Plasminogen Activators and Tumor Development in the Human Colon: Activity Levels in Normal Mucosa, Adenomatous Polyps, and Adenocarcinomas

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ABSTRACT

Malignant changes are often accompanied by alterations in activity and composition of the plasminogen activators (PA). To study the relationship between PA expression and the development of colorectal cancer, we determined urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) activity in normal mucosa (n = 80), adenomatous polyps (n = 76), and adenocarcinomas (n = 71) of the colon. Tissues obtained from surgical resection or polypectomy were analyzed for t-PA and u-PA activity in a specific enzymatic assay using plasminogen, a chromogenic substrate, and selective quenching with monospecific antibodies to both activators.

The plasminogen activator activities were found to be changed in adenocarcinomas as compared to normal mucosa. The relative contribution of u-PA (expressed as percentage of u-PA) was raised from 6 to 50% for, respectively, normal mucosa and adenocarcinoma. This change could be attributed to a 3-fold decrease in t-PA activity and a 5-fold increase in u-PA activity in the carcinomas.

Adenomatous polyps as a group showed percentages of u-PA [20.2 ± 1.3 (SE)] which were intermediate as well as significantly different (P < 0.001) from those of normal mucosa and adenocarcinomas. This observation was strengthened by a gradual rise in the relative contribution of u-PA in four resection specimens containing both adenomatous polyps and adenocarcinomas.

Zymography showed the presence of minor quantities of PA-PA inhibitor complexes in the tissue extracts studied.

The present study shows that the sequence of normal mucosa-adenomatous poly-adenocarcinoma in the colon is associated with a parallel change in plasminogen activator activity. Thus, change in the regulation of plasminogen activator activity is an early event in the development of colorectal cancer.

INTRODUCTION

Plasminogen activators are serine proteases which are found in blood plasma as well as in other body fluids and in organs (1). Their role is not restricted to the fibrinolytic cascade but they are also involved in processes such as tissue degradation and repair, inflammation, and malignant transformation (2-4). Two types of PA have been identified on the basis of their molecular weight and their serological and enzymatic properties. One of these, u-PA (M, 55,000), had first been found in urine, while the other, t-PA (M, 70,000), was extracted from tissues and probably originated from endothelial cells. The activity of t-PA is enhanced by fibrin whereas that of u-PA is not (5).

Malignant transformation of cells, in vivo as well as in vitro, is often found to be associated with changes in PA production and activity (2). In this respect, treatment of cells with tumor promoters (6) or tumor viruses (7) enhances the secretion of PA from cells in vitro. Furthermore, in a large number of solid tumors of lung (8), prostate (9, 10), breast (11, 12), stomach (13), and colon (12, 14-16), an increased expression or secretion of u-PA is seen, compared to their normal tissue counterparts. In a previous study (16) we documented that measurement of u-PA and t-PA activity levels in colorectal tumors and normal colonic mucosa enabled a discrimination between normal and malignant tissue which completely parallels the pathological diagnosis. In the colon, adenomatous polyps are the deemed precursor lesions of colorectal carcinoma (17, 18) and therefore represent an intermediate stage between normal mucosa and carcinoma. In the present study, we analyzed the activities of u-PA and t-PA in normal mucosa, adenomatous polyps, and carcinomas in order to elucidate the relation of tumor development and PA activity levels.

MATERIALS AND METHODS

Tissue Specimens. One hundred five patients (62 males and 43 females; mean age, 63 years, range, 9-88) underwent 76 colorectal resections and 30 endoscopic polypectomies at the University Hospital Leiden. This provided us with 80 fragments of normal colonic mucosa, 76 fragments of adenomatous polyps, and 71 fragments of adenocarcinoma. Of the carcinomas, 18 were at Dukes stage B1, 21 at B2, 1 at C1, 25 at C2, and 5 at stage D, while of one tumor the staging was unknown [staging modified by Astler and Coller with added stage D (19)].

The investigated adenomas and carcinomas originated from different parts of the colon, as shown in Table 1. Fragments of normal colonic tissue were excised from surgically removed colon specimens at least 10 cm from the neoplastic lesion, if present. The mucosa was then separated from muscle layer, serosa, and subserosal fat. All samples were stored frozen at −70°C until extraction. The nature of the lesions was in all cases confirmed by pathological examination of directly adjacent tissue.

Tissue Extracts. Tissue fragments (50-100 mg) were homogenized and extracted as described previously (16). In brief, the weighed samples were transferred to homogenizing tubes and 1 ml of 0.1 M Tris-HCl (pH 7.5) with 0.1% (v/v) Tween 80 per 60 mg of wet tissue was added. Tissue was homogenized for 2 min in melting ice with a motor-driven Teflon pestle, the homogenates were centrifuged twice at 8 x 10^3 x g at 4°C, and the extract supernatants were stored at −70°C. Protein concentrations were determined according to the method of Lowry et al. (20).

Assay of Plasminogen Activator. Enzymatic activities of u-PA and t-PA in the tissue extracts were determined by the sensitive spectrophotometric assay of Verheijen et al. (21). This assay enables simultaneous measurement of u-PA and t-PA by the addition of specific inhibiting antibodies (1), while t-PA is stimulated by CNBr fragments of fibrinogen. The conversion of plasminogen into plasmin is monitored using the chromogenic plasmin substrate S-2251 (Kabi, Stockholm, Sweden). When both antibodies were added, a residual amidolytic activity was still present and could be quantified (16). PA activities were expressed as (10^3 x ΔA_{405})/(Δdh^2 x μg protein). In the case of u-PA this unit conforms to 3.57 mlU of u-PA/μg protein, while for t-PA it conforms to 0.96 mlU of t-PA/μg protein, as determined by the inclusion of standard u-PA and t-PA preparations in the assays (Batches 66/46 and 83/517, respectively, of the National Institute of Biological Standards and Control, London, United Kingdom). The RA activity was expressed as (10^3 x ΔA_{405})/(Δdh^2 x μg protein).
PLASMINOGEN ACTIVATORS IN NEOPLASIA OF THE COLON

Table 1 Origin of the colonic tissue samples studied

<table>
<thead>
<tr>
<th></th>
<th>Normal mucosa</th>
<th>Adenomatous polyp</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum or ascending colon</td>
<td>18</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Transverse or descending colon</td>
<td>8</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Sigmoid or rectum</td>
<td>46</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>Unknown</td>
<td>8</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>76</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 2 Activities of the urokinase-type (u-PA), the tissue-type plasminogen activator (t-PA), and the residual amidolytic activity (RA) in normal mucosa, adenomatous polyps, and adenocarcinomas of the human colon

The relative contribution of u-PA in the total activator activity is expressed as percentage of u-PA.

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<th>Adenomatous polyp</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
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<tr>
<td>u-PA*</td>
<td>0.13 ± 0.01a</td>
<td>0.20 ± 0.02abcd</td>
<td>0.54 ± 0.05a*</td>
</tr>
<tr>
<td>t-PA*</td>
<td>2.21 ± 0.16</td>
<td>0.80 ± 0.05c*</td>
<td>0.64 ± 0.06a*</td>
</tr>
<tr>
<td>RA*</td>
<td>2.95 ± 0.18</td>
<td>1.74 ± 0.13c*</td>
<td>0.89 ± 0.08c*</td>
</tr>
<tr>
<td>% of u-PA</td>
<td>6.4 ± 0.5</td>
<td>20.2 ± 1.3c*</td>
<td>50.0 ± 2.5c*</td>
</tr>
</tbody>
</table>

* (10^3 x A/AA)/(AA x mg protein); conversion factor to nM u-PA/mg protein, 280; conversion factor to nM t-PA/mg protein, 1040.

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Zymographic Analysis. To visualize PA activities, tissue extracts were subjected to electrophoresis on 10% polyacrylamide-SDS slab gels, which were subsequently washed 3 times for 15 min each in 2.5% Triton X-100 and transferred to fibrin and plasminogen containing agarose underlays (22). Prior to electrophoresis, tissue extracts were incubated in 2% SDS for 5 min or 1 h at 37°C to activate PA-PAI complexes (23). The zymograms were incubated at room temperature in fibrin and plasminogen containing agarose underlays (22). Prior to electrophoresis, tissue extracts were incubated in 2% SDS for 5 min or 1 h at 37°C to activate PA-PAI complexes (23). The zymograms were incubated at room temperature and photographed every 24 h. Free plasminogen activator inhibitors (PAI, M, 40,000) were visualized using a reverse zymogram technique and photographed every 24 h. Free plasminogen activator inhibitors (PAI, M, 40,000) were visualized using a reverse zymogram technique (24). For this purpose, the same method is used as described above, but 0.1 IU of t-PA is added to the underlay gel, and incubation is at 37°C. Slow fibrinolysis occurs throughout the gel, except at sites where free PAI is present. Conditioned medium of human endothelial cells containing high quantities of free PAI served as positive control in this analysis.

Calculations and Statistics. The percentual u-PA activity (act) was calculated for each sample according to

\[
\% \text{ u-PA} = \frac{100 \times u-PA \text{ act}}{u-PA \text{ act} + t-PA \text{ act}}
\]

For comparison between the three tissue groups, Student's t test was used, or when standard deviations were significantly different, the separate variance analysis. To avoid the occurrence of spurious significances when comparing three groups, differences were considered significant only below \( P = 0.05/3 \) (25). Student's paired t test was applied to the analysis of PA levels in intraindividual pairs of normal tissue and tumor. By \( \chi^2 \) analysis, the relationship between activity levels on zymograms of free and complexed PA was studied.

RESULTS

The average activity of u-PA in adenocarcinomas was increased 4-fold, when compared to normal colonic mucosa while t-PA activity was more than 3-fold decreased. The RA activity in carcinomas was only one-third of the RA activity in normal mucosa (Table 2). The contribution of u-PA to the total plasminogen activator activity in the adenocarcinomas, expressed as percentage of u-PA activity, was 50%, whereas in normal mucosa this was found to be only 6%. All the differences observed between the adenocarcinomas and normal mucosa were statistically highly significant.

From 61 patients of the studied group, a fragment of adenocarcinoma as well as a fragment of normal mucosa were obtained. The ranges for the percentages of u-PA were 0.0-22.7% for the normal fragments and 8.8-100.0% for the carcinomas. All tissue pairs except one showed a marked increase of percentage of u-PA between the normal and the tumor samples. This increase was highly significant (\( P < 0.001 \)) according to the paired Student t test.

Within the whole investigated carcinoma group, no correlation could be observed between u-PA, t-PA, or RA activity on the one hand and Dukes stage of growth on the other.

In the adenomatous polyps, PA activities as well as the RA activity had mean values which were intermediate to those of normal mucosa and carcinomas (Table 2). As compared to normal mucosa, polyps showed more u-PA activity and less t-PA and RA activity. The activities in the polyps all tended to change in the direction of the activities found in the adenocarcinomas. This intermediate expression of plasminogen activator activity in polyps was also illustrated by the percentage of u-PA which turned out to be 20%, significantly more than in normal mucosa (6%; \( P < 0.001 \)) and significantly less than in adenocarcinomas (50%; \( P < 0.001 \)).

The distribution of all tissue fragments studied according to percentage of u-PA is shown in Fig. 1. Of the 80 normal mucosa fragments, 79 had a percentage of u-PA below 20%. On the contrary, 68 of 71 carcinomas had a percentage of u-PA higher than 20%.
samples, PA-PAI was either not or hardly detectable in the cubation in 2% SDS for l h at 37°C. PA-PAI complexes were readily detectable with concomitant high free PA activity. The remaining 2% contained PA-PAI complexes and low free PA activity. Obviously, most PA-PAI was found in extracts in which free PA activity also was high. \( \chi^2 \) analysis showed that there is indeed a significant \( (P < 0.01) \) relationship between activity levels of free PA and SDSA.

**DISCUSSION**

In this study we report significant differences in PA activities found in colonic adenomatous polyps when they are compared to both normal colon mucosa and adenocarcinomas. The evolution of the normal mucosa to a malignant tumor, with the adenoma as an intermediary stage, is paralleled by a decrease of tissue-type PA (t-PA) activity and an increase of urinary-type PA (u-PA) activity. Adenomatous polyps of the colon and rectum are characterized by properties which place them between normal mucosa and adenocarcinomas. Although of neoplastic origin, they usually do not show invasive growth, while histologically they show a scale of intermediate forms, related to the grade of epithelial cell dysplasia (17, 18, 26). At the genomic level increasing ploidy with increasing size and histological type has been observed (27). Together with the not unusual finding of a carcinoma in situ in polyps and the strong association of polyp occurrence and carcinoma, these are strong indications of the malignant potential of adenomatous polyps, and they are believed to be the main precursor of colorectal carcinoma (18, 28). This view is supported by the results of the present report, showing levels of u-PA and t-PA activities in adenomatous polyps that are intermediate to those in normal mucosa and tumors, thus perfectly reflecting their place in the colonic adenoma-carcinoma sequence. The four cases presented here, in which adenomas were also found in resection specimens with a carcinoma, show that these conclusions are also valid when individual cases are considered. This report shows that change in PA activity levels is an early event in the development of colorectal cancer and thus seems to be associated with early transformation steps.

Hypotheses about the possible role of enhanced PA expression in tumors focus on the specific property to activate degradation of basal membrane and pericellular matrix (3, 4), which would enable malignant cells to invade the surrounding tissue, to be released into the circulation, and to form metastases (2). Indeed, activity inhibiting antibodies to human u-PA have been found to prevent metastasis formation, because dissemination of the human carcinoma HEp3, growing on the chorioallantoic membrane of a chicken embryo, was inhibited by the administration of u-PA antibodies (29). Another hypothesis links PA and the evolution of cells towards malignancy. Secretion of PA may be a factor in the loss of gap junctions between adjacent cells, thereby isolating them and making their growth characteristics independent of neighboring cells (26). Interestingly, one of the functional domains in the molecules of both u-PA and t-PA is a fragment homologous to epidermal growth factor (3). This opens the possibility of an autocrine growth stimulation mechanism.

Plasminogen activator activities in smaller series of colonic adenomas have been investigated by others. Elliott et al. (30) analyzed 11 polyps using the semiquantitative zymogram technique and identified u-PA activity in most of the polyps. However, information on t-PA expression and more quantitative data are not presented. In a recent study on the relationship between plasminogen activators and colorectal neoplasia, Gelister et al. (31) evaluated a series of only 8 polyps. They found not only a u-PA increase but also a t-PA decrease in adenomatous polyps and carcinomas, confirming our findings as presented in a preliminary report (32) and extended in this paper.
As to the occurrence of PAI in tissues, several groups report contradictory results. In contrast to our findings and to those of Tissot et al. (12), complexes of u-PA or t-PA with fast acting inhibitors (PA-PAI) were not visible on the zymograms of Gelister et al. (31), possibly due to different extraction procedures or to insufficient activation by SDS. PA-PAI does not dissociate in SDS, but incubation for 1 h at 37°C reverses, at least partially, the inhibition of plasminogen activation in the complex. In this way, the presence of PA-PAI complexes can be visualized on zymograms at M, 100,000 (23). We observed that minor quantities of PA-PAI were present in some of the studied tissue samples. Extracts in which complexes were clearly present also contained, with few exceptions, a high “free” PA activity. In most cases in which PA activity was low, complexes were hardly detected. Therefore, it is unlikely that the low PA activity was caused by a high inhibitor concentration. Furthermore, because these inhibitors are not specific for one of the two PA types, both PA activities would be partially inhibited and the calculated percentage of u-PA would not be severely affected. For the same reason, the observed diminished t-PA activity in tumors can, in our opinion, hardly be explained by an increased PAI level, for u-PA activity is simultaneously inhibited and the calculated percentage of u-PA would not be.

In conclusion, adenomatous polyps of the colon exhibit both u-PA and t-PA activities intermediate to those of normal colonic mucosa and adenocarcinomas, thus mirroring their deemed role as precursor lesions to carcinomas. The combined expression as percentual u-PA activity may be a useful parameter for the early detection of colorectal cancer.

ACKNOWLEDGMENTS

The authors wish to thank Marij Mieremet-Onns, Annie van der Zon, and Wim van Duijn for their technical assistance and Karlien Krujier for typing the manuscript. We also like to thank the Department of Surgery (head, Professor A. Zwaveling) for providing us the intestinal specimens and the Department of Pathology (head, Professor P. J. Hoedemaeker) for histological evaluation of the tissues.

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