Plasmin-catalyzed Proteolysis in Colorectal Neoplasia

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ABSTRACT

The expression of different components of the plasminogen activator (PA)/plasmin system was explored in a series of colorectal neoplasia. We have found that urokinase (uPA) and urokinase receptor (uPA-R) gene expression is upregulated in adenomas and carcinomas, and that uPA/uPA-R production is confined to stromal cells in the proximity of epithelial proliferations. In addition, in adenomas, the focal increase in uPA mRNA is not systematically coupled to detectable enzymatic activity, whereas in carcinomas, uPA mRNA accumulation is consistently associated with detectable but variable levels of enzymatic activity. In contrast, in the tumor vasculature, tissue-type plasminogen activator-mediated proteolysis is considerably reduced when compared to normal mucosal and submucosal vessels; this reduction in plasmin formation appears to result from the highly increased production of plasminogen activator inhibitor type 1 by endothelial cells. Our observations demonstrate that colorectal neoplasia are associated with marked alterations in the extracellular proteolytic balance controlled by the PA/plasmin system. They show that contrasting disturbances in plasmin formation take place in distinct stromal compartments but not in epithelial cells, and that these disturbances are maximal during invasive neoplasia. Altogether, our results raise the possibility that alterations in plasmin formation should not be exclusively regarded as promoters of cancer cell invasiveness.

INTRODUCTION

In the past few years, considerable progress has been achieved in our understanding of the molecular mechanisms that lead to neoplastic transformation of intestinal epithelial cells. Genes involved in colorectal carcinogenesis have been identified and shown to encode proteins that participate in the control of vital cellular processes, such as DNA repair, cell proliferation, cell adhesion, and apoptosis (1–4). In addition, distinct genetic alterations have been ascribed to each of the various proliferative lesions that constitute the adenoma-carcinoma sequence. A succession of genetic lesions appears, therefore, to account for the progressive alterations observed between benign and dysplastic adenomas, whereas the accumulation of such alterations culminates in carcinomas. These molecular findings corroborate the morphological observations suggesting that carcinomas arise from a continuum of precursor lesions; however, limited information is presently available concerning late stages of tumor progression, such as the functional transition that allows noninvasive neoplastic cells to become invasive.

A large body of experimental evidence supports a role for secreted proteolytic enzymes in neoplastic cell invasiveness and metastasis formation (5–8). A variety of proteolytic systems (proteases-antiproteases) is known to be produced by tumoral tissues. In some instances, correlations have been drawn between enzyme content in primary tumors and clinical behavior (9, 10), lending further support to the contention that extracellular proteases may mediate tissue invasion in vivo. In that context, PAs are thought to play a prominent role; PAs are serine-proteases that catalyze the conversion of plasminogen into plasmin, a proteolytic enzyme of broad substrate specificity capable of degrading, directly or indirectly, most extracellular proteins (11, 12). The PA/plasmin system has been studied extensively in human neoplasia. Most carcinomatous tissues contain elevated amounts of uPA when compared to their nonmalignant counterparts, and in some tumors uPA content appears to be a prognostic factor (13–15). Similarly, disturbances of the PA/plasin system have been shown to occur during colorectal neoplasia; in particular, increased amounts of uPA are found in colorectal carcinomas when compared to normal intestinal mucosa and adenomas (16–18). Recent studies have localized the production of the enzyme in stromal cells (19), whereas the expression of the uPA-R has been allocated to invading malignant cells (20), raising the possibility that carcinoma cells may derive their proteolytic potential from adjacent nonmalignant cells. However, the contribution of excessive uPA production to the clinical behavior of colorectal neoplasia remains to be demonstrated.

Using methods whereby sites of enzymatic activity and of enzyme production can be localized, we have analyzed a series of colorectal neoplasia. We report here that progressive and contrasting alterations in plasmin-mediated proteolysis take place in distinct compartments of the tumor stroma in response to colorectal neoplasia. In particular, we show that increased uPA and uPA-R expression is restricted to nonepithelial cells, raising the possibility that excessive plasmin production in colorectal neoplasia may play other roles than directly promoting cancer cell invasiveness.

MATERIALS AND METHODS

Tissue Sampling and Histological Assessment. Tissue specimens were collected prospectively and immediately after surgical or endoscopic resection. One half of each specimen was frozen in precooled methylbutane and stored at −70°C. The other one-half was fixed in 10% formalin and embedded in paraffin for histological examination. All specimens were classified according to standard histological criteria. Fifteen specimens were classified as adenomas. Three specimens were devoid of morphological features of dysplasia, whereas 12 displayed variable degrees of dysplasia (hyperchromasia, inversion of the nucleo-cytoplasmic ratio, increased mitotic index, loss of nuclear polarity, decreased mucus production, disorganization of crypt structure). Twenty-eight specimens exhibited invasive adenocarcinomas that were classified according to Dukes: 5 were classified as a Dukes A, 12 as Dukes B, and 11 as Dukes C.

Fifteen specimens of morphologically normal mucosa were obtained from colectomies performed for carcinoma resection at a minimal distance of 5 cm from the carcinoma borders.

Plasmid Constructions and in Vitro Transcriptions. The uPA antisense and sense probes were prepared from pSP64-hUK (21) and pSP65-hUK containing the 610-bp EcoRI/PstI fragment isolated from pPHUK, respectively. The huPA-R antisense and sense probes were prepared from pBSKS-huPAR (22) containing a 584-bp BamH1/XbaI fragment of the human uPA-R cDNA. The IPA antisense and sense probes were prepared from pBSKS-hTA (23) containing the 614-bp BglII/EcoRI fragment isolated from pHW349F. The PAI-1 antisense and sense probes were prepared from pBSKS-hPAI-1 (24)

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3 The abbreviations used are: PA, plasminogen activator; uPA, urokinase; uPA-R, uPA receptor; IPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1.
containing the 1.4kbp EcoRI/BglII fragment isolated from pPAI-Cl. The PAI-2 antisense probe was prepared from pDB4707 (25) containing the 794-bp EcoRI/XhoI fragment of the human PAI-2 clone 1.7.

Linearized plasmids were transcribed in vitro in the presence of 12.5 mM of \([^{32}P]UTP\) (400 Ci/mmol; Amersham International, Amersham, United Kingdom), or \([^{33}P]UTP\) (2–3000 Ci/mmol; DuPont-NEN Products, Germany) or 30 mM of \([^{3}H]UTP\) and 30 mM of \([^{3}H]CTP\) (40 and 20 Ci/mmol, respectively; Amersham International). \(^{3}H\)-labeled probes were reduced to an average size of 50–100 nucleotides by mild alkaline hydrolysis as described previously (26).

RNA Analyses. In situ hybridizations were carried out on 5-μm cryostat tissue sections as described previously (26). Macroscopic mRNA localizations were performed by hybridizing \(^{32}P\)- or \(^{33}P\)-labeled probes to tissue sections and were revealed by film autoradiography. Microscopic mRNA localizations were performed by hybridizing \(^{3}H\)-labeled cRNA to adjacent tissue sections and were revealed by emulsion autoradiography.

Total RNA extractions from frozen colorectal specimens and Northern blot analysis were performed as described elsewhere (27).

Immunohistochemistry. Immunohistochemistry was performed on 10-μm cryostat tissue sections fixed in methanol with the avidin-biotin-horseradish peroxidase system from Dakopatts using mouse monoclonal antihuman CD68 (Dako A/S, Denmark), mouse monoclonal antihuman factor VIII (Dako A/S), and goat polyclonal antihuman tPA-purified immunoglobulins (American Diagnostica, Inc., Greenwich, CT). The primary antibody was detected with either biotinylated rabbit antigoat or biotinylated sheep antimouse antibodies.

Enzymatic Assays. Histological zymographies were performed on 10-μm cryostat tissue sections as described elsewhere (28). Briefly, tissue sections were overlayed with 50 μl of a mixture containing 2% nonfat dry milk, 0.9% agar, and 40 μg/ml of purified human plasminogen in PBS (with 0.9 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\)). The slides were then incubated in a humid chamber at 37°C for 3–6 h. Control experiments were carried out with overlay mixtures from which plasminogen was omitted; although other proteases are known to be present in the specimens analyzed, no proteolytic zones were observed, indicating that the lytic activities revealed by this histological assay are due to PAs.

To distinguish uPA from tPA, we added to the substrate 1 mM amiloride, a specific inhibitor of uPA catalytic activity (29), or antihuman tPA immunoglobulins (0.2 mg/ml; American Diagnostica, Inc.). Photographs were taken using dark-ground illumination.

For SDS-PAGE zymographies, tissue protein extracts were prepared and analyzed as described elsewhere (30). After separation of total proteins on a 10% SDS-PAGE, zymographies were performed on a casin underlay containing the same mixture as mentioned above for histological zymographies.

RESULTS

The PA/Plasmin System in Nonproliferating Colorectal Mucosae. In all specimens analyzed (15 of 15), histological zymograms revealed zones of plasminogen-dependent caseinolysis that predominated over the mucosae but were also observed in the submucosa (Fig. 1). These areas of caseinolysis were inhibited by anti-tPA antibodies but were not affected by the addition of amiloride, an inhibitor of uPA enzymatic activity, indicating that the observed caseinolysis was due to tPA. Immunohistochemical staining with anti-tPA antibodies revealed immunoreactivity in endothelial cells of mucosal and submucosal vessels (not shown). To determine whether PAs could be synthesized locally, Northern blot analyses of total RNAs were performed. In all samples analyzed, single-sized tPA mRNA transcripts were detected (not shown). Macroscopic mRNA localization studies, performed on adjacent cryostat tissue sections with \(^{32}P\)-labeled cRNA probes, revealed in all specimens (15 of 15) an accumulation of tPA mRNA in the mucosae (Fig. 1). In contrast, no signal was detected for uPA or PAI-1 mRNAs (Fig. 1). Cellular mRNA localizations, performed by hybridizing adjacent cryostat tissue sections to \(^{3}H\)-labeled cRNA probes, revealed an accumulation of tPA mRNA that predominated in mucosal endothelial cells lining the small vessels adjacent to the crypts. Faint uPA mRNA (in 3 of 15 specimens) and uPA-R mRNA (in 5 of 15 specimens) accumulations were noticed in pericryptal cells (not shown). In 3 of 15 specimens, PAI-1 mRNA was detected occasionally in endothelial cells (not shown).

These results indicate that, in morphologically normal-appearing colon mucosae, endothelial cells produce large amounts of tPA that are enzymatically active. In addition, in a minority of normal colon.
of plasminogen-dependent caseinolytic activity. In all cases (15 of 15) was never coupled to detectable uPA-mediated proteolytic activity in mucosae specimens displaying discrete signs of inflammation, dispersed inflammatory cells containing uPA and uPA-R mRNAs were observed, as well as endothelial cells containing PAI-1 mRNA. However, this focal accumulation of cells containing uPA/uPA-R mRNAs was never coupled to detectable uPA-mediated proteolytic activity in normal colonic tissues.

The PA/Plasmin System in Colorectal Adenomas. In 15 of 15 specimens analyzed, histological zymograms revealed discrete zones of plasminogen-dependent caseinolytic activity. In all cases (15 of 15), areas of caseinolysis were observed in the central regions of adenomas that were abolished by anti-tPA antibodies but not by amiloride, indicating that this enzymatic activity was due to tPA (Fig. 2).

In 8 of 15 cases, additional areas of caseinolysis were observed at the periphery of the adenomas; they were inhibited by the addition of amiloride in the substrate but were not modified by anti-tPA antibod-
ies, indicating that these circumscribed zones of caseinolysis were due to uPA (Fig. 2).

Macroscopic mRNA localization studies performed on adjacent cryostat tissue sections with 3H-labeled cRNA probes revealed (in 12 of 15 specimens analyzed) a coupled accumulation of uPA and uPA-R mRNAs in stromal cells. Both mRNAs colocalized at the periphery of the lesions (Fig. 2). In most specimens analyzed (13 of 15), PAI-1 mRNA was also detected (Fig. 2). Cellular mRNA localizations were performed by hybridizing adjacent cryostat tissue sections to 3H-labeled cRNA probes; uPA and uPA-R mRNAs were found to be expressed by stromal cells in the vicinity of dysplastic crypts. Immunohistochemistry for CD68 performed on adjacent cryostat tissue section confirmed the presence of numerous inflammatory cells that had a similar distribution to uPA and uPA-R mRNA-containing cells (not shown). In accordance with macroscopic localizations, uPA/uPA-R mRNA-positive cells were found mainly at the periphery of the lesions, in areas displaying marked inflammatory cellular infiltrations as well as high epithelial proliferative activity. In the majority of cases (13 of 15), PAI-1 mRNA was detected in endothelial cells from small vessels that predominated in the vicinity of dysplastic crypts (Fig. 3B). Factor VIII immunoreactivity confirmed the endothelial nature of these PAI-1 mRNA-containing cells (not shown). Finally, macroscopic and microscopic mRNA localization studies showed the presence of tPA mRNA in endothelial cells in all specimens analyzed (15 of 15; Fig. 3A). However, anti-von Willebrand factor immunohistochemistry, performed on adjacent cryostat tissue sections, revealed that some tPA-containing cells were not immunostained (not shown), a discrepancy that may reflect vessel neoformation.

Altogether, these results indicate that in the majority of adenomas, inflammatory cells accumulate uPA and/or uPA-R mRNAs in the immediate vicinity of proliferating epithelial cells. These accumulations are not systematically coupled to a corresponding increase in uPA-mediated catalytic activity. However, because morphological quantifications of dysplasia are difficult to ascertain, due to the recognized heterogeneous distribution of dysplastic zones within adenomas, no strict correlations could be drawn between detectable uPA enzymatic activity and the amount of dysplasia. In all cases, tPA mRNA was present in endothelial cells. Although histological zymographies do not provide quantitative estimations, tPA-catalyzed proteolytic activity in the vascular compartment appeared weaker when compared to the normal mucosa; this relative reduction was correlated to the concomitant expression of PAI-1 by endothelial cells.

The PA/Plasmin System in Adenocarcinomas. In most cases of adenocarcinomas (27 of 28), histological zymograms revealed 2 types of plasminogen-dependent caseinolytic activity: (a) zones of caseinolytic activity were noticed over tumoral tissues; they were inhibited by the addition of amiloride in the mixture but were not modified by anti-tPA antibodies, indicating that the caseinolytic activity associated with tumoral tissues was mediated by uPA (Fig. 4-); and (b) zones of caseinolytic activity were observed on adjacent nontumoral tissues; they were inhibited by the addition of anti-tPA antibodies in the substrate but not by the addition of amiloride, indicating that the observed catalytic activity was due to tPA (Figs. 4 and 5).

As for other specimens, tPA immunoreactivity was predominantly found in endothelial cells surrounding tumoral tissues (not shown). Representative carcinomas shown in Fig. 4 (cases I-IV) illustrate the marked heterogeneity in the amount and distribution of uPA-catalyzed proteolysis that was observed between tumors. Macroscopic localization studies revealed in all specimens (28 of 28) an accumulation of tPA, uPA, and uPA-R mRNAs over tumoral tissues (Fig. 5). Furthermore, macroscopic in situ hybridizations demonstrated the presence of PAI-1 mRNA over tumoral tissue in all carcinomas (28 of 28; Fig. 5), whereas PAI-2 was not detected. To identify the cellular sites of uPA,
Fig. 3. Microscopic mRNAs localization. Cryostat tissue sections of an adenoma (A and B), and an adenocarcinoma (C and D) were hybridized to 3H-labeled cRNAs and revealed by emulsion autoradiography as described in "Material and Methods." tPA (arrowheads in A) and PAI-1 (arrowheads in B) mRNAs are localized in endothelial cells lining submucosal vessels. uPA (C) and uPA-R (D) mRNAs accumulate in stromal cells, whereas epithelial cells (e) remain unlabeled. Slides were exposed for 12—16 weeks at 4°C and counterstained with methylene blue. A and B, × 600; C and D, × 1200.

uPA-R, tPA, and PAI-1 production, adjacent cryostat tissue sections were hybridized to 3H-labeled cRNA probes; uPA and uPA-R mRNA accumulations were observed in stromal cells that exhibited the topological and morphological features of inflammatory cells (Fig. 3, C and D). Immunohistochemistry on adjacent cryostat tissue sections with anti-CD68 antibodies identified stromal cells that shared similar morphology and distribution, providing further evidence that uPA- and uPA-R-producing cells are inflammatory cells (not shown). In contrast, tPA and PAI-1 mRNA accumulations were found in endothelial cells adjacent to tumoral crypts (not shown). Immunohistochemical staining with anti-von Willebrand factor antibodies on adjacent cryostat tissue sections displayed only a weak labeling (not shown). As discussed in adenomas, this may reflect neovessel formation. To investigate the apparent discrepancy between the strong tPA mRNA signal and the absence of tPA-mediated catalytic activity in the tumoral tissue, we analyzed total protein extracts by SDS-PAGE and zymography; we observed the presence of high molecular weight complexes, suggesting that tPA is complexed to PAI-1 in tumoral tissues (not shown). Finally, perhaps because the number of specimens analyzed was limited, we were not able to draw correlations between levels of uPA-mediated proteolytic activity and Dukes stages.

These results demonstrate that stromal cells within colorectal carcinomas contain high amounts of uPA mRNA that are in most cases associated with detectable, although variable, levels of uPA-mediated proteolytic activity. In contrast, endothelial cells lining peri- and intratumoral vessels produce large quantities of tPA that appear to be partly inactivated by a concomitant increase of PAI-1 production.

DISCUSSION

Extracellular proteases and antiproteases closely cooperate in the control of extracellular metabolism (8). Broad experimental evidence supports the concept that, in physiological conditions, interactions...
Fig. 4. Macroscopic visualization of PA enzymatic activities on tissue sections of four different adenocarcinomas. Histological zymograms were performed as described in Fig. 1. They show circumscribed zones of caseinolysis. Most of these activities are due to uPA because they are inhibited by amiloride (tPA activity) and not by anti-tPA antibodies (uPA activity). Notice the variable amounts of proteolytic activity between the different tumors: case I displays no uPA activity, whereas cases II, III, and IV display zones of uPA-mediated catalytic activity. In some cases, zones of proteolytic activity are localized predominantly at the periphery of tumoral sheets (case II, black areas, arrows, right). Of note, case I was a Dukes C tumor, with serosal infiltration. H & E, haematoxylin and eosin coloration (left). Micrographs of zymographies were taken after 3 (uPA + tPA activities) and 4 (uPA activity; tPA activity) h of incubation at 37°C. ×3.

between proteolytic enzymes, their inhibitors, and constituents of the extracellular milieu are required to achieve a finely tuned proteolytic equilibrium. Disruptions of this equilibrium occur frequently during pathological processes and are thought to account for inappropriate extracellular protein deposition or degradation (8, 31). Explorations of the PA/plasmin system give credit to such a concept; secreted PAs interact with specific extracellular components that help maintain a tight temporospatial control of plasmin-catalyzed proteolysis during
physiological processes, whereas alterations of the plasmin-catalyzed proteolytic balance have been described in a whole variety of disorders (11, 12, 31). For example, numerous experimental and clinical studies suggest that in neoplasia excessive plasmin formation can promote tissue invasion and metastasis formation through the degradation of nonmalignant tissues (11, 12).

We show in the present study that the plasmin-catalyzed proteolytic balance is altered in colorectal neoplasia and that opposite alterations take place in two distinct cellular compartments (see Table 1). uPA-catalyzed proteolytic activity was detected exclusively in adenomas and carcinomas but not in the normal colorectal epithelium; in both instances, stromal cells were identified as the predominant sites of uPA and of uPA-R production, indicating that in colorectal tissues, uPA and uPA-R are produced by the stromal and not by carcinomaous cells. Colorectal neoplasia, therefore, differ from other types of carcinomas in which neoplastic cells have been shown to synthesize uPA (28, 32). As evoked for other proteases that have been localized in peritumoral tissues (33, 34), these findings suggest that enzyme production occurs in response to epithelial proliferation. Cytokines known to be produced by epithelial cells in vitro are candidate mediators for this postulated paracrine mechanism (31); however, molecules capable of inducing uPA/uPA-R synthesis by nontumoral cells have not been formally identified in vivo. In the present analysis, stromal cells, including monocytes/macrophages, were found to be the main source of uPA-R synthesis. Our observation contradicts previous work performed in colon adenocarcinomas that has allocated uPA-R production to invading malignant epithelial cells (20); by analogy to murine spermatozoa that bind uPA secreted by epithelial cells of the vas deferens (35), this has led to the proposal that uPA produced by stromal cells may bind to the surface of cancer cells and thereby facilitate their invasion and migration through nonmalignant tissues. We have no definite explanation for this apparent discrepancy in the distribution of uPA-R-expressing cells; however, we cannot exclude that a minority of epithelial cells can express uPA-R because their formal identification would require combined in situ hybridization and immunohistochemistry on the same tissue section. In any event, all available results agree in demonstrating enhanced levels of uPA activity on or around tumor cells. Given that no other uPA-binding sites have yet been identified on mammalian cells, our observations suggest that the functional consequences of plasmin formation in colon carcinomas may not be directly involved in promoting cancer cell invasiveness. For instance, excessive plasmin production in the vicinity of proliferating epithelia may provide a permissive environment for the migration of cancer cells endowed with invasive properties not related to their own capacity of producing uPA. Alternatively, or in addition, plasmin can activate latent forms of growth factors and promote the release of growth factors from extracellular matrix constituents (31, 36, 37); uPA-catalyzed proteolysis may, therefore, contribute positively and/or negatively to the control of tumor growth by influencing the local availability of growth factors. Because immunometric determinations of constitutents of the PA/plasmin system in neoplasia of the digestive tract have been reported to carry prognostic value (15, 38), prospective explorations of larger series of colorectal specimens by a method that assesses net proteolytic activities of both PAs in tumor samples, such as described in the present study, should provide additional information with regard to the biological significance of PA production by colorectal carcinomas.

In the normal colorectal mucosa, the major source of plasmin formation is vascular and is due to uPA produced by endothelial cells. In carcinomas, abundant tPA mRNA was localized in endothelial cells lining neovessels; however, tPA-catalyzed proteolysis was reduced when compared to normal epithelium and adenomas. In accordance with previous studies (39), we attribute this diminution of tPA-catalytic activity to the concomitant production of PAI-1 by endothelial cells. PAI-1 mRNA upregulation could represent a differentiation feature of endothelial cells lining neovessels or, alternatively, result from endothelial interactions with tumor cells and/or their secretory products.
products. Indeed, experimental evidence indicates that PAI-1 is produced by endothelial cells during the “late phase” of angiogenesis (40). The influence on tumor growth and/or dissemination of reduced plasmin formation in the neovessels remains speculative. Because tPA, which may remain bound to the cells and/or be released in the blood, prevents inappropriate clot formation (41), inadequate fibrinolysis within tumor vessels may favor the constitution of tumor cell emboli. In this context, one should remember that high amounts of PAI-1 have been shown to be associated with an unfavorable prognosis in diverse carcinomas (14, 15). Conversely, the relative lack of plasmin formation may interfere with the systemic dissemination of neoplastic cells.

Finally, we observed that both alterations of the PA-mediated proteolytic balance (i.e., increased uPA-catalyzed activity and decreased tPA-catalyzed activity) are progressive in colorectal neoplasia, as noticed in other types of tumors in which PAs are produced by cancer cells (32). In adenomas, focal accumulations of uPA mRNA (and uPA-R) were not systematically coupled to detectable levels of enzymatic activity. In contrast, in adenocarcinomas, uPA (and uPA-R) mRNA-producing cells were more abundant and were consistently associated with detectable, although variable, levels of uPA catalytic activity. The uncoupling of uPA mRNA accumulation and uPA-catalyzed proteolytic activity suggests the local presence of specific inhibitors in the peritumoral stroma (42). Because PAI-i was predominantly localized in endothelial cells and no PAI-2 mRNA accumulation was detected, the participation of other inhibitors must be envisaged, such as protease nexin I and α2 antiplasmin (12). Conversely, vascular tPA-catalyzed proteolytic activity was maximal in the normal intermediate, immediate in adenomas, and nearly abolished in carcinomas; this progressive reduction was linked to an increasing accumulation of PAI-1 mRNA in endothelial cells.

Altogether, these observations document complex alterations of the plasmin-catalyzed proteolytic balance in colorectal carcinomas. By demonstrating that plasmin generation is modulated in opposite ways within distinct nonmalignant cellular compartments, we suggest that plasmin formation should not be regarded exclusively as a mediator of cancer cell invasiveness. In particular, the consequences of increased uPA and decreased tPA activities on the clinical behavior of colon adenocarcinomas remain to be explored.

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