GASTROINTESTINAL CANCER

Plasminogen activators in normal tissue and carcinomas of the human oesophagus and stomach

C F M Sier, H W Verspaget, G Griffioen, S Ganesh, H J M Vloedgraven, C B H W Lamers

Abstract

Carcinogenesis in the human colon is associated with a marked increase of urokinase type plasminogen activator and a decrease of tissue type plasminogen activator. This study was performed to determine the concentrations of urokinase type plasminogen activator and tissue type plasminogen activator in normal tissue and carcinomas along the upper part of the gastrointestinal tract. Activity and antigen levels of both activators were determined in homogenates of endoscopically obtained biopsies from normal and carcinomatous tissues. Although the concentrations of tissue type plasminogen activator and urokinase type plasminogen activator in normal squamous epithelium of the oesophagus were low compared with those in columnar epithelium from the stomach, the urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio of the different locations showed hardly any difference. Significant but heterogeneous increases were found in urokinase type plasminogen activator concentrations of biopsy specimens originating from carcinomas of both epithelial cell types. A decrease in tissue type plasminogen activator concentrations, as found in human colon carcinomas, could only be shown in carcinomas of columnar epithelium origin but not in squamous cell carcinomas of the oesophagus. The increase of urokinase type plasminogen activator and urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio and the decrease of tissue type plasminogen activator in the carcinomas did not show a significant correlation with known prognostic determinants as differentiation grade, TNM classification, intestinal metaplasia, inflammation, and ulceration. The heterogeneous increase of urokinase type plasminogen activator in oesophageal and stomach carcinomas, together with the recently described association of urokinase type plasminogen activator in tissue extracts of breast carcinomas with aggressiveness and prognosis, may be of relevance to prognostic studies in oesophageal and gastric cancer.

(Gut 1993; 34: 80–85)

Plasminogen activators are involved in many protein degrading processes by converting plasminogen into the active enzyme plasmin. Tissue type plasminogen activator (t-PA) is a key enzyme in thrombolysis, while urokinase type plasminogen activator (u-PA) plays a major role in extracellular matrix breakdown related activities like tissue remodelling and tumour invasion. Tumour extracts of different origin such as breast, lung, prostate, stomach, and colon were found to have raised concentrations of plasminogen activator activities. Immunological characterisation revealed that most malignant cells and tissues predominantly produce urokinase type plasminogen activator rather than tissue type plasminogen activator depending on their origin. Little is known, however, about plasminogen activators in solid tumours of the upper gastrointestinal tract. Early studies using crude fibrinolytic activity assays and histochemical fibrin slide techniques revealed plasminogen activators to be present in cancer tissue and cell lines obtained from the oral cavity and stomach. Moreover, increased secretion of urokinase type plasminogen activator by gastrointestinal tumours has been shown by raised plasma and urine urokinase type plasminogen activator concentrations in patients with carcinomas of the pancreas, stomach, and colorectum. We have previously shown that adenocarcinomas of the colon have a five to tenfold increase in urokinase type plasminogen activator content accompanied by a three to fivefold decrease of tissue type plasminogen activator, based on activity and antigen measurements. Comparison of plasminogen activator content between colon carcinoma resection tissue and endoscopically obtained biopsies of the same patients showed good agreement. In the same study a small group of six carcinomas of the stomach showed comparable results. The aim of the present study was to determine the concentrations of tissue type plasminogen activator and urokinase type plasminogen activator in carcinomas along the upper part of the gastrointestinal tract using endoscopical biopsies. For that purpose a comparison was made between plasminogen activator content of normal and carcinomatous biopsies of the oesophagus aligned with squamous epithelium, and of the distal oesophagus and different parts of the stomach aligned with columnar epithelium.
Plasminogen activators in normal tissue and carcinomas of the human oesophagus and stomach

Also the relation of plasminogen activator concentrations with histologically scored parameters such as differentiation grade, TNM classification, inflammation, ulceration, and intestinal metaplasia of the carcinomas was investigated.

Methods

PATIENTS

BIOPSIES

Normal mucosal biopsy specimens from three different locations of the upper gastrointestinal tract (squamous oesophagus, corpus/antrum, bulbus/duodenum) were taken during endoscopy from six dyspeptic patients without demonstrated pathology (two men, four women, age 45–48 years). These biopsy specimens were confirmed endoscopically and histologically to have no signs of underlying disease. From patients with a carcinoma in the oesophagus or stomach (37 men, 10 women, age 49–88 years) biopsy specimens were obtained from macroscopically suspected tissue and from normal mucosa 5–10 cm distal and/or proximal to the tumour. One male patient had two separate oesophageal carcinomas. The biopsies were immediately frozen at −70°C. For routine diagnostic purposes and for reference, adjacent biopsies were histologically examined by the pathologist and scored for inflammation, ulceration, intestinal metaplasia, and grade of differentiation. TNM classification of the carcinomas was performed, when possible, according to Hermanek and Sobin7 based on routine clinical evaluation. All patients included were histologically confirmed to have a carcinoma. The carcinomas were divided into two groups—squamous cell carcinomas of the oesophagus and adenocarcinomas of the lower oesophagus and stomach, and for distinction called respectively oesophageal and stomach carcinomas.

TISSUE EXTRACTION AND PROTEIN CONCENTRATION

Biopsy specimens were homogenised in 1 ml 0.1% (v/v) Tween 80; 0.1 M Tris-HCl (pH 7.5) per 25 mg wet tissue as described before.8 The homogenate was centrifuged twice at 8000 × g for 2.5 minutes, 4°C. Protein concentration of the supernatants was determined by the method of Lowry et al.9

ENZYME LINKED IMMUNOADSORBENT ASSAY (ELISA) FOR UROKINASE TYPE PLASMINOGEN ACTIVATOR

The sandwich ELISA for urokinase type plasminogen activator was carried out according to Binnema et al.10 Rabbit anti-urokinase type plasminogen activator was used as catching antibody and after incubation of the samples, affinity-purified goat anti-urokinase type plasminogen activator IgG (0.8 μg/ml) was added and incubated overnight. After washing, 100 μl optimal dilution of rabbit anti-IgG conjugated with alkaline phosphatase was added and 100 μl p-nitrophenyl-phosphate (1 mg/ml) was used as substrate. The amount of urokinase type plasminogen activator antigen in the samples was calculated from an eight point standard curve of urokinase type plasminogen activator (0–5 ng/ml).

ELISA FOR TISSUE TYPE PLASMINOGEN ACTIVATOR

Tissue type plasminogen activator antigen was measured essentially as described by Rijken et al.10 Rabbit anti-tissue type plasminogen activator was used as catching antibody, an anti-tissue-type plasminogen activator horse-derish peroxidase conjugate (Biopool, Sweden) as second antibody and 3,3′,5,5′ tetramethyl-benzidine was used as substrate. Absolute quantities of tissue type plasminogen activator antigen in the samples were calculated from an eight point standard curve of tissue type plasminogen activator (Biopool, Sweden, 0–4 ng/ml).

PLASMINOGEN ACTIVATORS ACTIVITY ASSAY

Urokinase type plasminogen activator and tissue type plasminogen activator activities were measured by a spectrophotometric enzyme assay as described previously.11 In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen and the chromogenic plasmin substrate S-2251 (Kabi, Stockholm) to detect total plasminogen activator activity. Tissue type

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Plasminogen activators in endoscopical biopsies from squamous oesophageal and stomach carcinomas compared with normal tissue biopsies from patients without [controls] or with [normal mucosa] carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA (n)</td>
<td>Antigen ng/mg protein (n)</td>
</tr>
<tr>
<td>Squamous oesophagus</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>[5]</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>[12]</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>[16]</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>[12]</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>[32]</td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
</tr>
</tbody>
</table>

Results shown are mean values (SEM).

Significance of difference from control tissue: *p<0.05; †p<0.01; ‡p<0.005 normal mucosa: *p<0.005; ††p<0.0005 (comparable) oesophageal tissue: *p<0.05; **p<0.001; ††p<0.0005 stomach tissue: ‡‡p<0.05; ‡‡‡p<0.01.

t-PA = tissue type plasminogen activator. u-PA = urokinase type plasminogen activator.

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plasminogen activator and urokinase type plasminogen activator activities were determined by adding specific inhibiting antibodies against tissue type plasminogen activator and urokinase type plasminogen activator, rabbit anti-human tissue type plasminogen activator IgG and goat antihuman urokinase type plasminogen activator IgG respectively, to parallel incubations and calculating the amount of inhibition. Urokinase type plasminogen activator and tissue type plasminogen activator standard preparations (National Institute of Biological Standards and Control, London, UK, batch nos. 66/46 and 83/517 respectively) were included. The inhibiting antibodies used were monospecific, showed no cross reactivity, and blocked maximum standard urokinase type plasminogen activator and tissue type plasminogen activator activity completely.

CALCULATIONS AND STATISTICAL ANALYSIS
Activator activities were expressed as mIU urokinase type plasminogen activator or tissue type plasminogen activator per mg protein. Antigen concentrations were expressed as ng antigen per mg protein. Results are given as mean (SEM). Difference between group means were statistically tested for significance using paired and unpaired Student's t test with separate variance estimate if the standard deviations were significantly different according to the F-test. Differences were considered significant when p<0.05.

Results
The antigen and activity levels of both plasminogen activators in normal tissue showed a steady increase going from squamous oesophagus to the stomach and duodenum. Urokinase type plasminogen activator and tissue type plasminogen activator in squamous oesophagus were significantly lower than in the stomach. Duodenal tissue contained more tissue type plasminogen activator than stomach tissue, but the concentration of urokinase type plasminogen activator in duodenal mucosa was found to be similar to that of the stomach (Table I). In general, the normal mucosa of the patients with a carcinoma showed a similar pattern although normal mucosa of squamous carcinomas contained higher concentrations of plasminogen activators, in particular based on activity, than corresponding normal mucosa of control patients. For urokinase type plasminogen activator antigen in the stomach the opposite was observed.

Urokinase type plasminogen activator antigen in carcinomas showed a six to seven fold increase in the stomach and a more than 13 fold higher concentration in oesophageal tissue compared with normal mucosa. Although in both carcinomas and normal mucosa urokinase type plasminogen activator antigen and activity showed a significant correlation (respectively, R=0.38, p<0.01 and R=0.41, p<0.005) and the activity of urokinase type plasminogen activator in carcinomas was higher than in normal mucosa, this difference did not reach statistical significance (Table I). Also with respect to tissue type plasminogen activator, antigen and activity were in general significantly correlated in carcinomas (R=0.58, p<0.0001) and normal mucosa (R=0.32, p<0.05). Moreover, tissue type plasminogen activator antigen as well as tissue type plasminogen activator activity in oesophageal carcinoma tissue were not different from normal tissue concentrations. Tissue type plasminogen activator in stomach carcinomas, however, showed a significant reduction in activity compared with normal stomach tissue, which was not seen in the tissue type plasminogen activator antigen concentration. The urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio showed no differences between normal and control mucosa independent of the origin of the tissue. Carcinomas of the oesophagus and stomach had respectively 14 and 10 times higher ratios than normal tissues but were not different from each other (Fig 1). Urokinase type plasminogen activator antigen and the urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio of carcinoma tissue and the corresponding normal mucosa of the same patient are shown in Figure 2. Although all carcinomas contained more urokinase type plasminogen activator antigen than the matching normal tissues, the individual carcinoma samples showed great heterogeneity which was not associated with the localization. In 38 of the 39 tissue pairs tested the urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio of the carcinomas was higher than the corresponding normal mucosa. Again considerable heterogeneity within the carcinomas was observed.

The heterogeneity in urokinase type plasminogen activator antigen and urokinase type plasminogen activator antigen ratio of the carcinomas, however, was not found to be related to differentiation, inflammation, metastasis formation, and the presence of intestinal metaplasia (Table II), although some minor differences between sub-

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**Figure 1:** Urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio in endoscopic biopsies from squamous oesophageal and stomach carcinomas compared with normal tissue from patients without (controls) or with a carcinoma. Significance of difference from normal tissue: *p<0.01; # p<0.0001.

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**Figure 2:** Urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio in endoscopic biopsies from squamous oesophageal and stomach carcinomas compared with normal tissue from patients without (controls) or with a carcinoma. Significance of difference from normal tissue: *p<0.01; # p<0.0001.
same time a comparison was made of tissue type plasminogen activator and urokinase type plasminogen activator concentrations between normal mucosa and carcinoma tissue in endoscopical biopsies from patients with a carcinoma of the oesophagus or the stomach.

Oesophageal tissue was found to contain significantly less urokinase type plasminogen activator and tissue type plasminogen activator, both in antigen and activity, compared with stomach tissue. This remarkable difference in expression of plasminogen activators between both normal tissues is most likely caused by differences in the type of epithelium as squamous oesophageal tissue biopsies contain relatively more epithelium compared with columnar epithelial tissue types of the stomach because of its multilayer cell construction. The concentrations of urokinase type plasminogen activator in the duodenum resemble the quantities found in normal stomach tissue biopsy specimens. The increase of tissue type plasminogen activator antigen and activity in normal duodenal mucosa might be caused by a different vascularisation of the mucosa of this tissue, because tissue type plasminogen activator is expressed mainly in endothelial cells of vessels. In the normal mucosa of patients with a carcinoma a similar increase in urokinase type plasminogen activator and tissue type plasminogen activator from oesophagus to stomach was observed as in controls.

Surprisingly, plasminogen activator antigen and activity levels of oesophageal and stomach carcinomas did not show significant differences between the two epithelial tissue types. With regard to the corresponding normal tissue, urokinase type plasminogen activator antigen concentrations in both carcinoma types were significantly higher and urokinase type plasminogen activator activities were also increased but not significantly. The tissue type plasminogen activator antigen concentrations in both oesophageal carcinomas and stomach carcinomas were similar to those of the normal tissues. In an immunohistochemical study of squamous cervical epithelia with dysplasia and (pre)invasive squamous cell carcinoma an increase in tissue type plasminogen activator concentration has been reported throughout the whole thickness of the epithelium. In the present study tissue type plasminogen activator activity in columnar carcinomas of the stomach, however, was significantly decreased. This decline was comparable with what we previously found in colon carcinomas. The difference in alternation between tissue type plasminogen activator antigen and activity, and urokinase type plasminogen activator antigen and activity in columnar epithelial carcinomas has been seen before in colonic neoplasia and is a consequence of the complex regulation mechanism of the activity of both proteases. In colon carcinomas urokinase type plasminogen activator is predominantly found in the inactive proenzyme form (sc-u-PA), which can be activated in the presence of plasmin. An important role in the control of (pro)-urokinase type plasminogen activator catalyzed proteolysis is played by the urokinase type plasminogen activator receptor.

Discussion

In this study a survey was made of the activity and antigen concentrations of plasminogen activators in normal mucosa biopsies obtained from three different locations of the upper gastrointestinal tract in control patients. At the
TABLE II Urokinase type plasminogen activator activity and antigen, and urokinase type plasminogen activator/tissue type plasminogen activator antigen ratio in endoscopic biopsies from oesophagus and stomach carcinomas

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>u-PA activity mU/mg protein</th>
<th>u-PA antigen ng/mg protein</th>
<th>u-PA/u-PA antigen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well</td>
<td>4</td>
<td>65 (3-3)</td>
<td>5.3 (2-2)</td>
</tr>
<tr>
<td>Moderate</td>
<td>20</td>
<td>59 (19)</td>
<td>19 (3.7)</td>
</tr>
<tr>
<td>Poor</td>
<td>23</td>
<td>91 (18)</td>
<td>11 (1.3)*</td>
</tr>
<tr>
<td>Inflammation</td>
<td>15</td>
<td>73 (23)</td>
<td>11 (1.2)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>12</td>
<td>59 (19)</td>
<td>12 (1.2)</td>
</tr>
<tr>
<td>Ulceration</td>
<td>20</td>
<td>81 (21)</td>
<td>17 (1.3)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>48</td>
<td>40 (21)</td>
<td>40 (1.3)</td>
</tr>
<tr>
<td>Absent</td>
<td>13</td>
<td>83 (44)</td>
<td>13 (2.3)</td>
</tr>
<tr>
<td>Present</td>
<td>16</td>
<td>65 (21)</td>
<td>12 (1.2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>17</td>
<td>71 (19)</td>
<td>16 (4.3)</td>
</tr>
</tbody>
</table>

Intestinal metaplasia
- Present: 32 (only stomach)
- Absent: 34
- Moderate: 30
- Poor: 23
- Inflammation: 31

Intestinal metaplasia and inflammation in gastric and esophageal cancers.

Results shown are mean values (SEM).

Significance of difference: *p<0.05

For abbreviations see Table I.

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8 Quax PHA, van Leeuwen RTJ, Verspaget WH, Verheijen JH. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res 1990; 50: 1488-94.
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