases and have similar catalytic properties – for example, optimum pH, substrate specificity, and susceptibility to various protease inhibitors – although they are distinct proteins. It has been suggested that cathepsins D and E may share some functions in intracellular protein catabolism. Very recently, immunochromatography and immunohistochemical analyses using the discriminative antibodies to cathepsins E and D have shown that the distribution of these two enzymes in various rat tissues and cells is noticeably different. Cathepsin D has a widespread distribution, whereas that of cathepsin E is relatively limited. Although the greatest accumulation of both cathepsins D and E has been observed in the human and rat gastric mucosa, their localisation in this tissue is noticeably different. Cathepsin E is mainly present in the foveolar epithelium, while cathepsin D is found largely in the fundic gland epithelial cells, especially in the deeper parietal cells. This difference may be associated with the functions of the specially differentiated cells in which the respective enzymes are found. It was therefore considered of interest to determine the biological behaviour of these two enzymes in abnormal gastric tissue in order to understand their pathophysiological roles in gastric epithelia.

In this study, we carried out an immunohistochemical comparison of cathepsins E and D in normal, inflamed, or ulcerative mucosa; intestinal metaplasia; dysplastic epithelium; and carcinomatous mucosa of the human stomach.

Methods

MATERIALS

Gastrectomy specimens from 21 patients with gastric carcinoma, 10 with gastric ulcer, were collected at Nagasaki University Hospital between 1987 and 1988. All specimens were fixed in formalin and embedded in paraffin. The specimens collected from patients with carcinoma consisted of papillary adenocarcinoma (1), well differentiated tubular adenocarcinoma (6), moderately differentiated tubular adenocarcinoma (6), poorly differentiated adenocarcinoma (4), mucinous adenocarcinoma (2), and signet ring cell carcinoma (2). Tissue blocks of normal mucosa were also obtained from each patient with carcinoma. Most patients also had gastritis and atypical epithelial lesions were found in four.

ANTIBODIES

The antisera against cathepsin E purified from the rat spleen, cathepsin E (erythrocyte membrane aspartic protease, EMAP) purified from the human erythrocyte membranes, and cathepsin D purified from the rat spleen were raised in rabbits as described previously. The IgG fractions from these antisera were prepared by passing them through columns of antigen-coupled Sepharose 4B (Pharmacia, Uppsala, Sweden).

IMMUNOHISTOCHEMICAL STAINING

The serial sections (5 μm thick) were blocked with 5% milk proteins in phosphate buffered saline and subsequently immunostained by the peroxidase-antiperoxidase (PAP) technique of
The primary antibodies were diluted to a concentration of 50 μg/ml. Before incubation with the antibodies, endogenous peroxidase activity was blocked by the method of Isobe et al.\textsuperscript{12} using periodic acid and sodium borohydride. The reaction products were developed by 3,3′-diaminobenzidine. As a control, the first antibodies were replaced by normal rabbit IgG. Nuclear counterstaining was carried out with haematoxylin.

HISTOCHEMICAL STAINING
To differentiate goblet cells in the mucosa with intestinal metaplasia, high iron diamine (HID)-Alcian blue staining was performed on another set of sections, according to the method of Matsukura et al.\textsuperscript{13} Deparaffined sections were washed in deionized water and then incubated with a reagent consisting of 120 mg N,N-dimethyl-m-phenylenediamine 2HCl and 20 mg N,N-dimethyl-p-phenylenediamine HCl in 50 ml deionized water with 1·4 ml of 40% ferric chloride. After incubation for 18 hours at room temperature, the sections were destained in ethanol, followed by an incubation with 1% Alcian blue in 3% acetic acid for 30 minutes at room temperature. With this high iron diamine positive mucin stained dark purple and high iron diamine negative mucin stained blue.

Results
Antibodies against rat cathepsins D and E cross-reacted with the human gastric tissue. Since the antibodies against human cathepsin E and rat cathepsin E did not show any prominent differences in the stainings, we grouped the results obtained with these two kinds of antibodies together. The Table summarises the incidence of immunoreactivity for cathepsins D and E in each gastric lesion.

NORMAL GASTRIC TISSUE
The distributions of cathepsins D and E were

Figure 1: Normal mucosa in the gastric body. Immunoperoxidase staining for cathepsin D (A, C) and cathepsin E (B, D). Counterstained with haematoxylin. In the foveolar epithelium, cathepsin D is localised at the free end and cathepsin E is diffusely distributed in the cytoplasm. Parietal cells in the neck or middle portion of the fundic gland show more intensive staining for cathepsins D and E. Bar, 100 μm.
distinct in the normal gastric mucosa. In the fundic gland, the staining of cathepsin D was granular and limited to the free surface of foveolar epithelial cells (Fig 1(A)), whereas cathepsin E was diffusely and intensively found in the subvacuolar region of the epithelial cells (Fig 1(B)). Parietal cells gave fine granular stainings for cathepsin D and cathepsin E (Fig 1(C) and (D)). More intensive stainings were obtained in the parietal cells in the neck of the gland than in other locations. Occasionally, mucous covering the mucosal surface contained cathepsin E positive material that was sometimes associated with cell debris. Macrophage like cells which had cathepsin D positive granules in the cytoplasm were sometimes recognised in the interstitium and lymphoid nodules of the lamina propria. Ganglion cells in the myenteric plexus of Auerbach were strongly positive for cathepsin D.

In the pyloric gland, cathepsin E was clearly positive in the surface epithelial cells located in the upper portion of the deep foveae. The staining became less intensive in the deeper portion. The pyloric gland cells were weakly positive for cathepsin E. The staining was finely granular and limited to the basal region of the cytoplasm of the mucous cells. These cells also contained coarse granules which were positive for cathepsin D in the apical end. Similar findings were observed in the cardiac glands.

CHRONIC GASTRITIS AND GASTRIC ULCER
Chronically inflamed gastric mucosa showed basically the same immunohistochemical distribution of these enzymes as normal mucosa, and erosive or ulcerative changes did not influence the basic staining patterns of either cathepsin. Single layerd regenerating epithelial cells which stretched from the intact mucosa to the edge of the ulcer showed evident immunoreactions for cathepsins D and E. In the ulcer base, there was an infiltration of cathepsin D positive macrophage like cells, which were located in a row just below the fibrin clot layer.

INTESTINAL METAPLASIA
Intestinal metaplasia was found in unaffected mucosa of 20 subjects. Metaplastic foveolar or glandular epithelium tended to lose immunoreactivity for cathepsins D (Fig 2(A)) and E (Fig 2(B)). Disappearance of the immunoreaction was particularly evident in the goblet cells with prominent mucinogen droplets in the cytoplasm. The disappearance did not, however, seem to be related to the release of droplets in apocrine fashion. Histochemically, high iron diamine positive goblet cells of the incomplete type of intestinal metaplasia did not show positive reactions for cathepsins D and E (Fig 2(C)) nor were most of Alcian blue positive cells (complete type of intestinal metaplasia) positive for both cathepsins. Among these Alcian blue positive cells, however, those in the surface epithelium showed partially positive reactions for cathepsins D or E (35% of subjects, respectively). The immunoreactivities for cathepsins D and E were not always obtained in the same cells or in the same subjects.

DYSPLASIA
There were no immunoreactions to cathepsins D and E in the surface and glandular epithelial cells when dysplastic changes were seen. In particular, pseudostratified epithelium with hyperchromatic and elongated nuclei, tall and basophilic cytoplasm, and scanty mucous content did not show any positive stainings for either cathepsin. Nor did the flattened epithelial cells of dilated glands in deeper positions show any positive staining, (Fig 3(A) and (B)).

ADENOCARCINOMA
No labelling for either cathepsin D or E was seen in the cells of well differentiated tubular or papillary adenocarcinoma. Only a stromal infiltration of cathepsin D positive macrophage like cells was noticeable in the carcinoma foci (Fig 4(A) and (B)). There was no positive staining either in the tumour cells of mucinous adenocarcinoma.
carcinoma with an active mucous secretion into the extracellular matrix. However, in more than half of the subjects with moderately differentiated adenocarcinoma, cathepsins D (three of six) and E (four of six) were found in some of the cells which formed bizarre ductal structures. Cathepsin E was found diffusely in the cytoplasm, whereas the immunoreaction products for cathepsin D were always in coarse granules which were evenly distributed in the cytoplasm but not in the apical surface. In all subjects with poorly differentiated adenocarcinoma, intensive and diffuse cytoplasmic stainings for both cathepsins D and E were observed in most of the tumour cells (Fig 5(A) and (B)). The cells of signet ring cell carcinoma also gave a prominent staining for both cathepsins D and E. Their stainings were diffuse but limited to the surroundings of mucinogen droplets, giving a ring shape appearance (Fig 6(A) and (B)). Macrophage like cells which had cathepsin D positive granules infiltrated the tumour stroma, although there was no definite relation between differentiation of the carcinomas and the degree of infiltration by these cells.

### Discussion

Immunohistochemical distributions of cathepsins D and E in human gastric lesions were analysed and compared with those in normal

<table>
<thead>
<tr>
<th>Gastric conditions</th>
<th>No of patients</th>
<th>No of cathepsin D positive (%)</th>
<th>No of cathepsin E positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>21</td>
<td>21 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Gastritis</td>
<td>31</td>
<td>31 (100)</td>
<td>31 (100)</td>
</tr>
<tr>
<td>Peptic ulcer</td>
<td>10</td>
<td>10 (100)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>20</td>
<td>7 (35)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>4</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Adenocarcinoma papillary</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tubular, well differentiated</td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tubular, moderately differentiated</td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Signet ring cell</td>
<td>2</td>
<td>2 (100)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

![Figure 4: Well differentiated tubular adenocarcinoma. Immunoperoxidase staining for cathepsin D (A) and cathepsin E (B). Counterstained with hematoxylin. The tumour cells show no positive reaction for enzymes, while macrophages in the stroma are heavily labelled with anticathepsin D. Cathepsin E is barely shown in atrophic epithelial cells of the foveolae. Bar, 100 μm.](http://gut.bmj.com/)
gastric mucosa. The two enzymes showed different intracytoplasmic distributions in normal mucosa. Punctate or granular staining of cathepsin D in the foveolar epithelial cells and parietal cells suggests its localisation in lysosomes, while rather diffuse staining of cathepsin E in these cells is indicative of its cytosol distribution. Cathepsin D has been well characterised as a typical lysosomal enzyme. Cathepsin E has been shown to exist diffusely in the cytoplasmic matrix but was not associated with lysosomes in our recent investigation with immuno-electron microscopy (unpublished data). This different distribution of cathepsins D and E within a cell indicates that these enzymes play different roles in the same cells. Their limited distribution in foveolar epithelial and parietal cells also suggests that they are closely related to specialised cellular functions such as secretion of mucous and gastric acid.

The differential distribution of cathepsins D and E in the normal mucosa was lost in cancerous and precancerous lesions. The two enzymes disappeared in parallel in intestinal metaplasia, dysplasia, and well differentiated adenocarcinoma and both occurred with a similar staining pattern in poorly differentiated adenocarcinoma and signet ring cell carcinoma. Epidemiological and histopathological methods have shown that intestinal metaplasia of the gastric mucosa is closely related to the development of well differentiated adenocarcinoma. In particular, the incomplete type of intestinal metaplasia where the epithelial cells have high iron diamine positive mucin is regarded as an incipient phase in the development of gastric carcinoma. In the present study, high iron diamine positive goblet cells were always shown to be negative for cathepsins D and E. Less intensive immunostaining for slow moving protease (SMP, now called cathepsin E) in intestinal metaplasia has already been reported. This coincidence in staining patterns seems to add new evidence for the close relation between intestinal metaplasia and the well differentiated form of gastric adenocarcinoma.

The preliminary study on the immunohistochemical distribution of cathepsin E in gastric cancer was reported by Shiraiishi et al. They showed that the frequency of cathepsin E (SMP) staining was higher in diffuse type (75%) than in intestinal type (46%). The intestinal type subjects reported by these authors presumably included all types of tubular adenocarcinoma, whereas we could have classified patients with tubular adenocarcinoma into three grades. The 46% positive in their intestinal type adenocarcinoma might correspond with the 67% positive in our patients with moderately differentiated tubular adenocarcinoma. Reid et al reported that in some subjects with gastric carcinoma, cathepsin D was mainly confined to the stromal cells, although they did not state the histopathological types of carcinoma in their subjects. This description might suggest that their findings were similar to ours in well differentiated tubular adenocarcinoma.

It is difficult to explain why both cathepsins are absent in precancerous lesions and well differentiated adenocarcinoma. One important fact, however, is that intracellular processing of some proteins is at least suppressed during the early stages of malignant transformation, and
that this dysfunctioning of cathepsins D and E could play a part in the development of carcinoma in the gastric mucosa. Depletion of enzymatic activity of acid protease has been shown in gastric carcinomas by Kühn and Bezuidenhout, although they neither discriminated between cathepsins D, E, and pepsin activities nor indicated the histological subtype of adenocarcinoma. However, less frequent positive immunostaining for pepsinogens I (5%), II (31%), and cathepsin E (SMP) (54%) has been shown in both the intestinal and diffuse types of gastric carcinoma. One of the other hand, in our series, in poorly differentiated adenocarcinoma and signet ring cell carcinoma, the intracytoplasmic staining for cathepsin D became diffuse in the same way as in cathepsin E. It is obvious that expression of cathepsins D and E in these tumour cells is controlled by different factors from those in the normal foveolar epithelial or parietal cells.

In conclusion, immunostaining for cathepsins D and E in gastric tissue offers not only an objective indicator for histopathological diagnosis of malignancy but also useful histochemical evidence for further pathophysiological studies on gastric carcinoma.
