The Plasminogen Activation System in Human Colon Cancer: Messenger RNA for the Inhibitor PAI-1 Is Located in Endothelial Cells in the Tumor Stroma

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ABSTRACT

Fourteen human colon adenocarcinomas were examined by in situ hybridization for the presence of mRNA for plasminogen activator inhibitor type 1 (PAI-1).

All specimens contained PAI-1 mRNA in endothelial cells of some vessels in the stroma immediately surrounding the invasive tumor glands, in granulation tissue, and in some capillaries located under the free luminal surface of carcinomatous epithelium. In addition, a limited number of stromal cells in the cancerous areas located at the periphery of newly formed capillary networks, and presumably representing sprouting endothelial cells, contained PAI-1 mRNA. Cancer cells were devoid of detectable PAI-1 mRNA in all cases. PAI-1 mRNA was not seen in three biopsies of normal colon.

Together with previous findings of urokinase-type plasminogen activator and its mRNA being located in fibroblast-like cells in the tumor stroma and mRNA for the urokinase receptor in the cancer cells at invasive foci, these results indicate a complex cooperativity among several cell types in regulation of plasminogen activation in colon cancer. A possible role of PAI-1 in protecting the extracellular matrix in the tumor tissue against degradation and a role in tumor-induced angiogenesis are discussed.

INTRODUCTION

Degradation of the extracellular matrix is an important feature of cancer cell invasion. It appears to be caused by a concerted action of several enzyme systems, including the urokinase pathway of plasminogen activation (1–4), different types of collagenases and other metalloproteinases (5, 6), and other matrix-degrading enzymes (5, 7). Plasminogen activation results in formation of the serine protease plasmin that can degrade many matrix proteins and also activate the latent forms of some metalloproteinases. It is catalyzed by two different plasminogen activators, u-PA and t-PA, the latter being a key enzyme in thrombolysis.

The u-PA pathway of plasminogen activation is a complex cascade reaction that is regulated at different levels. u-PA is synthesized as a virtually inactive proenzyme (pro-u-PA) and requires activation before it, in turn, can activate plasminogen (9–11). pro-u-PA activation is efficiently catalyzed by plasmin, leading to a strong amplification of the overall reaction. It is not known how the cascade is initiated. u-PAR localizes the enzyme to cell surfaces (12–15). The binding of pro-u-PA to this receptor and of plasminogen to as yet unidentified binding sites strongly enhances plasmin generation (Refs. 16 and 17; Footnote 5).

Two specific plasminogen activator inhibitors, PAI-1 and PAI-2, react with both free and receptor-bound u-PA, but not with pro-u-PA (18–20). Both inhibitors are fast acting and belong to the serpin superfamily. They are encoded by different genes and located on different chromosomes, and they are differentially expressed in cultured cells and in tissues (21–23). The synthesis of u-PA, its receptor, and its inhibitors is regulated by a variety of hormones, cytokines, and growth factors (24, 25).

Few immunohistochemical investigations of the two inhibitors have been reported. Both PAI-1 and PAI-2 have been localized in human placenta in the trophoblastic epithelium (26, 27). PAI-1 is also present (together with t-PA) in the noradrenalin-containing cells of the rat adrenal glands (28). In the transplantable murine Lewis lung carcinoma, PAI-1 was seen in the malignant cells. The PAI-1-positive cells were heterogeneously distributed in the tumors. Whereas PAI-1 was absent in peripheral areas characterized by a high u-PA content and tissue degradation, it was present in areas with high u-PA content but no histological signs of tissue destruction, indicating that the inhibitor may protect the tissue against the effect of u-PA (29).

The u-PA content is strongly increased in colon cancer tissue compared with normal colon tissue (30–36). Recently, we found that both u-PA immunoreactivity (36) and u-PA mRNA (37) are located in fibroblast-like cells in the tumor stroma of colon adenocarcinomas, but they are not present in detectable amounts in the cancer cells. In contrast, mRNA for the u-PA receptor is found in the cancer cells at the tumor-stromal interphase of invasive foci (37). With the use of a previously isolated cDNA for PAI-1 (38), we have now found that PAI-1 mRNA is located in still another cell type in the colon adenocarcinomas, namely, endothelial cells in the tumor stroma, indicating a complex interaction among several different cell types in regulation of plasminogen generation in this type of cancer.

MATERIALS AND METHODS

Materials. The following materials were obtained from the indicated sources: T7 and T3 polymerase, pBluescriptKS(+) plasmid vector (Stratagene, La Jolla, CA); RNasin and DNase I (Promega, Madison, WI); [35S]UTP (1300 Ci/mmol) (Amersham DK, Birkerød, Denmark); diethiothreitol and restriction endonucleases (Boehringer Mannheim, Mannheim, Germany); K5 autoradiographic emulsion (Ilford, Cheshire, England); formamide (Fluka, Buchs, Switzerland); and salmon sperm DNA (type III; Sigma, St. Louis, MO). All other materials were as described previously (29).

Tissue Preparation. Routinely processed, formalin-fixed, and paraffin-embedded specimens from 14 adenocarcinomas of the colon, operated during 1989 and 1990, were drawn from the files of the Department of Pathology at the Righospital. The specimens were assessed in accordance with standard criteria. Four cases were classified as Dukes'..
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B, moderately differentiated; eight cases as Dukes' B, highly differentiated; one case as Dukes' C, moderately differentiated; and one case as Dukes' C, highly differentiated. In addition, three biopsies from normal colon (sampled at routine colonoscopy) were examined. These biopsies were processed as the carcinoma specimens.

Preparation of 35S-labeled RNA Probes. Two nonoverlapping fragments of human PAI-1 cDNA (38) were subcloned using standard techniques (39) in the transcription vector pBluescriptSK(+). Plasmid constructs pHPAI04 contained the Parl(1110)-Parl(1613) fragment, and construct pHPAI05 contained the BamHI(12)-Parl(1109) fragment, base pair numbers referring to the sequence as listed in the EMBO database (Acquisition No. M16006). The plasmids were linearized for transcription using restriction endonucleases, and 5 µg of the linearized plasmids were extracted with phenol and with chloroform:isoamylalcohol (25:1), precipitated with ethanol, and redissolved in water. Each transcription reaction contained 1 µg of linearized DNA template, and transcriptions were performed essentially as recommended by the manufacturer of the polymerases. The RNA was hydrolyzed in 0.1 M sodium carbonate buffer, pH 10.2, containing 10 mM DTT to an average size of 100 bases. Probe preparations always contained more than 2 x 10^6 cpm/µl, and the amount of trichloroacetic acid-precipitable material was usually above 90%. For pHPAI05, RNA probes transcribed from opposite strands of the same plasmid template, yielding sense and antisense transcripts, were adjusted to the same radioactivity concentration. Probes were stored at -20°C until used.

In Situ Hybridization. In situ hybridization was performed using a method adapted from a number of published procedures (37, 40). In brief, paraffin sections were cut, placed on gelatinized slides, heated to 60°C for 30 min, deparaffinized in xylene, and rehydrated through graded alcohols to PBS [0.01 M sodium phosphate buffer (pH 7.4) containing 0.14 M NaCl]. The slides were then washed twice in PBS, acid treated in 0.2 M HC1 for 20 min, and washed for 5 min in PBS. This was followed by incubation in 5 µg/ml of proteinase K in 50 mM Tris-HCl (pH 8.0) with 5 mM EDTA for 7.5 min, washing twice in PBS (2 min), and fixation in 4% (w/v) paraformaldehyde in PBS for 20 min. Fixative was removed by washing with PBS, and slides were immersed in 0.2% (v/v) acetic acid anhydride in 100 mM triethanola- mine in a beaker on a magnetic stirrer for 30 min. Finally, the slides were washed in PBS (5 min), dehydrated in graded ethanol, and air dried before the RNA probe was applied (80 pg/µl), in a solution of deionized formamide (50%), dextran sulfate (10%), t-RNA (1 µg/µl), Ficoll 400 [0.02% (w/v)], polyvinylpyrrolidone [0.02% (w/v)], bovine serum albumin Fraction V [0.02% (w/v)], 10 mM DTT, 0.3 M NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, and 10 mM NaPO4 (pH 6.8). Sections were covered by alcohol-washed and autoclaved coverslips, and they were hybridized at 47°C overnight (16 to 18 h) in a chamber humidified for 15 min and treated with RNase A (20 µg/ml) in NTE at 37°C for 30 min. This was followed by washing in NTE at 37°C (twice for 30 min) and in 2 liters of 15 mM sodium chloride:1.5 mM sodium citrate (pH 7.0) with 1 mM EDTA for 30 min at room temperature with stirring. Sections were then dehydrated and air dried. Finally, autoradiographic emulsion was applied following the manufacturer's recommendations, and sections were stored in black air-tight boxes at 4°C until developed after 1 to 2 wk of exposure. Sections were lightly counterstained with Mayer's haematoxylin/eosin.

Immunohistochemistry. Endothelial cells were identified by peroxidase-antiperoxidase immunostaining, a modification of the method of Sternberger et al. (41), using a polyclonal rabbit antibody against Factor VIII-related antigen (DAKO A/S, Code A 82).

RESULTS

In all 14 tumor specimens, hybridization to PAI-1 mRNA was confined to cells that were clearly identified as endothelial cells and to some adjacent stromal cells (see below). The malignancy of PAI-1 mRNA-containing stromal cells were frequently seen in cancerous areas in which neovascularization was evident (Fig. 1, c and h). The identity of these cells could not be established with certainty. It is possible that at least some of them represented migrating endothelial cells. Two observations support this view. (a) By immunostaining of adjacent sections for Factor VIII-related antigen, the PAI-1 mRNA-positive stromal cells were seen to be located at the periphery of capillary networks positive for Factor VIII-related antigen (Fig. 1, g and h). (b) In a few sections we observed strands of coherent PAI-1 mRNA-containing cells. These cells resembled endothelial cells with the exception that no apparent lumen could be seen (not shown).

Positive control experiments were performed by application of two different antisense probes from two nonoverlapping parts of the same cDNA. These probes were adjusted to the same radioactivity and were applied to adjacent sections of five of the tumors. In all cases, the two probes showed identical hybridization patterns (Fig. 1, d and e). As negative controls, sense RNA probes transcribed from one of the two cDNA fragments (PHPAI05; see “Materials and Methods”) were applied to adjacent sections of the 14 tumors. All of these had a low and even distribution of silver grains above the tissue sections (Fig. 1f).

DISCUSSION

We have investigated the localization of PAI-1 mRNA in human colon cancer by in situ hybridization. The results indicate that this mRNA is present in readily detectable amounts in endothelial cells in cancerous areas. By contrast, no hybridization was seen in cancer cells or in normal colonic tissue. To ensure that the hybridization signals obtained represented genuine PAI-1 mRNA, we performed positive control experiments using two nonoverlapping cDNA sequences as templates for the 35S-labeled probes used. In addition, we used as a negative control a sense transcript probe adjusted to the same radioactivity.

To our knowledge, no previous reports are available on the histological distribution of PAI-1 mRNA in human tumors. In a recent immunohistochemical investigation of the transplanted murine Lewis lung carcinoma, PAI-1 protein was found in the malignant cells in areas of the tumors where u-PA immunoreactivity could also be detected. In these areas, no or only minimal tissue destruction was seen. By contrast, PAI-1 protein was absent in other u-PA-containing areas in which tissue destruction was evident (29). These findings have later been confirmed at the mRNA level* and support the view that PAI-

* P. Kristensen, unpublished results.
Fig. 1. In situ hybridization of colon cancer for PAI-1 mRNA (a to h) and immunostaining with antibodies to Factor VIII-related antigen (g). Endothelial cells of capillaries located just under the free luminal surface of cancerous epithelium (a) contain PAI-1 mRNA (arrows). In another case (b), both an arteriole (curved arrow) and two postcapillary venules (straight arrows) located in the connective tissue adjacent to an advancing tumor contain PAI-1 mRNA. Endothelial cells of a postcapillary venule (c) as well as cells located in the surrounding stroma that appear not to be part of any vessel (curved arrows) show a hybridization signal for PAI-1 mRNA. Granulation tissue with PAI-1 mRNA in the endothelium of newly formed capillaries (d). Hybridization to sections adjacent to d of an antisense probe (e) and a sense probe (f) transcribed from a part of the PAI-1 cDNA nonoverlapping with the part used in d. Immunostaining of capillary network in one tumor with a polyclonal antibody to Factor VIII-related antigen (g). The substrate for the peroxidase reaction was H2O2/diaminobenzamidine (brown color). Section is lightly counterstained with Mayer's haematoxylin. The open arrow in g marks the area shown in h, where an adjacent section has been hybridized with an antisense probe for PAI-1 mRNA. Note the intense signal in cells located at the periphery of the capillary network (at open arrow). Note also the weaker signal in endothelial cells in a large number of the capillaries seen in this area (curved arrows). Bars are equal to 25 μm in a and b; 40 μm in c; 17 μm in d to f; 170 μm in g; and 100 μm in h.
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1 may act to confine plasminogen activation to areas of invasive growth.

The present study contrasts with the above findings with respect to the cell type in which PAI-1 mRNA is located. It may reflect differences between transplanted and spontaneous tumors or species differences, but most likely it is related to a difference between individual tumor types. In a preliminary in situ hybridization study of human squamous cell carcinomas of the skin, PAI-1 mRNA was thus detected in the cancer cells, while no detectable signal could be seen in endothelial cells in these tumors. In vitro studies have shown that PAI-1 expression is regulated by a variety of hormones, cytokines, and growth factors, such as glucocorticoids, tumor necrosis factor alpha, interleukin 1, transforming growth factor beta, and epidermal growth factor (see Ref. 23). The effect of each of these on the PAI-1 expression varies strongly among cell types. The different kinds of PAI-1 expression seen in various tumor types may therefore reflect differences in both the occurrence of PAI-1 regulatory factors and the susceptibility of the respective tumor cells to these factors. Which regulatory factors are responsible for the induction of PAI-1 in endothelial cells in colon cancer tissue will be an interesting topic for future investigations.

The lack of detectable PAI-1 mRNA in endothelial cells in normal colon tissue is in good agreement with a previous immunohistochemical study that showed that PAI-1 immunoreactivity could not be detected in endothelial cells in normal mouse lung (29). However, the findings are in apparent contrast with the fact that cultured endothelial cells often produce PAI-1 (see Ref. 23). Most likely this represents still another example of cultured cells not necessarily being representative of cells in situ with respect to production of components of the plasminogen activation system (see Ref. 1), relevant previous examples being the production of u-PA by many cultured endothelial cells, which contrasts with the lack of u-PA immunoreactivity in endothelial cells in the normal organism (42), and production of u-PA by many human colon cancer cell lines (43), in contrast to the absence of detectable u-PA immunoreactivity and u-PA mRNA in colon cancer cells in situ (36, 37).

We have previously reported that u-PA mRNA (and protein) and u-PAR mRNA are located in different cell types in colon cancer. u-PA is produced by fibroblast-like connective tissue cells in the tumor stroma at invasive foci adjacent to cancer cells that contain u-PAR mRNA and presumably protein (36, 37). The present finding of PAI-1 mRNA in a third cell type adjacent to the above mentioned indicates a complex cooperation between the cells in generation of plasmin activity and in the regulation of this generation. Binding of u-PA to its receptor strongly enhances cell surface plasmin generation (Refs. 16 and 17; Footnote 5), and surface-bound plasmin is inaccessible for its main inhibitor, alpha2-antiplasmin (17, 44). In contrast, receptor-bound u-PA is accessible for inhibition with PAI-1 (20). It therefore appears likely that u-PA (secreted from the stromal cells) exerts its action after it has been bound to its receptor on the cancer cells, and that PAI-1 (released from the endothelial cells) regulates the activity of the receptor-bound u-PA. PAI-1 binds with a high affinity to vitronectin in the extracellular matrix (45–49). Vitronectin-bound PAI-1 is active and may serve to protect the tumor stroma against a proteolytic degradation that can destroy the matrix in normal colon tissue in which PAI-1 mRNA is virtually absent.

In addition to fibroblast-like cells, u-PA protein is present in some endothelial cells in capillaries and venules in the tumor stroma in colon cancer (36). These endothelial cells do not contain detectable amounts of u-PA mRNA (37), suggesting that the u-PA seen in these cells is not produced by them but by stromal fibroblast-like cells and subsequently bound to the endothelial cells; mRNA for the u-PA receptor was not found in these cells (37), but PAI-1 may be important in the binding of u-PA. PAI-1 secreted by cultured endothelial cells is built into the extracellular matrix around the cells and, so positioned, it is capable of binding the active form of u-PA (50–53). Furthermore, it has been shown that endothelial cells can internalize u-PA/PAI-1 complexes (54). It is noteworthy that PAI-1 only binds active u-PA and not pro-u-PA (18). The above interpretation therefore implies that the u-PA detected in this location has at a certain stage been enzymatically active.

It is likely that at least some of the stromal PAI-1 mRNA-containing cells seen in this study represent migrating endothelial cells located at the tips of capillary loops and involved in tumor neovascularization. These cells were in some cases seen to consist of strands of coherent cells with no lumen. The existence in vivo of such solid strands of immature endothelial cells has been confirmed by ultrastructural studies of colorectal carcinomas, showing that capillary buds (consisting of strands of endothelial cells without a lumen) are abundant in these malignancies (55).

Extracellular proteolysis, and particularly the u-PA pathway of plasminogen activation, is thought to play a role in the early stages of angiogenesis, which is similar to the one in cancer invasion, facilitating the penetration of endothelial sprouts through the vascular basement membrane into the interstitial extracellular matrix (see Refs. 6 and 56). In a recent work with u-PA-producing mouse endothelial cells expressing the polyoma virus middle T-oncogene, aberrant hemangioma-like cystic structures formed by these cells when grown in vitro could be corrected by the addition of serine protease inhibitors (57). PAI-1 produced by the endothelial cells during angiogenesis may therefore play a critical role by preventing excessive degradation of the extracellular matrix.

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