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In Situ Stromal Expression of the Urokinase/Plasmin System Correlates with Epithelial Dysplasia in Colorectal Adenomas

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An increase of urokinase-type plasminogen activator (uPA) and a decrease of tissue-type PA (tPA) have been associated with the transition from normal to adenomatous colorectal mucosa. Serial sections from 25 adenomas were used to identify PA-related caseinolytic activities by in situ zymography, blocking selectively uPA or tPA. The distribution of uPA, tPA, and type 1 PA inhibitor mRNAs was investigated by nonradioactive in situ hybridization, and the receptor for uPA was detected by immunostaining. Low- and high-grade epithelial cell dysplasia was mapped histologically. Results show that 23 of 25 adenomas expressed uPA-related lytic activity located predominantly in the periphery whereas tPA-related activity was mainly in central areas of adenomas. In 15 of 25 adenomas, uPA mRNA was expressed in stromal cells clustered in foci that coincided with areas of uPA lytic activity. The probability of finding uPA mRNA-reactive cells was significantly higher in areas with high-grade epithelial dysplasia. uPA receptor was mainly stromal and expressed at the periphery. Type 1 PA inhibitor mRNA cellular expression was diffuse in the stroma, in endothelial cells, and in a subpopulation of α-smooth muscle cell actin-reactive cells. These results show that a stromal up-regulation of the uPA/plasmin system is associated with foci of severe dysplasia in a subset of colorectal adenomas. (Am J Pathol 1997, 150:283–295)

A majority of colorectal carcinomas is thought to develop from premalignant adenomatous lesions considered as intermediate stages of varying malignant potential.[1] Consistent with an adenoma to carcinoma progression, these stages represent benign and heterogeneous tumors expressing with increasing size, villous content, and dysplasia grade an increasing risk of malignant conversion.[2] Both genomic instability and cumulative alterations in oncogenes, tumor suppressor and DNA damage recognition, and repair genes may underlie colorectal carcinogenesis.[3,4] These genetic changes are associated with perturbations in epithelial cell growth and differentiation and in cellular interactions with extracellular matrices.[4]

Extracellular proteolysis involves multiple enzymatic activities and is a hallmark of malignant invasion and metastasis.[5] Proteases and their inhibitors have been shown to mediate the degradation of tissue matrices, a process required for invasive cells to migrate and cross anatomical barriers.[6,7] Marked changes in components of the plasminogen activator (PA)/plasmin system were found in solid tumor tissues including colorectal cancer.[8] Two types of PAs, the urinary (urokinase or uPA) and the tissue (tPA) types, have been identified. uPA can bind to a cell surface receptor (uPAR), and receptor expression has been associated with the invasive phenotype of colon carcinoma cells both *in vitro*[9,10] and *in vivo*.[11] Earlier findings showed, in extracts of colorectal tissues, that the uPA levels were consistently higher in carcinomas when compared with

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mucosa adjacent to the tumors.[12,13] Additional results have established that a stepwise increase in PA-related proteolytic activity and uPA expression is found in the course of the mucosa-adenoma-carcinoma sequence.[14–16] Moreover, recent clinical studies have indicated that increased tissue levels of uPA and PA inhibitors together with a decreased level of tPA may have prognostic value in colorectal cancer patients[17] and that a high level of uPAR antigen can be an independent prognostic factor for 5-year survival.[18]

In colorectal carcinomas, several studies have reported that uPA and type-1 plasminogen activator inhibitor (PAI-1) can be produced by cells found in stromal regions.[11,19–21] In contrast to that of carcinomas, the tissue distribution of components of the PA/plasmin system in adenomas has received limited attention.[22] A recent study indicated that no correlation could be drawn between uPA expression and epithelial dysplasia in adenomas.[21] Furthermore, the use of homogenates in previous studies precluded the identification and the tissue distribution of PA/PAI-expressing cell types in parallel with the grading of epithelial cell dysplasia.

We have investigated the expression of uPA, tPA, and PAI-1 mRNAs and uPA- and tPA-mediated activity and scored focal dysplasia in serial sections of colorectal adenomas. *In situ* hybridization using digoxigenin-labeled RNA probes was applied to provide a high degree of cellular resolution,[23] and immunostaining was used to localize the uPAR protein. Our results show that both uPA and PAI-1 mRNAs are found in cells located in the stroma and not in the adenomatous epithelium. A majority of areas of caseinolytic activity mediated by uPA corresponded to clusters of uPA mRNA- and uPAR protein-expressing stromal cells. Moreover, foci of uPA mRNA-positive cells and uPA-mediated caseinolysis were closely associated with severe dysplastic changes in the epithelium. A subpopulation of PAI-1 mRNA-reactive cells was identified as expressing α-smooth muscle cell actin, a marker of myofibroblasts and vascular smooth muscle cells in the stromal reaction. These results indicate that adenomatous lesions in the colorectum contain areas where dysplastic alterations in epithelial cells can be linked to an up-regulation of the uPA/plasmin system in the stroma.

Materials and Methods

Tissue Samples and Histopathological Analyses

Twenty-five colorectal adenomas were collected from patients referred for colonoscopy at the Division of Gastroenterology (Centre Hospitalier Universitaire Vaudois, Lausanne). Adenomas of 1.0 to 2.2 cm in size were collected during endoscopy, and the median part, containing the stalk, was fixed and embedded for histological assessment. Peripheral parts were snap-frozen in 2-methylbutane precooled with liquid nitrogen and stored at −70°C.

Serial 7-μm cryostat adenoma tissue sections were prepared for histological grading of dysplasia and expression of PA-related caseinolytic activities by *in situ* zymography/histozymography and nonradioactive *in situ* hybridization of uPA, tPA, and PAI-1 mRNAs. The scoring of epithelial cell dysplasia was performed on hematoxylin and eosin (H&E)-stained frozen tissue sections by three pathologists (L. Guillou, L. Mazzucchelli, and P. Chaubert) as described elsewhere.[24,25] Mild and moderate dysplasia were categorized as low-grade dysplasia, and severe dysplasia and carcinoma *in situ* were categorized as high-grade dysplasia. The level of agreement between the three pathologists was tested using the pairwise κ statistics.[26]

Statistical Correlations

Correlation between dysplasia score and uPA mRNA expression was analyzed by Pearson's χ^2 test.

Histozymography

Histozymographies were performed on unfixed 7-μm cryostat adenoma tissue sections adjacent to those used for dysplasia scoring and for uPA mRNA expression, as described by Sappino et al.[27] Zymographic analyses on agar-casein overlay, with or without plasminogen, respectively, with the addition of 1 mmol/L amiloride, a specific inhibitor of uPA, or of 0.1 mg/ml anti-human melanoma tPA IgG immunoglobulins (Biopool, Umeå, Sweden) were performed. Caseinolysis was monitored by photographs taken at 30-minute intervals using a stereomicroscope equipped with an annular dark-field type of illumination.

Preparation of Digoxigenin-Labeled Riboprobes

Fragments from the human tPA, uPA, and PAI-1 cDNA sequences, subcloned in pBluescriptKS phagemids, were provided by Dr. A.-P. Sappino (Division of Oncology, University Hospital, Geneva, Switzerland). The sequences of interest were amplified by polymerase chain reaction (PCR) using primers

outside the T3 (antisense probe) and T7 (sense probe) promoters (A: GAAACAGCTATGACCATG; B: GTAAAACGACGGCCAGT). The PCR products were purified by separation on a 1% NuSieve (FMC Bioproducts, Rockland, ME) agarose gel, extracted from the gel using the Magic-prep system (Promega, Madison, WI), and used as templates for synthesis of the digoxigenin-labeled riboprobes. Each transcription reaction contained 250 ng of purified PCR products and 40 U of either T3 or T7 RNA polymerase. Transcription was performed using reagents from the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's specifications. To check the efficiency of the reaction, the transcripts were analyzed on a 1% agarose gel and transferred to a nylon membrane, and digoxigenin was detected using standard protocols recommended by the manufacturer.

Nonradioactive in Situ *Hybridization*

In situ hybridization was performed using a method adapted from a number of published procedures.[23] The 7-μm cryostat adenoma tissue sections were mounted on glass 22 × 22 mm coverslips coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO), fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at 4°C, rinsed in PBS for 5 minutes, and stored in 70% ethanol at 4°C until use.

All steps were performed at room temperature unless otherwise specified. Fixed sections were rinsed with PBS (0.1 mol/L, pH 7.2), incubated in 0.1 mol/L PBS, pH 7.2, 0.1 mol/L glycine for 3 minutes, treated with 0.3% Triton X-100 in 0.1 mol/L PBS (pH 7.2), and rinsed again with PBS. Samples were treated with 0.2 μg/ml proteinase K (Boehringer Mannheim) in 0.1 mol/L Tris, 0.05 mol/L EDTA (pH 8.0) at 37°C for 20 minutes. They were fixed in 4% paraformaldehyde in PBS for 5 minutes at 4°C, rinsed in PBS, and acetylated (0.25% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0) for 10 minutes. The sections were rinsed twice (5 minutes each) in 2× standard saline citrate (1× SSC contains 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) before prehybridization in 2× SSC, 50% formamide at 52°C for 15 minutes. A 20-ng amount of digoxigenin-11-UTP-labeled cRNA probe was applied to each section in 30 μl of hybridization mixture containing 2× SSC, 50% formamide, 5% dextran sulfate, 1 mg/ml yeast tRNA (Boehringer Mannheim), 10 mmol/L dithiothreitol, 1 mg/ml RNAse-free bovine serum albumin (Sigma) at 52°C for 3 hours. Slides were subsequently washed in 2× SSC, 50% formamide at 52°C for 10 minutes and 30 minutes, respectively. Unhybridized transcripts were digested with 100 μg/ml ribonuclease A (Boehringer Mannheim) in 2× SSC at 37°C for 30 minutes. Slides were washed again in 2× SSC, 50% formamide at 52°C for 10 minutes. Sections were then washed with digoxigenin buffer 1 (100 mmol/L Tris/HCl, pH 7.5/150 mmol/L NaCl) and incubated with 1:360 polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (750 U/ml; Boehringer Mannheim) in buffer 1 with 0.3% Triton X-100 and 1% normal sheep serum for 4 hours. Slides were washed with buffer 1 (twice for 10 minutes each) and with buffer 2 (100 mmol/L Tris/HCl, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$) for 10 minutes. Tissue-bound alkaline phosphatase activity was visualized with a chromogen solution (45 μl of nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide; Boehringer Mannheim), 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate 50 mg/ml in 100% dimethylformamide), 1 mmol/L levamisole (2.4 mg) in 10 ml of buffer 2) at room temperature in the dark for 16 hours in a moist chamber. The enzymatic reaction was stopped by rinsing with buffer 3 (10 mmol/L Tris/HCl, pH 8.1, 1 mmol/L EDTA). Sections were counterstained with methyl green and fixed with an aqueous mounting solution (Dako Glycergel, Dako, Glostrup, Denmark). Specificity controls included the use of sense probes and pretreatment of tissue sections with RNAse. Sections of HT1080 human fibrosarcoma xenografts grown subcutaneously in nude mice were used as control tumors expressing high levels of uPA, tPA, and PAI-1 mRNA.

Immunohistochemistry

The 7-μm cryostat adenoma tissue sections were fixed with 4% paraformaldehyde in PBS for 10 minutes at 4°C, rinsed in PBS, and stored in 70% ethanol at 4°C. A monoclonal antibody (supernatant from hybridoma clone 1A4, diluted 1:10) directed against an epitope of the α-smooth muscle cell actin (α-sm-1)[28] was kindly provided by Dr. G. Gabbiani (Department of Pathology, Geneva, Switzerland). The α-sm-1-expressing cells were detected using rabbit anti-mouse antibodies (Dako) and the mouse alkaline phosphatase anti-alkaline phosphatase complex (Dako) subsequently incubated with a Fast Red substrate-chromogen-levamisole mixture (Fast Red tablets, Boehringer Mannheim). The alkaline phosphatase anti-alkaline phosphatase expression system was also used to detect stroma-infiltrating inflammatory cells reactive to the monoclonal antibody MAC 387 (Dako) labeling tissue histiocytes, granulocytes,

and macrophages. Immunostaining for the uPAR, CD87, was performed using a mouse monoclonal antibody recognizing soluble, surface-expressed, and intracellular uPAR (Nr. 3936, subclass IgG2a, 50 μg/ml, American Diagnostica, Greenwich, CT). Frozen tissue sections were air dried and fixed in acetone for 10 minutes. The primary antibody was detected by fluorescence using biotinylated horse anti-mouse antibodies (25 μg/ml, Vector Laboratories, Burlingame, CA) and a streptavidin-fluorescein isothiocyanate system (Extravidin-FITC conjugate, 10 μg/ml; Sigma). Controls included the use of the second and third layers omitting the primary antibody as well as the streptavidin-FITC conjugate alone.

Results

Twenty-five colorectal adenomas were investigated for uPA, tPA, and PAI-1 mRNA expression by nonradioactive *in situ* hybridization, uPAR antigen by immunocytochemistry, PA-related caseinolytic activity by histozymography, and epithelial cell dysplasia by histological independent examination. Serial cryostat sectioning was performed to insure the consecutive analysis of tissue mRNA expression, PA-mediated caseinolysis, dysplasia grading, and uPAR antigen tissue localization. Clinical and histopathological characteristics of adenomas used in this study were as follows: adenoma size, taken as the largest diameter of the polyp head (ranging from 1.0 to 2.2 cm), histological type (13 tubular and 12 tubulovillous), age and gender of patients referred for colonoscopy (9 females and 16 males, 41 to 86 years old).

Histozymographic Analyses

All adenomas (25 of 25) revealed areas of plasminogen-dependent caseinolysis developing in the overlay as circumscribed dark zones. Initial lysis was first detected within 0.5 to 1.5 hours of incubation at 37°C. Figure 1 illustrates five adjacent sections of 1 representative adenoma visualized after 1 hour. In Figure 1A, in the absence of plasminogen in the overlay, no lysis was detected (up to 5 hours of incubation at 37°C). In Figure 1B, in the presence of plasminogen, PA-related lysis areas were scattered over the entire surface of the section. In Figure 1C, anti-tPA antibodies or, in Figure 1D, amiloride have been added in the overlay to distinguish uPA-mediated (Figure 1C) from tPA-mediated (Figure 1D) caseinolysis. UPA-related caseinolysis areas were predominant in 23 of 25 samples and were localized in peripheral regions whereas tPA activity appeared to be associated with central regions in the adenomas. Only 2 of the 25 samples expressed marked tPA-related activity (not shown). The majority of adenomas did not show tPA- and uPA-related overlapping activities. In Figure 1, both uPA plus tPA activities (Figure 2B) and tPA activity only (Figure 1D) are compared with the adjacent H&E-stained section (Figure 1E); initial lysis induced by uPA or tPA is associated with the stromal compartment in adenomas.

Cellular Expression of uPA mRNA

Digoxigenin-based nonradioactive *in situ* hybridization was carried out on paraformaldehyde-fixed cryostat tissue sections using alkaline phosphatase and nitroblue tetrazolium/X-phosphate to produce a dark blue precipitate giving a good signal-to-noise ratio. Figure 2 illustrates the expression of uPA mRNA in sections of one adenoma using the digoxigenin-labeled uPA antisense (Figure 2, A and C) or sense (Figure 2, B and D) RNA probes. Sense probes did not produce hybridization signals over the noise level. In contrast, antisense probes detected a uPA message in the cytoplasm of cells localized within the stromal compartment of the adenomas. uPA mRNA was essentially found within peripheral regions. Clusters of 10 to 20 uPA-positive cells were distributed at the tip of tubules in the lamina propria (Figure 2A, arrows; see also Figure 4, A, B, and C). The uPA signal was found in the cytoplasm of both round cells or spindle-shaped, fibroblast-like cells (Figure 2C). The MAC387 immunoprobe, which reacts with a cytoplasmic antigen expressed in granulocytes, histiocytic cells, and tissue macrophages, revealed in adjacent sections stromal inflammatory cells infiltrating the periphery of adenomatous tubules. We observed that a fraction of stromal cells only expressed both uPA mRNA and the MAC387 antigen (not shown).

Altogether, 15 of the 25 adenomas expressed a stromal uPA message. There was no uPA expression over the noise level within adenomatous epithelial cells or in endothelial cells. tPA mRNA signal could not be detected above the noise level. As positive control, the tPA riboprobe, however, detected a tPA message in frozen sections of human fibrosarcoma HT1080 xenografts grown in nude mice (not shown).

As both uPA-related caseinolytic activities and clusters of uPA mRNA-expressing stromal cells have been found mainly in peripheral regions of adenomas, we have mapped the areas containing foci of uPA mRNA-expressing cells over the uPA-related caseinolytic histozymograms revealed in the presence of anti-tPA antibodies. Figure 3 illustrates the distribution of uPA

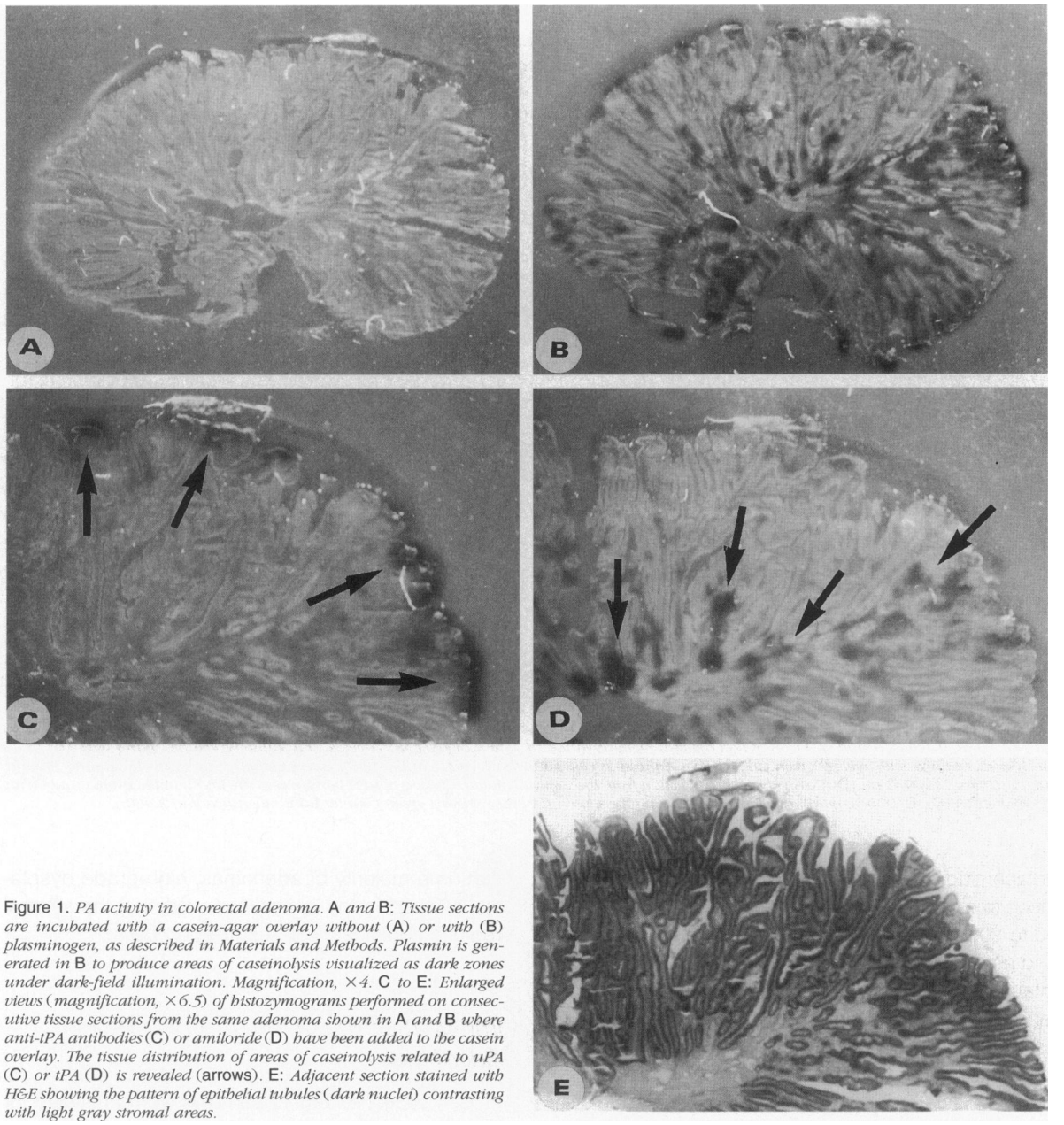

Figure 1. *PA activity in colorectal adenoma.* A *and* B: *Tissue sections are incubated with a casein-agar overlay without* (A) *or with* (B) *plasminogen, as described in Materials and Methods. Plasmin is generated in* B *to produce areas of caseinolysis visualized as dark zones under dark-field illumination. Magnification,* ×4. C *to* E: *Enlarged views (magnification,* ×6.5) *of histozymograms performed on consecutive tissue sections from the same adenoma shown in* A *and* B *where anti-tPA antibodies* (C) *or amiloride* (D) *have been added to the casein overlay. The tissue distribution of areas of caseinolysis related to uPA* (C) *or tPA* (D) *is revealed* (arrows). E: *Adjacent section stained with H&E showing the pattern of epithelial tubules (dark nuclei) contrasting with light gray stromal areas.*

activity and uPA message in four representative adenomas. Circles indicate the localization of cellular uPA mRNAs drawn over uPA-related caseinolytic areas. All uPA cellular foci are superimposed over the areas of uPA activity (Figure 3A). In adenomas shown in Figure 3, B, C, and D, few areas of uPA activity were free of detectable uPA mRNA signal. In contrast, all clusters of cellular uPA mRNA-positive cells corresponded to zones of caseinolytic activity. This finding is further documented in Figure 4, which shows two histozymograms from an adenoma developed in the presence of plasminogen (Figure 4a) and with amiloride (Figure 4b). Amiloride clearly affected three peripheral areas of lysis, which have been circled as A, B, and C. In the adjacent section, these three areas overlapped with three clusters of uPA mRNA-positive cells located in the lamina propria of tubular tips (Figure 4, A, B, and C).

Stromal Expression of uPA mRNA Co-Localizes with Epithelial Dysplasia

To assess a likely association between the stromal expression of uPA and the grade of dysplasia in

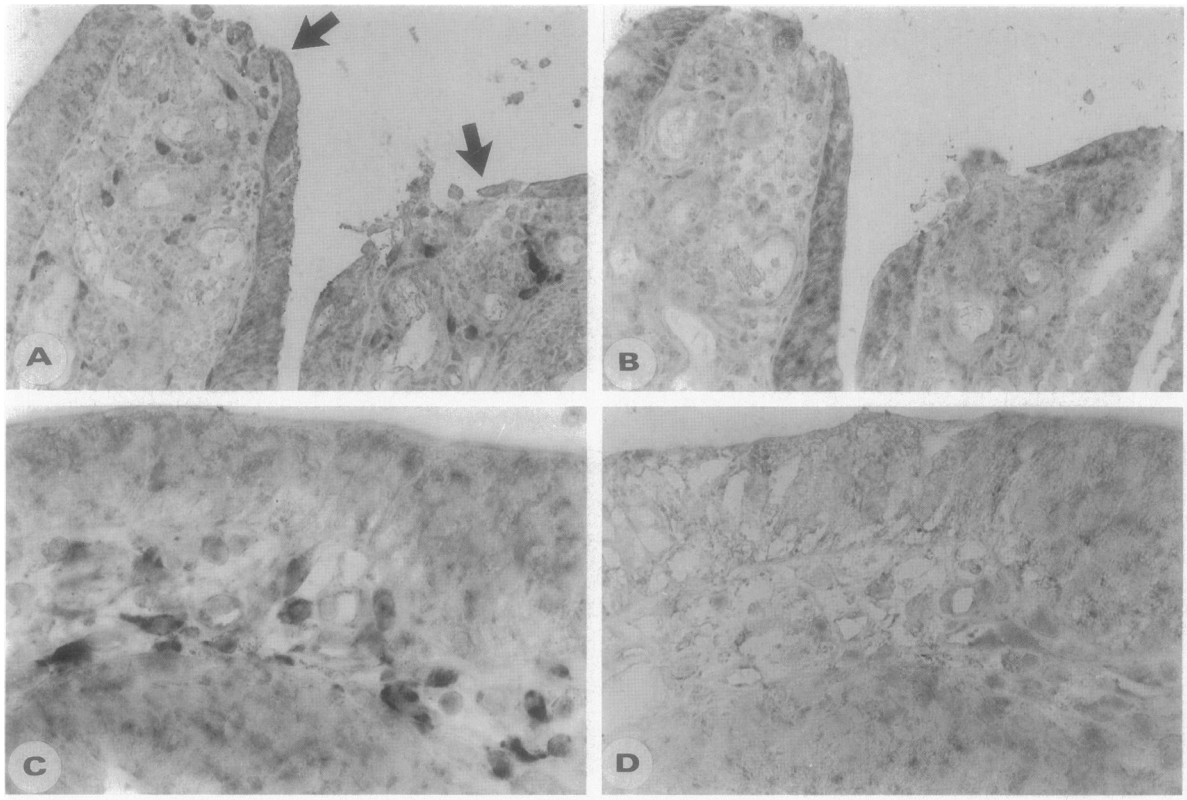

Figure 2. *Cellular expression of uPA mRNA in adenomas. Nonradioactive* in situ *hybridization was performed on tissue sections, as described in Materials and Methods.* **A** *and* **B**: *Views of a peripheral region hybridized with a digoxigenin-labeled uPA antisense* (**A**) *or control sense* (**B**) *probe and counterstained with methyl green. uPA mRNA-expressing cells* (**arrows**) *localize at the tip of adenomatous tubules in the lamina propria. Magnification, ×200.* **C** *and* **D**: *Detailed views of a section from the same adenoma. Clusters of uPA-positive cells are found in the stroma underlying the epithelium* (**C**). **D** *is the control adjacent section using the sense uPA probe. Methyl green counterstain; magnification, ×400.*

adjacent adenomatous epithelium, a total of 35 areas taken from 15 adenomas, each containing clusters of 10 to 20 uPA-positive stromal cells, were delineated and mapped over adjacent H&E-stained sections. A total of 57 areas lacking uPA mRNA expression were randomly taken as controls. A total of 92 areas (2 × 2 mm per area and 4 to 8 areas per section of adenoma) were analyzed and scored for epithelial cell dysplasia independently by three pathologists, as described in Materials and Methods. A complete agreement between the three reviewers was found in 88 of the 92 areas. As determined by the κ statistics, the level of agreement between L.G. and L.M., L.G. and P.C., and L.M. and P.C. was significantly better than by chance (L.G./L.M., $P < 0.0001$; L.G./P.C., $P < 0.001$; L.M./P.C., $P < 0.0001$).

Table 1 shows that 13 of 35 areas (37.8%) containing uPA mRNA-positive cells and 3 of the 57 negative areas were found to express high-grade dysplasia. The probability of finding uPA mRNA-expressing cells was significantly higher in areas with high-grade epithelial cell dysplasia ($P = 0.0001$, Pearson's χ^2 test) (Table 1). These findings indicate that, in a majority of adenomas, high-grade dysplasia is found in the epithelium overlining the stromal regions containing uPA-expressing cells.

Immunostaining of the uPA Receptor in Adenomas

Frozen sections from 12 samples were analyzed for specific uPAR immunoreactivity. In a majority of cases (9 of 12), the uPAR protein was associated with various cell types infiltrating the lamina propria. Receptor-reactive cells were found both scattered in the stroma over the entire section area and, conspicuously, as groups of cells accumulating under the adenomatous epithelium in peripheral regions (Figure 5A, arrow). In parallel, immunoreactivity for the MAC387 antigen performed on adjacent sections identified stromal inflammatory cells displaying a tissue distribution similar to that of uPAR-reactive cells (not shown). Besides monocytic/macrophage-like cells exhibiting a pronounced reactivity, spindle-shaped fibroblasts scattered in the stroma or closely

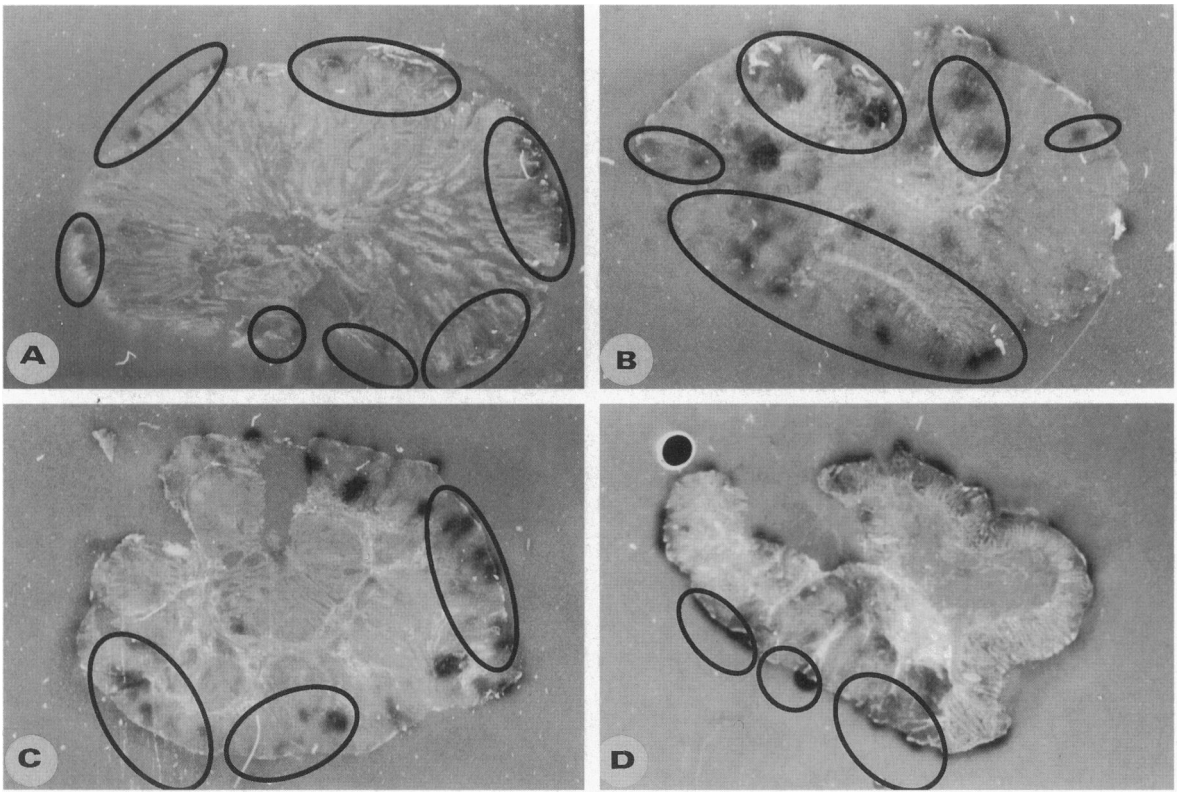

Figure 3. *Mapping of uPA activity and uPA mRNA:* **A** *to* **D**: *Histozymograms prepared from four individual adenomas and revealed after 1 hour of incubation at 37°C in the presence of plasminogen and anti-tPA antibodies. uPA-related activity is shown as circumscribed dark areas. Regions where clusters of uPA mRNA-positive cells were found in hybridized sections have been circled over the corresponding histozymograms. Magnification, ×4* (**A** *to* **D**).

apposed to the epithelium (pericryptal fibroblasts) were also found to express uPAR. A weak luminal staining was observed in some of the vessels lining the crypts. In 3 of 12 specimens, a marked uPAR reactivity was also associated with epithelial cells in the crypts. As illustrated in Figure 5B, the 3936 monoclonal antibody decorates the lateral side and the intercellular spaces of apical, mostly mucin-producing, colonic cells. Epithelial uPAR immunostaining was distributed over a majority of the crypts (2 cases) or confined to a few crypts (1 case).

Cellular Expression of PAI-1 mRNA

PAI-1 mRNA reactivity was found in 20 of 25 adenomas. As shown in Figure 6, the antisense probe (Figure 6A) revealed PAI-1 mRNA-expressing cells in vascular structures and extravascular cell types localized within the stromal compartment of adenomas. PAI-1-positive cells were identified as endothelial cells lining the wall of small arterioles (Figure 6, C, E, and F) or venules (Figure 6D). In addition, perivascular cells and cells scattered in the stroma were reactive for the antisense PAI-1 probe. Some larger cells lining the sections of the vessel walls exhibited a strong hybridization signal for PAI-1 (see Figure 6, C, E, and F, arrows). Consecutive sections probed by immunocytochemistry for α-sm-1 showed that these large elongated perivascular PAI-1-positive cells also expressed the differentiation marker, identifying them as vascular smooth muscle or myofibroblastic cell types (not shown). Other PAI-1-expressing cells, located in the stroma or in the vicinity of vessels, were faintly positive or negative for the α-sm-1 antigen. Cells reactive for both PAI-1 and the MAC387 antigen were not detected. In contrast to uPA, cellular PAI-1 mRNA expression was diffusely distributed within the peripheral and central regions of adenomas. The signal-to-noise ratio indicates that epithelial cells do not express PAI-1 mRNA when applying antisense or sense probes (Figure 6). As PAI-1-expressing cells were distributed diffusely in sections of adenomas, a selection of areas positive or negative for PAI-1 mRNA could not be achieved. The majority of the 92 areas scored for uPA and dysplasia contained PAI-1-expressing cells in the stroma. Therefore, no correlation could be estab-

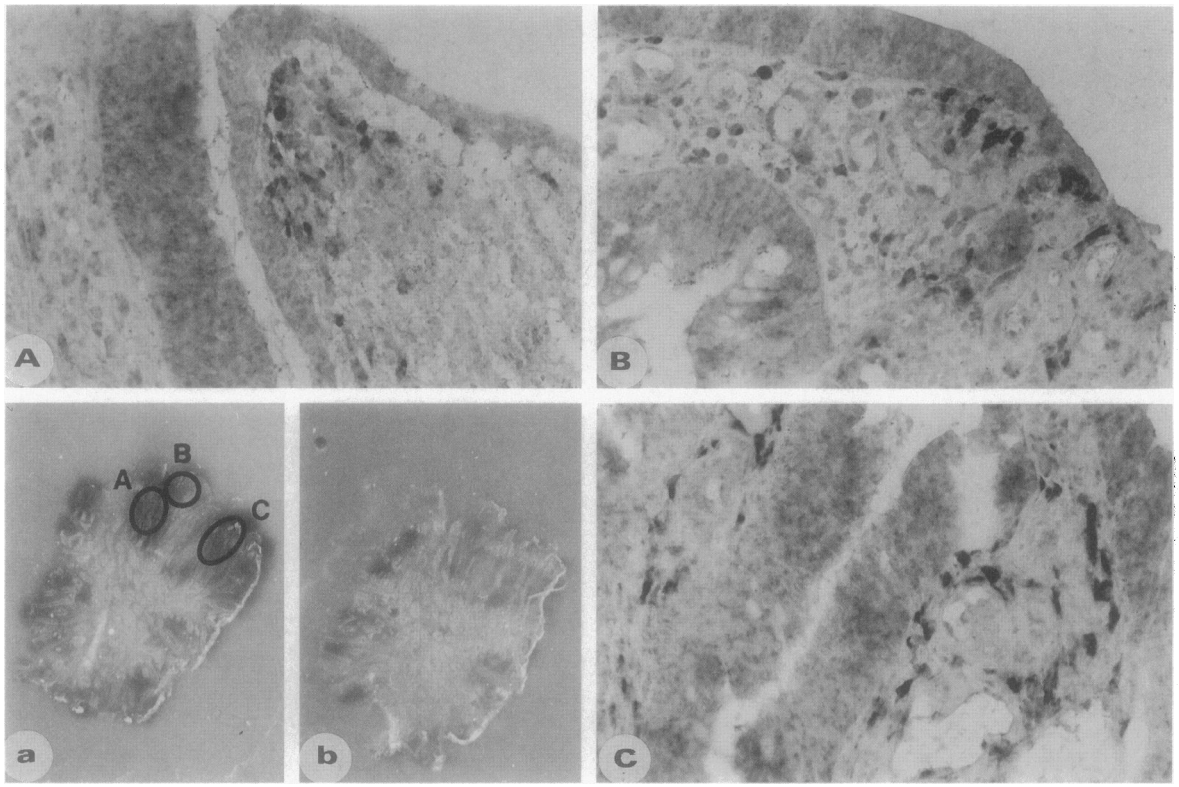

Figure 4. *Correlation between uPA mRNA signal and uPA activity.* **a** *and* **b**: *Histozymograms as revealed with plasminogen* (**a**) *and with plasminogen and amiloride* (**b**) *in the overlay. Three areas of caseinolysis, not developed in the presence of amiloride* (**b**), *are uPA-mediated and are circled and labeled as* **A, B,** *and* **C.** *These areas correspond in adjacent sections hybridized for uPA mRNA to foci of stromal uPA-positive cells shown in* **A, B,** *and* **C.** *uPA-positive cells appear to accumulate underneath the adenomatous epithelium in the lamina propria. Hybridized sections are counterstained with methyl green. Magnification,* ×*200* (**A** *to* **C**).

lished between PAI-1 mRNA expression and the distribution of epithelial cell dysplasia.

Discussion

Clinical and experimental evidence associates the altered expression of plasminogen activators, in particular uPA, with the invasive properties of malignant tumor cells.[5,29]

In the present study, we assessed the contribution of components of the PA/plasmin system in serial sections of colorectal adenomas combining immunocytochemistry, histozymography, and nonradioactive *in situ* hybridization with a precise mapping of dysplasia grade. Our results demonstrate first a prevalence of uPA-mediated caseinolytic activity in the majority of adenomas (23 of 25). This catalytic activity is mainly expressed within peripheral regions of adenomas and is derived from the stromal compartment. In contrast, tPA-mediated caseinolytic activity is scattered within central regions of adenomas and associated with vessels. Our findings assessing net PA-mediated catalytic activity in tissue sections are in agreement with previous studies reporting, in tissue extracts, an increase of uPA activity in colorectal adenomas when compared with adjacent mucosae.[14–16]

Analysis of uPA mRNA in adenomas reveals that transcripts are expressed focally in stromal cells adjacent to the adenomatous epithelium. These results

Table 1. *Dysplasia Grade and uPA mRNA Expression in 92 Microscopic Areas from Adjacent Sections of 15 Adenomas*

Grade of epithelial dysplasia	Number of areas	
	With uPA mRNA-expressing stromal cells	Without uPA mRNA-expressing stromal cells
High	13	3
Low	22	54

Dysplasia grade was scored on H&E-stained sections serial to those prepared for nonradioactive *in situ* hybridization for uPA mRNA. Areas of mild and moderate epithelial cell dysplasia were categorized as low grade, and severe dysplasia and *in situ* carcinoma were categorized as high grade. The Pearson's χ^2 test was used to validate the correlation between the grade of dysplasia and the presence of uPA mRNA ($P = 0.0001$).

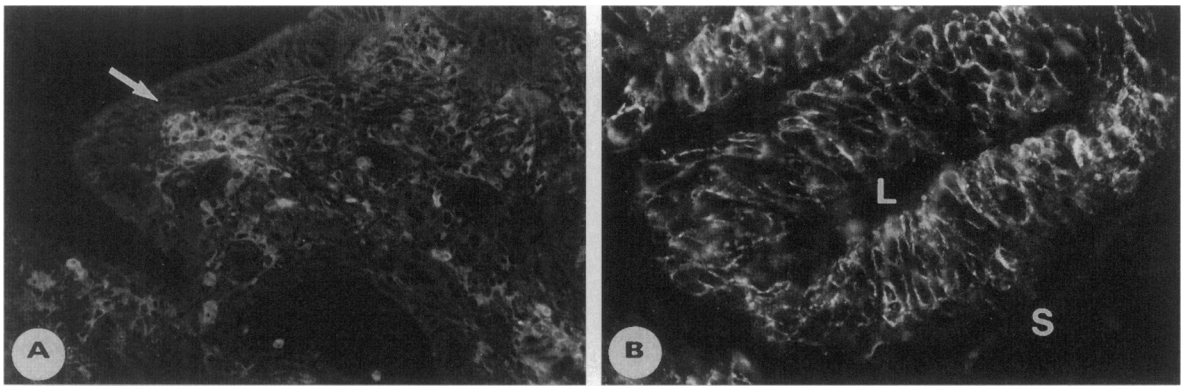

Figure 5. *Immunostaining of uPAR, using a monoclonal antibody and a biotin-avidin-FITC technique as described in Materials and Methods.* **A:** *A group of stromal cells reactive for uPAR is located beneath the epithelium at the tip of an adenomatous crypt* (**arrow**). *Additional uPAR-positive cells are scattered in the lamina propria.* **B:** *A minority of adenomas expresses the uPAR antigen in the epithelium. Staining is seen in the apical region lining the lateral side of epithelial cells and the intercellular space. L, crypt lumen; S, stromal side. Magnification, ×200* (**A**) *and ×270* (**B**).

suggest that the areas of epithelium associated with enhanced uPA gene expression in the stroma may contain precursors of more advanced lesions and that uPA could contribute to their progression. There is experimental evidence to indicate that the level of uPA expression could contribute to early stages of tumor progression. Indeed, in a model of dimethylhydrazine-treated rats, enhanced uPA production and plasmin generation are features of both the premalignant and the malignant colonic lesions.[30]

Our inability to detect a pattern of uPA mRNA expression in all samples examined in the present study may reflect heterogeneity among adenomas. Naitoh et al[31] showed in samples of colon cancer that the sensitivity of the uPA riboprobes labeled with digoxigenin-UTP was comparable to that of techniques using radioactivity. In our study and in contrast to uPA, the tPA probe did not produce a signal above the noise level. A decrease in tPA expression from normal mucosa to adenoma was previously reported.[14,15,32] Recent findings obtained in our laboratory using tissue extracts of frozen sections substantiate the down-regulation of tPA activity and antigen from adjacent mucosa to adenoma. (P. Protiva, I. Sordat, P. Chaubert, E. Saraga, C. Trân-Thang, B. Sordat, A. L. Blum, and G. Dorta, submitted for publication). Both tPA activity and antigen were found markedly reduced, suggesting a mechanism independent in part of PA inhibitors.

Our results show that stromal foci of uPA mRNA-reactive cells superimpose with areas of uPA-related caseinolytic activity. uPA is known to be expressed by a variety of cell types.[33] Besides fibroblast-like cells, uPA-reactive cells contribute to the inflammatory monocyte/macrophage cell population stained by the MAC387 (this study) or the CD68[21,34] monoclonal antibodies. In colorectal carcinomas, uPA mRNA[11,21] or uPA protein[19,35] was identified in stromal fibroblast-like cells surrounding invading tumor cell nodules. Other investigators have also shown in colon cancer that uPA can be detected both in fibroblasts and in neoplastic cells at the invasive edge of the tumor.[31,36] Our data in adenomas support uPA gene expression being confined to the focal accumulation of stromal fibroblast-like cells and of a subpopulation of inflammatory cells.

A significant correlation was found between the distribution of the cellular uPA mRNA signal in the stroma and the occurrence of high-grade dysplasia in the adjacent epithelium. In adenomas, high-grade dysplasia categorizes the most severe cytological abnormalities and glandular disorganization,[25] features viewed as markers of the potential for malignant transformation. Interestingly, severe dysplastic changes found in inflammatory bowel diseases[37] are also associated with alterations in the PA/plasmin system such as an increase in the level of uPA and a decrease in tPA.[38] Earlier studies using tissue homogenates showed no significant correlation between PA content and the grade of dysplasia.[14,16] There has been only one previous indication that the occurrence of dysplastic cells in adenomas may correspond to the high uPA antigen level found in tissue extracts.[15] In contrast to our findings, this correlation could not be established significantly in a recent analysis of colorectal adenomas.[21]

uPA can bind to a cell surface receptor, uPAR, which in malignant cells can be occupied by paracrine or autocrine mechanisms. In addition to the role of uPA/uPAR in extracellular proteolysis, a number of studies have suggested that uPAR may as well be involved in regulating cell adhesion and migration (reviewed in Ref. 39). In contrast to colorectal carcinoma, the analysis of uPAR expression

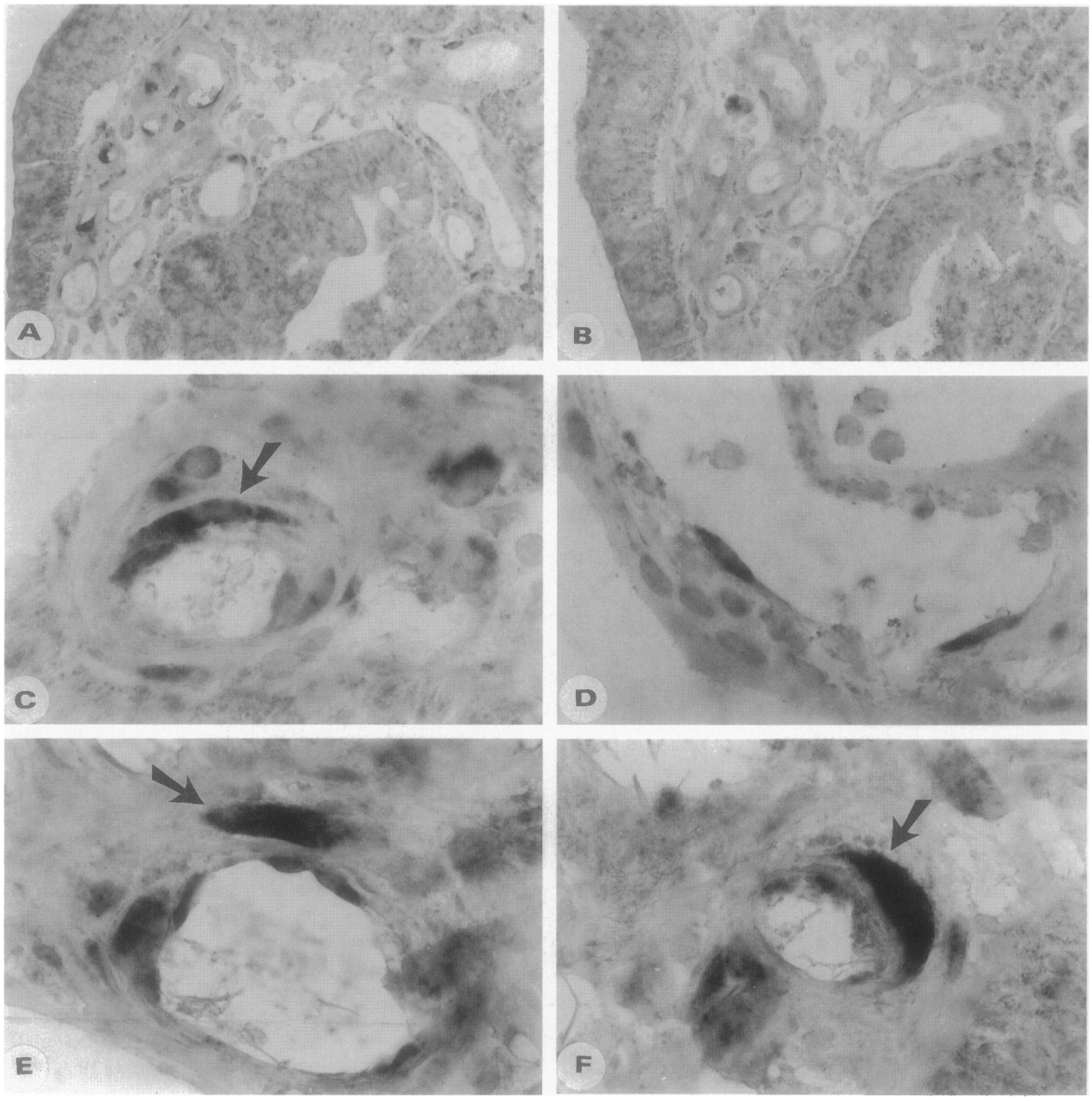

Figure 6. *Cellular expression of PAI-1 mRNA.* **A** *and* **B:** *Peripheral areas from sections of adenomas taken at low magnification and hybridized with the antisense* (**A**) *or control sense* (**B**) *PAI-1 digoxigenin-labeled probes. The PAI-1 signal is restricted to vascular endothelial cells and few stromal cell types. Methyl green counterstaining; magnification, ×200.* **C** *to* **F:** *Examples, detailed from several adenomas, of both luminal endothelial cells and of large and elongated perivascular PAI-1 mRNA-positive cells* (**arrows**). **C, E,** *and* **F:** *Arterioles* **D:** *Venule. Some extravascular stromal cells are also PAI-1 positive* (*in* **C**). *All enlarged fields are taken from hybridized sections stained with methyl green. Magnification, ×800.*

in adenomatous lesions has received a limited attention. Homogenates of polyps contained values of uPAR intermediate between normal mucosa and carcinomas.[40] In colorectal tumor extracts, high uPAR antigen is associated with a poor prognosis and identifies a group of high-risk patients.[18] In this study, immunostaining shows, in a majority of adenomas (9 of 12), that uPAR protein is expressed by various types of cells in the lamina propria. Interestingly, these stromal cells tend to accumulate as clusters at the tips of crypts where foci of uPA mRNA-reactive cells and uPA-mediated caseinolysis are co-localized. Using [^{3}H]cRNA probes, a recent study reported the co-expression of uPA and uPAR mRNAs in stromal cells both in carcinomas and in adenomas at the periphery of the lesions.[21] Earlier results from kinetic studies had shown that abnormal epithelial proliferation occurs in familial polyposis adenomatous crypts near the luminal surface and was sug-

gestive of a retrograde migration of cells downward in the crypts.[41] Interestingly, this shift of proliferative activity to the upper region of the crypts has also been reported in ulcerative colitis.[42] As shown here, high-crypt regions in adenomas correspond to the stromal accumulation of uPA mRNA near the dysplastic epithelium. Moreover, both uPA-related caseinolytic activity and the uPAR protein can be found at the periphery together with MAC387- or CD68-positive inflammatory cells. It is accepted that both uPA and uPAR expression is required for assembling a proteolytic system active at the cell surface. The uPA/plasmin system may participate in the remodeling of extracellular matrices in regions of the lamina propria and contribute to epithelial cell migration. The significance of uPAR epithelial staining found in a minority of adenomas (3 of 12), together with stromal reactivity, remains to be elucidated. In this context, a signal for uPAR mRNA has been detected in the mouse over the epithelium of the gastric mucosa. uPAR immunostaining was also reported on the duct epithelium in benign breast tumors and in the intestinal epithelium adjacent to colon cancer.[43]

In contrast to that of uPA, the signal for PAI-1 mRNA was diffusely distributed, suggesting that PAI-1 expression is independently regulated.[44] In the colonic normal mucosa, pericryptal fibroblasts express α-sm-1 and proliferate in conjunction with hyperplastic adenomatous epithelia.[45] In breast cancer, interstitial fibroblasts respond to the tumor environment by a partial conversion to a myogenic phenotype.[46] The morphological appearance of the PAI-1/α-sm-1 double-reactive cells and their association with the vascular wall in adenomatous stroma identify these cells as vascular smooth muscle cells. Additional PAI-1-expressing cells, also reactive for α-sm-1 and found in the stroma independent from the vessels, may correspond to myofibroblasts such as those reported in adenomas.[45]

As shown in cutaneous squamous carcinomas,[27] up-regulation of the PAI-1 gene may be linked to newly formed vessels. There is experimental evidence to indicate that PAI-1 expression is induced in migrating endothelial cells,[47] and previous studies have localized PAI-1 protein to the vascular basement membrane, the perivascular matrix, and in tumors, to regions with endothelial cell proliferation. In colon cancer, PAI-1 mRNA has been found restricted to endothelial cells in the tumor stroma.[20,21]

Patterns of uPA/PAI-1 expression different from those reported in colon cancer have been observed in cutaneous squamous carcinoma,[27] melanomas,[48] and glioblastoma.[49] To what extent these patterns reflect the occurrence of similar or different factors regulating uPA/PAI-1 expression in colonic adenomas remains to be investigated.

Focal interactions can take place between a dysplastic epithelium and cells in the lamina propria, with an increased production of uPA in the stroma. A likely explanation is that dysplastic proliferating cells elaborate chemokines or growth-factor-like activity that stimulates the expression of uPA/uPAR in surrounding stromal cells. The question of whether cytokines and/or growth factors, ie, transforming growth factor-β, produced by transformed cells and known to induce the production of uPA in certain cell types are involved in these interactions needs to be addressed. Transforming growth factor-β is known to play a role in controlling the growth of human colonic epithelium, and as cells progress from benign to more malignant types, they show a reduced responsiveness to the inhibitory effect of transforming growth factor-β.[50]

Our results suggest that an imbalance between a focal expression of uPA and uPAR and the diffuse tissue distribution of PAI-1 will result in regional variations in net uPA-mediated proteolytic activities, such as those observed at the periphery of adenomas. Extracellular proteolysis restricted to high-crypt regions may provide a microenvironment permissive for both epithelial cell migration, as indicated by results from kinetic studies, and the activation of latent or matrix-bound growth factors. Assuming that plasmin generation occurs in response to abnormal epithelial cell proliferation, a shift of the replicating zone to high-crypt regions would be consistent with uPA-mediated activity being detected in the periphery, as observed here and especially in the vicinity of dysplastic epithelial cells. Mediators of these complex interactions, including the recruitment of inflammatory cells *in situ*, have not yet been identified in adenomas. Additional investigations are needed to explore these issues and to identify the clinicopathological significance of plasmin generation, matrix remodeling, and inflammation associated with the development and the progression of colorectal adenomas.

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