Plasminogen Activator Profiles in Neoplastic Tissues of the Human Colon

Paul A. F. de Bruin, Gerrit Griffioen, Hein W. Verspaget, Jan H. Verheijen, Gerard Dooijewaard, Harry F. van den Ingh, and Cornelis B. H. W. Lamers


ABSTRACT

Plasminogen activator (PA) activity, in particular urokinase-type u-PA), has been shown to be markedly increased in adenocarcinomas of the colon. Adenomatous polyps were found to be intermediate in their PA activity to normal mucosa and adenocarcinomas. In the present study we evaluated the PA profile in relation to malignancy parameters of the adenomas.

Forty-eight adenomatous polyps, obtained by endoscopic polypectomy, were scored according to size, histological type, and grade of dysplasia. In extracts, tissue-type PA (t-PA) and u-PA were determined using a spectrophotometric enzyme assay, antigen assays, and a bioimmunoassay for u-PA. Twenty-five paired samples of normal mucosa and adenocarcinoma were used as controls. Additionally, four hyperplastic polyps were studied by the same methods. The presence of complexes of PA with PA inhibitors was assessed by zymography.

A 10-fold increase of u-PA antigen in carcinomas was found as compared to normal tissue. An increase was also noted in u-PA activity, although its extent was less, due to the fact that 74% of u-PA was in the inactive proenzyme form. Adenomatous polyps contained PA activities and antigens intermediate to those of normal mucosa and carcinomas, in accordance with the view that they are precursors in the development of colorectal cancer. Within the adenoma group, no relation was found between PA profile changes and histological type or polyp size. Surprisingly, in a group of four hyperplastic polyps, similar profiles of PA were found as in adenomas. When the u-PA/t-PA antigen ratio was taken as a parameter of developing malignancy, two discrete increases were seen during the adenoma-carcinoma sequence, the first at adenoma formation and the second accompanying the start of invasive growth in polyps with severe dysplasia.

Zymography showed that only t-PA was present in complex with specific PA inhibitors, explaining how the decrease of t-PA activity in adenomas and carcinomas could be stronger than the parallel decrease of t-PA antigen, when these were compared with normal mucosa, which contained hardly any complexes.

INTRODUCTION

PAs are proteolytic enzymes present in fluids and various tissues of the body. Their ability to activate plasminogen makes them important initiators of extracellular proteolysis. For its enzymatic activity, t-PA (M6, 70 x 10^3) is largely dependent on binding to fibrin, while the urinary type (u-PA, M6, 50 x 10^3) is not.

An increased activity (3-10) and expression (11) of u-PA have been found in tissues and cell lines of malignant origin. A role for this enzyme was speculated to be the initiation of pericellular proteolysis, thereby enabling invasive growth and metastatic potency of the malignant cells (12, 13). u-PA is synthesized as an inactive one-chain proenzyme, which can be activated by plasmin after transport to the extracellular fluid

(14). Recently, receptors for u-PA have been found on cultured (15) and malignant cells (16, 17), suggesting a possible "armament" of these cells for the destruction of surrounding tissue. u-PA was found to bind to these receptors by means of its growth factor-like domain (18). Moreover, u-PA has recently been shown to induce human epidermal tumor cell proliferation (19). As a second role in malignancy, u-PA could thus be a factor in an autocrine growth stimulation mechanism.

In a previously published study (9), we showed that the activity of u-PA in colorectal carcinomas was increased and t-PA activity was decreased when a comparison was made with normal colonic mucosa. In a second study (20), adenomatous polyps were shown to contain PA activities intermediary to and significantly different from normal mucosa and adenocarcinomas, which reflects their role as precursor lesions to colorectal adenocarcinomas (21). Both enzyme activities were measured using a sensitive spectrophotometric plasminogen activator assay (22).

The purpose of the present study was to reveal possible relationships between PA and polyp parameters such as diameter, histological type, and grade of dysplasia in the human colon. Besides the enzymatic assay, a zymogram technique, ELISAs for u-PA and t-PA, and a BIA were used, in order to obtain information about the presence of t-PA, u-PA, pro-u-PA, and PA inhibitors (17, 23) in the various tissues.

PATIENTS, MATERIALS, AND METHODS

Patients and Tissue Samples. Twenty-five patients (ages 52-84 years; mean, 69; 16 male and 9 female) were operated upon colorectal carcinoma at the University Hospital, Leiden. From each resection specimen a fresh sample of normal colon mucosa, taken at least 10 cm from the tumor, and a sample of tumor were obtained. After removing fat and muscle layer, the samples were frozen at —70°C until extraction.

In another group of 45 patients (ages 32-88 years; mean, 63; 26 male and 19 female) endoscopic polypectomy was performed, providing tissue samples of 4 hyperplastic and 48 adenomatous polyps, which were likewise stored at —70°C. Of all tissue samples, adjacent fragments were examined by the pathologist, confirming the nature of the tissues in all cases. Of the polyps, the maximal diameters were recorded and were subdivided into classes differing 5 mm.

Histology of Adenomatous Polyps. Paraffin sections of the adenomatous polyps were examined to determine the histological type [tubulopapillary, tubulovillous, or villous, according to the WHO classification] and the grade of dysplasia [mild, moderate, or severe (21)]. A fourth class was formed by severely dysplastic polyps containing one or more areas of invasive growth ("malignant polyp"). All adenomatous polyps were examined by the same pathologist (H. F. v. d. I.), to exclude inter-observer variations.

Tissue Extraction. Extracts were prepared from 50-100 mg wet tissue samples as described before (9). Essentially, the samples were homogenized at 0°C in 1 ml 0.1% (v/v) Tween 80-0.1 M Tris-HCl, pH 7.5, per 60 mg wet tissue. The supernatant was centrifuged twice at 8000 x g for 2.5 min.

Protein Concentrations. Protein concentrations in the extracts were determined according to the method of Lowry et al. (24).

ELISA for u-PA. u-PA antigen was determined by a sandwich ELISA using rabbit-anti-u-PA as first antibody and affinity-purified goat anti-u-PA as second antibody. After overnight absorption of the 1:10 diluted sample followed by the reaction with the second antibody, u-PA antigen
was detected using a rabbit anti-goat immunoglobulin-alkaline phosphatase conjugate and p-nitrophenylphosphate as substrate. A calibration standard of 0-5 ng u-PA/ml was included in the assay. This ELISA was described in detail by Binnema et al. (25).

ELISA for t-PA. t-PA antigen was measured essentially as described by Rijken et al. (26). Rabbit anti-t-PA was used as catching antibody, an anti-t-PA-horseradish peroxidase conjugate (Biopool, Sweden) was used as second antibody, and 3′,5′-tetrathylbenzidine was used as substrate. Standard t-PA (Biopool; 0-4 ng/ml) was included for calibration.

PA Activity Assay. u-PA and t-PA activities were measured by a spectrophotometric enzyme assay (22) as described previously (9). In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen, and the chromogenic plasmin substrate S-2251 (Kabi, Stockholm) to detect total PA activity. t-PA and u-PA activities were determined by adding specific inhibiting antibodies against t-PA and u-PA to parallel incubations and calculating the amount of inhibition. u-PA and t-PA standard preparations (National Institute of Biological Standards and Control, London, UK; Batch Nos. 66/64 and 83/517, respectively) were included in the assay.

Zymography. Tissue extracts were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and PA activities were visualized on fibrin/plasminogen containing agarose underlay gels according to the method of Granelli-Piperno and Reich (27), as described before (9). Prior to electrophoresis, samples were incubated for 1 h at 37°C in 2% sodium dodecyl sulfate to induce activator activity in the PA-PAI complexes (28). In a few unselected cases, activity-inhibiting antibodies to t-PA (29) or u-PA were included in the underlay gel to discriminate between PA activities of different origin.

BIA for u-PA and pro-u-PA. This BIA was performed as published by Dooijewaard et al. (30). In brief, rabbit anti-u-PA coated on microtitre plates was used as catching antibody for u-PA and pro-u-PA. After activation of pro-u-PA by a short plasmin incubation and thorough washing, plasminogen and the plasmin substrate S-2251 were added to measure immobilized u-PA activity, giving the sum of u-PA and pro-u-PA. Without prior activation by plasmin, only active u-PA was detected. Subtraction of these two measurements yielded the amount of pro-u-PA in the sample. A standard of 0-5 ng u-PA/ml was included for calibration.

Calculations and Statistics. Enzyme activities in the tissue samples were expressed as mIU u-PA or t-PA per mg protein. Antigen concentrations were expressed as ng antigen per mg protein. Differences between group means were statistically tested using Student's t test, with separate variance estimate if the standard deviations were significantly different according to an F test. To correlate PA parameters with polyp diameter, analysis of variance was used. Differences were considered as significant below P = 0.05.

RESULTS

The group of 48 colonic adenomas investigated was composed of 18 tubulose, 20 tubulovillous, and 10 villous adenomas. Eight of them showed mild, 20 moderate, and 14 severe dysplasia. The 6 remaining adenomatous polyps were not only severely dysplastic, but they contained also an area of invasive growth through the basal membrane. The distribution by diameter was as follows: smaller than 5 mm, 1; 5-10 mm, 8; 10-15 mm, 12; 15-20 mm, 11; and ≥20 mm, 15.

The results of the ELISAs are represented in Fig. 1. t-PA and u-PA antigens are shown for samples of normal colonic mucosa, hyperplastic and adenomatous polyps, and adenocarcinomas. The adenomas are subdivided into four groups of increasing dysplasia.

u-PA antigen was 10-fold increased when the carcinoma samples were compared with the corresponding normal mucosal group. In the polyp tissue groups, u-PA antigen showed intermediary values, which were significantly higher than in the normal tissue. In all groups, except for the adenomatous polyps with invasive growth, u-PA antigen was significantly lower than in the adenocarcinomas. In contrast with u-PA, t-PA antigen showed a tendency to decrease with developing malignancy. In all adenomatous polyps and in the carcinomas, t-PA is one-half of that in the normal tissue. Evidently, all studied abnormal tissues were characterized not only by an increased u-PA antigen content but also by a decreased t-PA antigen. Studying these tissues, the u-PA/t-PA ratio could thus be an interesting parameter. In Fig. 2, this ratio is shown for the four tissue groups, with the adenomas subdivided into the dysplasia groups. As expected, the antigen ratio is strongly increased in carcinomas when compared with normal tissue, while a lesser but significant increase is seen in neoplastic polyps. There is no difference between adenomas with mild, moderate, or severe dysplasia, nor do they differ from the four hyperplastic polyps. However, adenomas with invasion have a significantly (P = 0.005) raised antigen ratio compared with the other polyps, to a level not statistically different from that in the malignant tumor group.

The spectrophotometric enzyme assay results for u-PA and t-PA are shown in Table 1. The activity of u-PA in the adenocarcinomas is 3 times as high as in the corresponding normal tissue samples, while t-PA activity is 4 times lower. The activity of u-PA in the adenomatous polyps falls between those in normal tissue and in carcinoma. No differences in u-PA activity are found between the four dysplasia classes of adenomas, but there is a tendency to a decrease in t-PA activity going from mild dysplasia to adenomas with invasion. In the four hyperplastic polyps no difference of u-PA activity was found compared with normal tissue.
PLASMINOGEN ACTIVATORS IN THE HUMAN COLON

Table 2 Antigen of u-PA and t-PA in adenomatous polyps according to diameter and histological type

<table>
<thead>
<tr>
<th>Polyp diameter</th>
<th>n</th>
<th>u-PA</th>
<th>t-PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 mm</td>
<td>1</td>
<td>3.4 ± 0.0*</td>
<td>3.6 ± 0.0*</td>
</tr>
<tr>
<td>5-10 mm</td>
<td>8</td>
<td>5.4 ± 1.4</td>
<td>5.3 ± 0.9*</td>
</tr>
<tr>
<td>10-15 mm</td>
<td>12</td>
<td>3.4 ± 0.6</td>
<td>4.0 ± 0.2*</td>
</tr>
<tr>
<td>15-20 mm</td>
<td>11</td>
<td>5.2 ± 0.8</td>
<td>3.4 ± 0.2*</td>
</tr>
<tr>
<td>Larger than 20 mm</td>
<td>15</td>
<td>4.4 ± 0.8</td>
<td>3.1 ± 0.3*</td>
</tr>
</tbody>
</table>

Significance of difference from normal mucosa: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Table 3 Zymographic analysis of tissue samples from colonic normal mucosa, carcinomas, and adenomatous polyps

<table>
<thead>
<tr>
<th>Lysis</th>
<th>Normal mucosa</th>
<th>Adenomatous polyp</th>
<th>Adenocarcinoma</th>
<th>t-PA (M, = 70 x 10^3)</th>
<th>Normal mucosa</th>
<th>Adenomatous polyp</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not detectable</td>
<td>80*</td>
<td>31</td>
<td>60</td>
<td></td>
<td>0</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Hardly visible</td>
<td>20</td>
<td>34</td>
<td>24</td>
<td></td>
<td>0</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Readily visible</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td></td>
<td>72</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Strong</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Significance of difference from normal mucosa: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Table 1 Enzyme activities of plasminogen activators in samples of colonic tissue

<table>
<thead>
<tr>
<th>Type of Polyp</th>
<th>n</th>
<th>mU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>25</td>
<td>39 ± 6*</td>
</tr>
<tr>
<td>Hyperplastic polyp</td>
<td>4</td>
<td>24 ± 1.1*</td>
</tr>
<tr>
<td>Adenomatous polyp</td>
<td>48</td>
<td>59 ± 5.9*</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>8</td>
<td>64 ± 10.9*</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>20</td>
<td>55 ± 9*</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>14</td>
<td>61 ± 8*</td>
</tr>
<tr>
<td>Polyp with invasive carcinoma</td>
<td>6</td>
<td>59 ± 23</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>25</td>
<td>126 ± 21*</td>
</tr>
</tbody>
</table>

* n, number of samples.
* Mean ± SE.
* Significant decrease according to analysis of variance (P < 0.05).
* Significant decrease according to analysis of variance (P < 0.001).

40% of the carcinomas and 70% of the polyps did contain these complexes (Table 3). Activity of u-PA showed a parallel with the occurrence of PA-PAI complexes, in this respect that it was hardly detectable in normal mucosa, but easily in most polyps and carcinomas. As in activity and antigen, t-PA bands on the zymograms were strong in normal mucosa but decreased and were sometimes absent in polyps and carcinomas. Prior incorporation of inhibiting antibodies to u-PA in the indicator gel resulted in a disappearance of u-PA lysis areas, while t-PA and PA-PAI complexes remained unaffected. On the contrary, when antibodies to t-PA were added, not only t-PA lysis areas but also those of the PA-PAI complexes disappeared, suggesting that the type of plasminogen activator present in these complexes is mostly, if not exclusively t-PA. This could at least partially explain the observed decrease when compared with normal mucosa. Within the adenoma group, no differentiation could be made between various polyp parameters and the zymography results alone.

When the u-PA results of the antigen assay were compared with those of the enzyme assay, a remarkable discrepancy was seen between the levels of this enzyme in normal tissue and in carcinoma. The antigen level of u-PA is 10 times as high in carcinomas as in normal mucosal tissue, while the activity level is only three times increased. In order to investigate whether the presence of u-PA proenzyme could explain this difference, a BIA was performed on all tissue samples. The BIA enables independent measurement of u-PA proenzyme and active enzyme. The results of this assay (Table 4) confirm the supposition that the occurrence of inactive u-PA antigen could largely be ascribed to pro-u-PA. For the adenomatous polyps, the percentage proenzyme is 75; in the carcinoma group, it is 73.

pared with normal tissue. On the contrary, t-PA activity was significantly decreased, to a level similar to that in adenomatous polyps.

Subdivision of the 48 adenomatous polyps (Table 2) into groups of increasing diameter or groups of different histology (tubulovillous, tubulovillous, or villous) showed few correlations of these parameters on the one side and PA activities or antigens on the other side. Only a small but significant (P = 0.01) decrease in t-PA antigen content per mg of protein was seen going from small to large adenomas.

The PA activity changes in our study were less pronounced than the PA antigen changes. In order to investigate whether this discrepancy could be attributed to the action of specific PA inhibitors, zymographic analysis was performed on all tissue samples. By zymography, PA activities originating from u-PA, t-PA, and activated PA-PAI complexes are separated by their different molecular weight and made visible. As judged by the lysis areas, very little, if any, complexes of PA with PAI were present in the normal colonic mucosa samples. On the contrary,
normal tissue, the low total u-PA level did not permit an accurate calculation of the percentage proenzyme.

There are no significant correlations of this percentage within the polyp group with diameter, histological type, or grade of dysplasia, nor are there any significant differences among the different tissue groups, hyperplastic polyps, adenomas, and carcinomas.

**DISCUSSION**

In this study we identified plasminogen activator profiles in normal colonic epithelium, in colon malignancies, and in tissues which are precursors for these malignancies, using four different assay techniques.

t-PA activity levels in all polyps and in the carcinomas were found to be reduced to 25–40% of the level in the normal mucosa, while the levels of antigen showed a lesser reduction to 40–70%. When this is compared with the zymographic data, which indicate an increased PAI content in adenomas and carcinomas, binding of t-PA to specific inhibitors (23) may account for this discrepancy. The amount of PAI, however, is lower than that of the activators because free u-PA and t-PA can still be detected whereas free PAI was not found in reverse zymography as reported previously (20). We conclude that there is a substantial reduction of t-PA in polyps and malignant tumors of the colon and that approximately 50% of the remaining enzyme is inactivated by binding to inhibitors. Reduced activity of t-PA in adenomas and carcinomas has been reported previously by our group (20) as well as by others (10). To our knowledge it is a novel observation that this reduction is paralleled by a less pronounced antigen decrease.

The situation for u-PA is more complex because of the possible role of u-PA proenzyme (17, 31, 32). In normal tissue, the BIA detected only minor quantities of u-PA plus pro-u-PA; levels of active u-PA were close to the detection limit of the assay. According to the u-PA antigen assay, there is a strong (more than 10-fold) increase of u-PA in carcinomas compared with normal tissue. We also found an increase in u-PA antigen, although less pronounced, in adenomatous polyps. The increase in u-PA antigen could be predominantly attributed to the proenzyme form. Gelister et al. (10) recently examined u-PA and t-PA enzyme activities and antigens in normal colonic mucosa, colon carcinomas, and eight adenomatous polyps. Although using a BIA for both activators, proenzyme of u-PA in their study probably remained undiscovered because no plasmin activation step was applied. Secondly, the limited number of studied polyps did not permit an analysis of different PA profiles among adenoma subgroups. Apart from these limitations, the results from their and our studies show a good agreement.

The increase of u-PA antigen we found through the sequence normal mucosa-adenoma-carcinoma is much stronger than the parallel increase of u-PA enzyme activity in this sequence. This indicates that the spectrophotometric enzyme assay mainly detects u-PA already activated. However, it cannot be excluded that there is some activation of pro-u-PA by plasmin generated during the assay incubation.

The zymograms with anti-t-PA show complete quenching of activated PA-PAI complexes, indicating that these complexes consist mainly of t-PA and PAI. Pro-u-PA does not react with PAI (33), while activated u-PAs may be bound to receptors on the plasma membrane of malignant cells and are possibly protected against inactivation, as speculated by Blasi et al. (17).

Subdivision of the 48 adenomatous polyps into groups of different histology (tubulous, tubulovillous, or villous) showed no correlation of this parameter on the one hand, and PA activities or antigens on the other. However, when a subdivision was made according to diameter, a gradual decrease of t-PA antigen was found with increasing polyp size. A possible explanation might be a lesser vascularization in large adenomas, since t-PA is mainly a product of endothelial cells.

When an analysis was made of the relation between PA profiles and polyp dysplasia (mild, moderate, severe, or with invasive carcinoma), there was a remarkable rise in u-PA/t-PA antigen ratio in polyps with invasive growth in comparison with polyps without invasion. Thus, the appearance of malignancy in a polyp is connected with a change in PA content. u-PA increases, completely accordant with the deemed role of this enzyme in the degradation of the basal membrane, i.e., invasive growth (34). From these results, alteration in PA content during the sequence of colon carcinogenesis (normal mucosa-adenoma-carcinoma) does not seem to be a continuous process, but rather a process in which two discrete changes can be distinguished: (a) a decrease of t-PA and an increase of u-PA when an adenoma develops; (b) a second increase of u-PA at the appearance of invasive growth.

Hyperplastic polyps are usually small and are relatively seldom removed during endoscopy. In this study, only four hyperplastic polyps were included and the results should therefore be interpreted with some restraint. Surprisingly, their PA profiles are not equivalent with those of normal mucosa but rather with those of adenomatous polyps. u-PA antigen in this group is significantly higher than in the normal control group, and the resulting antigen ratio is similar to that for adenomas. In hyperplastic polyps, increased expression of gastric associated mucus antigen M4 and carcinoembryonic antigen (35) as well as the rare occurrence of adenomas within these polyps have been reported (36). In the present study also PA profiles in these polyps show more resemblance to those of adenomas than those of normal colonic mucosa. These facts may be arguments to reconsider the general view that hyperplastic polyps are innocent lesions without malignant potential.

In conclusion, adenomatous polyps and adenocarcinomas of the colon contain increased levels of u-PA antigen and activity. About 75% is present as an inactive proenzyme. Lesions with invasive growth (malignant polyps and carcinomas) contain significantly more u-PA antigen and have a significantly higher u-PA/t-PA antigen ratio than benign lesions. Antigen and activity of t-PA are lower in polyps and carcinomas than in the

| Table 4 | Activities of u-PA according to BIA in samples of colonic tissue |
|---------|-------------------|-------------------|-------------------|
|         | ng/mg protein     |
|         | u-PA active       |
|         | u-PA proenzyme    |
|         | u-PA total        |
| Normal mucosa | 25 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| Hyperplastic polyp | 0.6 ± 0.3 | 1.1 ± 0.6 | 1.7 ± 0.4 |
| Adenomatous polyp | 0.9 ± 0.2 | 2.3 ± 0.2 | 3.2 ± 0.3 |
| Mild dysplasia | 1.0 ± 0.3 | 2.7 ± 0.9 | 3.7 ± 1.2 |
| Moderate dysplasia | 1.0 ± 0.3 | 2.1 ± 0.4 | 3.0 ± 0.5 |
| Severe dysplasia | 1.6 ± 0.3 | 2.7 ± 0.4 | 3.4 ± 0.6 |
| Polyp with invasive carcinoma | 0.6 ± 0.6 | 2.0 ± 0.4 | 2.9 ± 0.8 |
| Adenocarcinoma | 25 | 2.0 ± 0.6 | 4.8 ± 0.6 | 6.8 ± 0.9 |

* n, number of samples.  
* ND, not detectable.  
* Mean ± SE.  
* Significance of difference from carcinoma: P < 0.05.  
* Significance of difference from normal mucosa: P < 0.001.  
* Significance of difference from normal mucosa: P < 0.01.  
* Significance of difference from carcinoma: P < 0.01.  
° n, number of samples.  
* ND, not detectable.  
° n, number of samples.  
* ND, not detectable.  
° n, number of samples.  
* ND, not detectable.
normal mucosa, and about 50% of this quantity is bound in PA-PAI complexes.

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