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ORIGINAL PAPER

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Plasminogen- and colony-stimulating factor-1-associated markers in bladder carcinoma: diagnostic value of urokinase plasminogen activator receptor and plasminogen activator inhibitor type-2 using immunocytochemical analysis

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Abstract The expression of plasminogen- and colony-stimulating factor-1-associated markers was first investigated in seven bladder carcinoma cell lines and in 15 primary bladder tumors using RT-PCR (mRNAs), zymography (protein activity), ELISA and immunocytochemistry analysis (ICC) (protein levels). The mRNAs expression, the activity and the levels of the secreted proteins were not informative. Only urokinase plasminogen activator receptor (uPA-R/CD87) and possibly plasminogen activator inhibitor type-2 (PAI2) antigen expression at the cellular levels seem to be useful markers. uPA-R antigen expression correlated with the secretion of hepatocyte growth factor (HGF) ($P=0.016$) and the motility of the bladder tumor cells ($P=0.014$), two markers associated with a poor prognosis in bladder carcinoma. To validate our technique and confirm these preliminary results, uPA-R and PAI2 antigen expression was determined in the imprints from 129 resected bladder carcinoma fragments. uPA-R correlated with the grade ($P=0.002$), tumor invasion ($P=0.003$) and the ploidy ($P=0.05$) of the bladder carcinomas and with the low overall survival ($P=0.045$) of the patients. PAI2 correlated only with the stage ($P=0.02$) and low overall survival ($P=0.038$). We conclude that in bladder carcinomas, studying the transcripts of PAs, PAIs, CSF-1 and its receptor, as well as measuring their concentration or activity in culture supernatants was of no clinical interest in terms of diagnostic or prognostic value. Only the ICC of uPA-R, which correlated with the major histopathological parameters of tumors and the low overall survival, proved to be a diagnostic and prognostic marker.

Keywords Transitional cell carcinoma · uPA-R expression · Immunocytochemistry

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Introduction

During several steps of the metastatic process, proteolytic degradation of the extracellular matrix (ECM) is required. Among the proteases that play an active role in the degradation of ECM are the plasminogen activation system [8]. This includes the serine protease tissue type (tPA) and urokinase type (uPA) plasminogen activators. uPA activity is influenced by the cell surface urokinase receptor (uPA-R/CD87). Binding of the protease uPA to uPA-R focuses proteolytic action to the surface of the tumor cells expressing this receptor. The uPA/uPA-R complex converts enzymatically inactive plasminogen into active plasmin that degrades proteins of the ECM, thus facilitating tumor cell invasion and metastasis. The proteolytic activity of the serine proteases in the ECM is controlled by two inhibitors, members of the serpin family designated plasminogen activator inhibitors 1 and 2 (PAI1 and PAI2), secreted by multiple cell types (endothelial cells, tumor cells, macrophagic cells, etc). These inhibitors may bind to the cell associated uPA/uPA-R complex that forms an enzymatically inactive trimeric complex (uPA/uPA-R/PAI) [31]. Strong

correlations have been reported between PAs, uPA-R or PAI1 and a poor prognosis and metastasis for several human carcinomas [1]. In contrast, the role of PAI2 in malignant disease is controversial: some investigators have shown that PAI2 can inhibit cancer cell invasion and metastasis [22] whereas others have shown that the presence of high levels of PAI2 antigen in cancer tissue is a poor prognostic factor [12].

The colony-stimulating factor-1 (CSF-1, also called macrophage colony-stimulating factor (M-CSF)) and its receptor (CSF-1R, the product of the *c-fms* proto-oncogene, with tyrosine kinase activity) are expressed by multiple cell types, either constitutively or after induction by various agents and cytokines that promote numerous biological functions [11, 27]. Their expression is associated with a poor prognosis in ovarian and breast cancer due to their effect on the promotion of metastasis. This may explain, in part, why bone is a preferential site of prostatic carcinoma metastases [3, 20, 30, 33]. As several investigations support the idea that uPA is a CSF-1 inducible gene [33], it is logical to hypothesize that its expression may exert a negative impact on bladder cancer prognosis.

The aim of this study was first to evaluate the expression of the PAs, PAIs, uPA-R, CSF-1 and its receptor in seven bladder carcinoma cell lines (BCCLs) and in 15 primary bladder tumors using reverse transcription polymerase chain reaction (RT-PCR), zymography (plasminogen activator activity), ELISA (secreted antigen levels), and immunocytochemistry (ICC) (cellular antigen levels). Their expression pattern was analyzed in several culture conditions and compared with either the grade of the BCCL or the grade and the stage of the bladder tumor. Only uPA-R and PAI2 antigen expression correlated with the grade of the bladder tumor cells ($P < 0.05$). Next, the expression of both uPA-R and PAI2 antigens was investigated at the cellular level, in tumor imprints from 129 patients with primary bladder carcinoma using the ICC technique. The aim of the secondary study was to confirm the preliminary results and to evaluate the clinical relevance of uPA-R and PAI2 antigen expression in patients with newly diagnosed bladder tumor and to correlate their antigen expression with clinicopathological parameters.

Materials and methods

Bladder carcinoma cell lines

The seven human adherent urothelial cell lines [RT4 (G1), RT112 (G2), CHA 89, T24, J82S, DAG-1 (G3), and TCCsup (G4)] were maintained in culture in 25-cm^2 flasks with RPMI 1640 medium with 10% inactivated fetal calf serum (FCS) (Gibco BRL, Eragny, France), antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml) and L glutamine (2 mM) (Boerhinger, Meylan, France). Optimal growth of the bladder tumor cells was determined when FCS varied from 0 to 10% (v/v). Total cell number and the percentage of apoptotic cells were determined. After 48 h of optimal culture (1% FCS), the cells were used to determine the expression of both uPA-R antigen (immunological method) and mRNAs (PCR method). The 48 h culture supernatants were harvested to measure the plasminogen activator activity (PAA) by zymography and the PA, PAI, and CSF-1 antigen levels by ELISA.

Bladder cancer patients

Preliminary experiments

Tumor cells were obtained from resection specimens of 15 patients with primary bladder carcinoma. The grade (G) and the tissue invasion (pT) of tumors were determined from histological sections according to the U.I.C.C. classification: well-differentiated (G1), less differentiated (G2) and poorly differentiated (G3) tumors; tumors restricted to the epithelium (pTa), invading either the lamina propria (pT1), or the superficial muscle layer (pT2), or the juxtavesical tissues (pT3). We studied nine pTa, two pT1, four pT2, four G1, four G2, and seven G3 tumors. Carcinoma cells were obtained after the mechanical dissociation of the tumor fragment, washed in RPMI 1640 medium, then cultured in RPMI 1640 with 1% FCS. After 48 h of culture, the cells and the culture supernatants were used as described above (bladder cancer cell lines).

Validation of preliminary results

Imprints from bladder patients were made from the resection specimens from 129 patients (mean age, 67 years; range, 38–92 years; 108 males and 21 females) with primary bladder carcinoma. None of the patients had previously received irradiation, chemotherapy, or BCG therapy. The carcinomas were classified as follows: 69 pTa, 36 pT1, 20 pT2, 4 pT3, 41 G1, 36 G2 and 52 G3.

Drugs and reagents

Mouse monoclonal antibodies were purchased from Biopool (Umeä, Sweden) (PAI2), American Diagnostic (Greenwich, Conn., USA) (uPA-R/CD87), and Santa Cruz Biotechnology (Santa Cruz, Calif., USA) (CSF-1R/CD115). ELISA kits were from Biopool for uPA, tPA, PAI1, and PAI2 determinations and from R and D system Kit (R and D System, Minneapolis, Minn., USA) for HGF determination.

Assessment of cell ploidy and apoptosis

The cell DNA content (CycleTest PLUS/DNA reagent kit: Becton Dickinson, San Jose, Calif., USA) allowed us to determine the ploidy of tumor cells and their percentage in the sub-G0 fraction of the cell cycle. Apoptosis was measured using the Vybrant Apoptosis Assay kit (Molecular Probes, Eugene, Ore., USA). Both were performed by flow cytometry (FACScalibur, Becton Dickinson) and blood lymphocytic cells from healthy donors were used as normal diploid cells.

SDS-PAGE zymography

SDS-PAGE zymography was used to identify the molecular species of plasminogen activators present in the culture supernatants [13]. Molecular weight calibration was performed with molecular weight tPA (68 kDa) and uPA (54 kDa and 33 kDa) standards migrating in the same conditions.

Immunological analysis

On BCCLs, cell-associated uPA-R and CSF-1R antigens were analyzed by indirect immunofluorescence using anti-uPA-R (American Diagnostic) or anti-CSF-1R (Santa Cruz Biotechnology). Fluorescence intensity was quantified by flow cytometry (FACScalibur). CSF-1 levels were measured in 48-h culture

supernatant by a specific ELISA developed in one of our laboratories (detection limit at 10 IU/ml) [10]. The levels of the tPA, uPA, PAI1, PAI2 and HGF antigens were measured with ELISA kits according to the manufacturer's instructions. uPA-R and PAI2 antigen expression at the cellular level were determined using the peroxidase anti-peroxidase (PAP) technique. Cytocentrifuged smears (from bladder tumor cultures) and imprints (from resection specimens) were fixed with acetone (5 min) before immunocytochemistry using the DAKO Envision+ System HRP kit (DAKO, Carpinteria, Calif., USA).

Molecular analysis

Total cellular RNA was isolated using TRIzol (GibcoBRL). The integrity of the RNA was confirmed by using GAPDH-specific primers after reverse transcription. All of the reverse transcription and PCR reactions were performed as previously described [5] using specific primers (Eurogentec, Liège, Belgium) (Table 1).

Transwell motility assay

Motility of bladder tumor cells from the seven BCCL was carried out using a Transwell chamber equipped with an uncoated polycarbonate filter membrane (8 μm pores, Corning Costar, Cambridge, Mass. USA). Tumor cells were plated at 10^5 cells/cm^2 in the upper compartment of the chamber and cultured for 24 h. Motility, expressed as a percentage, was the total number of cells that had moved through the filter divided by the total number of cells in the chamber × 100.

Statistical analysis

Statistical analysis was performed with the Statview software package. In the BCCLs and bladder tumors, the Mann-Whitney U-test was used to determine the relationship between protein levels in the culture supernatant and the grade of the cells. In the population of 129 bladder cancer patients studied, the continuous variables uPA-R and PAI2 were transformed into binary variables with a cut-off point at 5%. The statistical significance of the differences (significance level set at $P < 0.05$) was then analyzed using χ^2 tests to compare uPA-R and PAI2 ICC-positive or -negative tumors with their histopathological parameters (grade, stage, ploidy, tumor number and size, recurrence number, relapse-free survival). Kaplan-Meier survival curves were compared with the log-rank test.

Results

Determination of useful markers

After initial tests in which FCS was used at increasing concentrations (0%, 0.1%, 1% and 10%), we used it at 1% (v/v) in RPMI 1640 culture medium, since this concentration allowed a reproducible growth curve and the production of proteins with less than 20% apoptotic cells (instead of >50% in 0.1% and FCS-free culture medium) as determined by the Annexin V/FITC test.

Plasminogen-associated marker

Bladder carcinoma cell lines

The transcripts of the fibrinolysis markers uPA, tPA, uPA-R, PAI1 and PAI2 were detected by RT-PCR. All seven cell lines expressed uPA, tPA, PAI1 and uPAR mRNAs while only three (DAG-1, T24 and TCCsup) expressed PAI2 mRNA. When measured using ELISA, both the PAs (uPA and tPA) and PAI1 antigens were present in all of the BCCL culture supernatants whereas only DAG-1, T24 and TCCsup secreted detectable levels of PAI2. uPA-R antigen was expressed only on the G3+G4 grade BCCL (Table 2). Statistical analysis showed that the antigen levels secreted, as determined using ELISA, did not correlate with the BCCL grade, whereas uPA-R antigen expression, determined using immunofluorescence, did ($P = 0.02$) (Table 2). Zymography showed that PAs (uPA: 54 kDa and tPA: 68 kDa) were biologically active and were released together with

Table 1. Oligonucleotide primers for RT-PCR

Target	Primers	Size of the PCR product (bp)	References
GAPDH	5′-CCAGCCGAGCCACATCGCTC-3′ 5′-ATGAGCCCCAGCCTTCTCCAT-3′	359 bp	
uPA	5′-AGAATTCACCACCATCGAGA-3′ 5′-ATCAGCTTCACAACAGTCAT-3′	474 bp	[18]
uPA-R	5′-TTACCTCGAATGCATTTCCT-3′ 5′-TTGCACAGCCTCTTACCATA-3′	455 bp	[18]
tPA	5′-CTGCAGCTGAAATCGGATTCGT-3′ 5′-CTGATGATGCCCACCAAAGTC-3′	368 bp	[18]
PAI1	5′-ATGGGATTCAAGATTGATGA-3′ 5′-TCAGTATAGTTGAACTTGTT-3′	452 bp	[18]
PAI2	5′-GCGTGAAAAGCTAAACATTGG-3′ (bp 711–731) 5′-AGTTCTCCCTGTCATAACAC-3′ (bp 1123–1143)	432 bp	[18]
CSF-1-A, CSF-1-B	5′-CATGACAAGGCCTGCGTCCGA-3′ (bp 389–409) 5′-AAGCTCTGGCAGGTGCTCCTG-3′ (bp 762–782)	395 bp	[24]
CSF-1-C, CSF-1-E	5′-GCCGCCTCCACCTGTAGAACA-(bp 1547–1567) 5′-CAGCAAGAACTGCAACAACAGC-3′ (bp 501–522)	286 bp	[24]
c-fms P36 c-fms P37 c-fms Ex1	5′-GACAGAGTGTCCAAAAGCGTG-3′ (bp 17–37) 5′-ACGTGTGTCCAGTGTCCCGAT-3′ (bp 198–218) 5′-GCACGAAGTGAGAAGGTG-3′ (bp 41–51)	202 bp	[25]
c-fms P38 c-fms P47 c-fms Ex3	5′-TTGACATAGAGGTGGATGGCG-3′ (bp 585–605) 5′-AGCTGGTCGTGAAGCCAGGA-3′ (bp 386–405) 5′-ATGGCTCCAGCAGCATCCTCA-3′ (bp 488–508)	223 bp	[25]

Table 2. Markers expression in the seven bladder carcinoma cell lines. Protein expression was analyzed either using ELISA (secreted proteins) or using immunofluorescence (IF) (cellular proteins). ELISA results are expressed in IU/ml (CSF-1) and in ng/l (PAs and PAIs) for 10^6 cells and for 48 h. IF results are expressed in percentage of positive cells as determined using FACscalibur. The Mann-Whitney U-test was used for statistical analysis. NS, not significant; M: motility expressed in percentage of cells

	Secreted proteins (ELISA)					Cellular proteins (IF)			M
	tPA	uPA	PAI1	PAI2	CSF-1	PAI2	uPA-R	CSF1-R	
RT4/G1	0.05	22	9	<0.1	17	<1%	<1%	40%	0.28%
RT112/G2	3	0.1	84	<0.1	14	<1%	5%c7	5%	0.31%
DAG-1/G3	3	6	350	7	10	10%	30%	<1%	4.02%
T24/G3	10	2	52	2	38	<1%	40%	20%	10.89%
J82S/G3	0.1	0	80	<0.1	175	<1%	80%	<1%	4.86%
CHA89/G3	0.1	3	250	<0.1	133	<1%	45%	<1%	10.54%
TCCsup/G4	2	4	9	4	134	5%	70%	40%	2.03%
P for correlation with grade	NS	NS	NS	NS	NS	NS	0.02	NS	0.05

PAIs, as evidenced by the 100–110 kDa lytic band corresponding to PA/PAI complexes (Fig. 1). RT4, RT112, DAG-1, CHA89 and TCCsup exhibited related uPA fibrinolytic activity only at 54 kDa. For the J82S cell line, zymography confirmed the absence of PAs (uPA and tPA) activity in the culture supernatants (Fig. 1, column 9). When exogenous uPA was added to the J82S culture supernatant, a lytic band appeared at 100–110 kDa showing the formation of a PA/PAI complex (Fig. 1, column 10). This result indicated the presence of PAI in the J82S culture supernatant, confirming the ELISA determination (PAI1 = 80 ng/l) (Table 2). Motility of the tumor cells was investigated and the results were then analyzed in relation with the uPA-R antigen expression and the grade of the tumor cells. The low grade BCCL (G1/G2) did not express uPA-R antigen and did not migrate whereas the high grade BCCL (G3/G4) did both (Table 2). Statistical analysis showed a correlation between the uPA-R antigen expression (>10%) and the cell motility (> 0.5%) ($P = 0.014$).

Bladder tumors

uPA, tPA, uPA-R and PAI1 mRNAs were present in all 15 bladder tumors, but PAI2 mRNA was only present in six. Its presence was not related to the grade, stage or ploidy of the tumor. At the protein level, all of the 15 bladder tumors secreted variable levels of uPA and PAI1 (Table 3). These results indicate that these two proteins were found predominantly in the bladder carcinoma cells; however there was no correlation between their antigen expression and the grade, stage or ploidy of the tumor cells ($P > 0.05$). tPA and PAI2 antigens were found in only ten and four supernatants from bladder tumors, respectively. Only tPA antigen expression correlated with the grade of the tumor cells ($P = 0.05$). The cellular expression of uPA-R and PAI2 antigen, as determined using the PAP technique, correlated with grade, stage and ploidy of the tumor (Table 3). Zymography analysis indicated that 12 of 15 bladder tumors showed a lytic band at 54 kDa, related to free uPA. Moreover, only four bladder tumors (Nos.: 2, 3, 5 and 15) exhibited fibrinolytic activity at both 54 and 33 kDa. Three of the 15 showed a 68-kDa lytic band (free tPA) and five showed a lytic band at 100–110 kDa (PA/PAI complex) (Fig. 2). Tumors 8 and 10 did not show a lytic band due to the low levels of PAs (see Table 3) confirming the low sensitivity of zymography. HGF protein levels were then investigated in the culture supernatant from ten bladder tumors using ELISA. Table 4 shows that all of the G3 grade bladder tumor cells secreted elevated HGF levels (> 250 ng/l) whereas all of the low grade bladder tumor cells secreted low levels (< 200 ng/l). Moreover, four of five G3 grade bladder tumors (Nos. 1, 2, 7 and 10) expressed uPA-R antigen at the cellular level (PAP technique), were aneuploid (A) and secreted high levels of HGF (> 250 ng/l) (Table 4). uPA-R antigen expression

Fig. 1. Zymographic analysis of culture supernatants from BCCL. After 48 h of culture in the presence of 1% FCS, culture supernatants from the seven bladder carcinoma cell lines were used for zymographic analysis. *Column 1*, tPA: 68 kDa molecular weight tPA; *column 2*, T-: negative control (RPMI 1% FCS), *column 11*: uPA: mixture of high (54 kDa) and low (33 kDa) molecular weight uPA

Table 3. Marker expression in the 15 bladder tumors. Protein expression was analyzed either using ELISA (secreted proteins) or using peroxidase anti-peroxidase technique (PAP). ELISA results are expressed in IU/ml (CSF-1) and in ng/l (PAs and PAIs) for 10^6 cells and for 48 h. PAP results are expressed in percentage of positive cells. The Mann-Whitney U-test was used for statistical analysis. PAP, peroxidase anti-peroxidase; NS, not significant

	Secreted proteins (ELISA)					Cellular proteins (PAP)	
	tPA	uPA	PAI1	PAI2	CSF-1	uPA-R	PAI2
pTa/G2/D	< 0.1	5	14	< 0.1	< 10	10%	< 1%
pTa/G2/D	7	16	7	< 0.1	11	25%	< 1%
pTa/G2/D	< 0.1	11	4	< 0.1	< 10	< 1%	< 1%
pT2/G3/A	3	15	40	< 0.1	< 10	60%	10%
pT1/G3/A	2	5	2	< 0.1	< 10	10%	7%
pT2/G3/A	13	14	19	32	< 10	25%	40%
pTa/G1/D	< 0.1	6	13	< 0.1	< 10	< 1%	< 1%
pT1/G3/A	4	0.2	4	12	20	15%	8%
pTa/G1/D	< 0.1	21	6	< 0.1	< 10	5%	< 1%
pTa/G1/D	0.7	3	11	< 0.1	< 10	< 1%	< 1%
pTa/G2/D	< 0.1	6	30	12	< 10	< 1%	< 1%
pTa/G3/A	10	4	0.2	< 0.1	< 10	< 1%	< 1%
pT2/G3/A	2	89	23	< 0.1	13	90%	58%
pT1/G3/A	22	77	4	< 0.1	41	7%	16%
pT2/G2/D	< 0.1	30	90	3	15	< 1%	< 1%
P for correlation with stage	NS	NS	NS	NS	NS	0.02	0.02
P for correlation with grage	0.05	NS	NS	NS	NS	NS	0.03
P for correlation with ploidy	NS	NS	NS	NS	NS	0.04	0.04

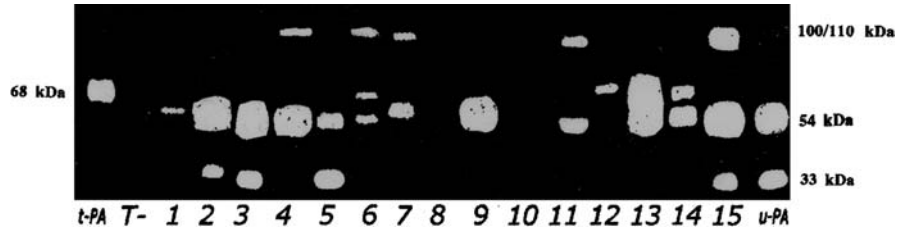

Fig. 2. Zymographic analysis of culture supernatants from bladder tumor cells. After 48 h of culture in the presence of 1% FCS, culture supernatants from the 15 bladder tumors were used for zymographic analysis. *Column 1*, tPA: 68 kDa molecular weight tPA; *column 2*, T-: negative control (RPMI 1% FCS), *column 3–17*: TU 1–TU 15; *column 18*: uPA: mixture of high (54 kDa) and low (33 kDa) molecular weight uPA

(> 5%) and HGF secretion (> 250 ng/l) were statistically correlated ($P = 0.016$).

CSF-1 and CSF-1R

Bladder carcinoma cell lines

Transcripts of the CSF-1 and *c-fms* (exon 1 and 3) were detected by RT-PCR in the seven cell lines (Fig. 3) which also produced CSF-1 in their culture supernatant as measured by ELISA. The three high-grade cell lines (J82S, CHA89 and TCCsup) produced high levels of CSF-1, 175, 133 and 134 IU/ml, respectively, while the G1 and G2 cell lines (RT4 and RT112) produced less than 20 IU/ml. However the two high grade bladder cell lines DAG-1 and T24 (G3 grade) secreted low levels of CSF-1: 10 and 38 IU/ml, respectively (Table 2). The CSF-1R antigen was present at the cell surface of 4%–40% of the cells in four out of seven BCCLs (RT4, T24, RT112, and TCCsup). Nevertheless, either the CSF-1 or the CSF-1R transcripts and their antigen expression did not correlated with the grade of the BCCL ($P > 0.5$).

Table 4. The relationships between uPA-R and HGF secretion, grade and ploidy of bladder tumors. HGF antigen expression was analyzed using ELISA and the results are expressed in ng/l for 10^6 cells and for 48 h. PAP results are expressed in percentage of positive cells. The Mann-Whitney U-test was used for statistical analysis. G: grade; A aneuploid; D: diploid

Characteristics of the tumor	HGF (ELISA)	uPA-R (PAP)
G3/A	640	25%
G3/A	520	7%
G1/D	102	0%
G1/D	70	0%
G1/D	58	0%
G3/A	250	0%
G3/A	805	15%
G1/D	140	0%
G1/D	160	0%
G3/A	248	6%

Bladder tumors

The exon 1 transcript of the *c-fms* was detected in all 15 tumors whereas only five bladder tumors secreted a detectable level of CSF-1 without relation to the grade, stage or ploidy of the tumor (Table 3).

Validation of the ICC technique

We first demonstrated that uPA-R and PAI2 antigens, preferentially expressed at the cellular level (membrane

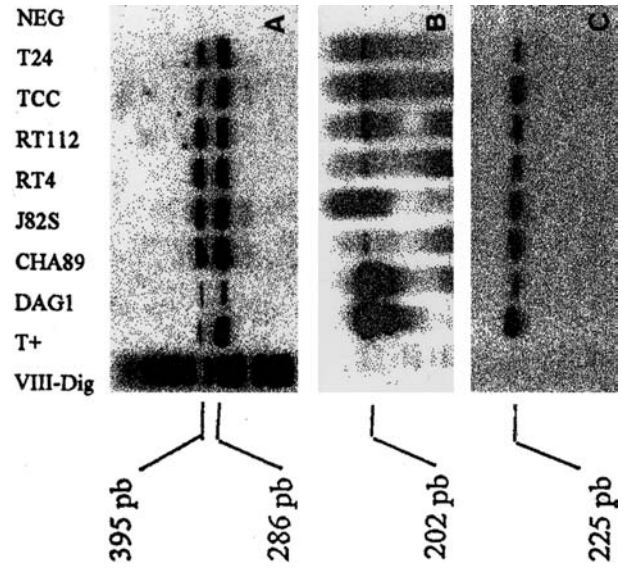

Fig. 3A–C. Expression of CSF-1 and *c-fms* mRNAs of BCCL. **A**: CSF-1; **B**: *c-fms* exon 1; **C**: *c-fms* exon 3; *Neg:* negative control; *T+*: positive Control; *VIII dig*: digoxygenin-labeled DNA molecular weight markers. CSF-1 and *c-fms* transcripts were detected by RT-PCR and Southern blot

and intracellular protein, respectively), correlated with the grade, stage and ploidy of the bladder tumor cells, whereas the other proteins (uPA, tPA, PAI1, CSF-1 and CSF1-R), did not (Tables 2, 3). In our preliminary experiment (seven BCCL and 15 bladder tumors), we also showed that uPA-R antigen expression correlated with the motility of the BCCL (Table 2) and the HGF antigen levels secreted by the bladder tumors (Table 4). To validate these results, we investigated both uPA-R and PAI2 antigen expression in the imprints from 129 bladder patients using the ICC technique. The uPA-R and PAI2 antigens were detected in 65 of 129 (50.3%) and 40 of 129 (37%) tumors, respectively, and their expression was correlated ($r = 0.517$, $P < 0.001$). Since uPA-R and PAI2, which were predominantly detected in carcinoma cells, were also present in stromal cells (macrophages, fibroblasts, and endothelial cells), only tumors with more than 5% labeled cells were considered to be positive. Using this threshold, 46 of 129 (35.6%) and 28 of 129 (21.7%) specimens were positive for uPA-R and PAI2, respectively. The uPA-R antigen expression correlated with the grade ($P = 0.002$), stage ($P = 0.003$), and ploidy ($P = 0.05$) of tumors but not with other clinicopathological factors (size and number of tumors, recurrence number, disease-free survival, age and sex) (Table 5). PAI2 antigen expression only correlated with the grade of tumors ($P = 0.005$) (Table 5). Moreover, positive uPA-R and PAI2 antigen expression was also associated with shorter survival: the 60-month overall survival rate of the positive group was significantly lower than in the negative group (Fig. 4).

Table 5. The relationship between uPA-R and PAI2 expression with histopathological parameters. The χ^2 test was used to analyze the difference in frequency between the histopathological parameter. F, frequency of positivity; D, diploid; A, aneuploid

	n	uPA-R (> 5%)		PAI2 (> 5%)	
		F	*P*	F	*P*
Grade					
G1 + G2	77	0.22	0.002	0.10	0.005
G3	52	0.56		0.38	
Stage					
pTa	69	0.23	0.003	0.16	0.10
pT1 + pT2	60	0.50		0.28	
Ploidy					
D (2n)	61	0.39	0.05	0.21	0.10
A (> 2n)	67	0.56		0.34	

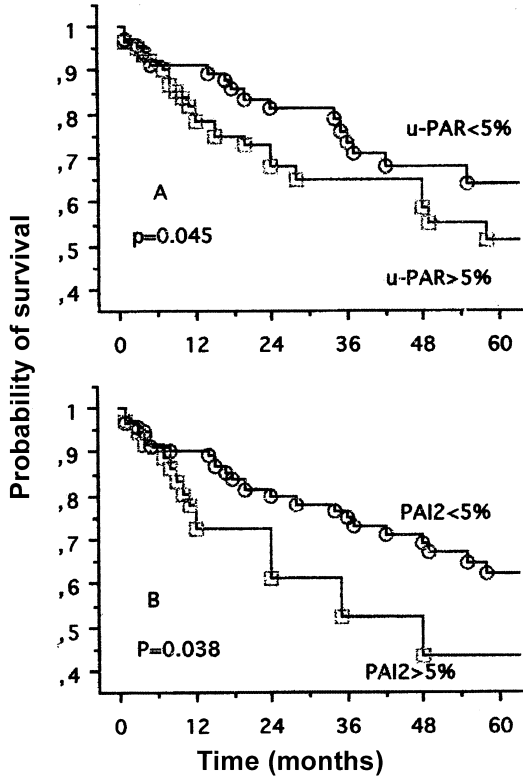

Fig. 4A, B. Sixty month survival of bladder cancer patients. Patients who had staining expression (> 5% of positive cells) for uPA-R **A** or PAI2 **B** had a significantly shorter survival than those who did not

Discussion

Since the expression of the transcripts and proteins of the plasminogen-associated markers and of CSF-1 and its receptor CSF-1R plays a role in the metastatic process of ovarian, breast and lung cancers [2, 4], we studied them in bladder cancer. Using seven bladder cancer cell lines (BCCL) and 15 freshly resected bladder tumors, neither the transcripts (RT-PCR analysis) nor the PAs,

PAIs, and CSF-1 antigen levels (ELISA determination) correlated with the grade of the BCCL or with the grade, stage and ploidy of the bladder tumors. Only uPA-R antigen expression at the cellular levels showed a correlation ($P = 0.02$). We thus questioned the role of the secreted PAs, PAIs, and CSF-1 antigens in bladder tumor invasion and metastasis. Only two of five high grade BCCL (T24 and TCCsup) secreted both CSF-1 and PAI2 antigen suggesting that CSF-1 did not stimulate PAI2 secretion as shown in ovarian carcinomas [4]. However, four of five G3 + G4 grade bladder tumor cell lines secreted high levels of CSF-1, showing that colony-stimulating factor may be part of a network of paracrine or autocrine loops that modulate carcinoma cell activity. Moreover, there is no correlation between CSF-1 secretion and motility of the cells from the BCCL. These results suggest that, in bladder carcinoma, CSF-1 is not associated with the metastatic process of the tumor cells. On the other hand, tumor associated overexpression of uPA and PAI1 antigens measured by ELISA was considered by others to be of poor prognostic value [15, 16, 31]. In this study, uPA and PAI1 production was demonstrated by all BCCL and all bladder tumors showing that they are major fibrinolytic markers in bladder tumor cells. However, uPA production did not predict tumor invasiveness (Table 2). Our study, examining the secreted PAs and PAIs, confirms reports already published on RT4 and T24 cell lines [17]. However, it is difficult to compare our results with the levels of cell associated PAs and PAIs in tissue extracts of bladder cancers [15]. It is possible that these discrepancies could be due to modifications in the release of these markers in culture supernatants related to the culture conditions, which in several respects are extraphysiological. FCS [29] and nutrients, but also the absent or disturbed relationships between tumor cells and stromal cells (fibroblasts, macrophages or endothelial cells), could modify the levels of the proteins studied. In a limited number of situations (homogeneous material with $> 95\%$ of tumor cells cultured without serum), ELISA can provide reliable quantitative results that are well in line with the quality-control procedures established in biology. However, in most tumors that are heterogeneous, the ELISA technique did not provide such results. Our data also show that all of the BCCL exhibited only 54 kDa uPA whereas 4 of 15 bladder tumors exhibited fibrinolytic activity both at 54 and 33 kDa. Such disagreement has already been described for tumor cells [26]. It was shown that cultured cells are not necessarily representative of the cells in the intact organism from which they are derived with respect to the production of PAs [6]. It is possible that the enzymatically active high-molecular-weight form of uPA (HMWuPA 54 kDa) has been degraded by plasmin into the low-molecular-weight form of uPA (LMW uPA 33 kDa). Plasmin, formed by the conversion of the plasma protein plasminogen, could bind either to the bladder tumor cells or the cells surrounding the tumor. This could explain the discrepancy observed between BCCL and bladder tumor cells.

In our preliminary study on the 15 bladder tumors, the secreted PAI2 antigen (ELISA determination) did not correlate with the grade and the stage of the bladder carcinoma, whereas the intracellular PAI2 antigen expression (ICC determination) did ($P = 0.03$ and $P = 0.02$, respectively). These discrepancies, observed between ELISA and ICC for this marker, have already been noted [9]. The latter finding is compatible with results from other studies showing that PAI2 is usually located intracellularly [7, 17], suggesting that PAI2 could be an inhibitor of intracellular protease. The ICC technique, which preserves cell morphology and tissue architecture, gives a semiquantitative analysis of the markers produced and present in fresh uncultured tumor cells. These arguments led us to use ICC in our study of imprints from 129 bladder cancer patients. Using a threshold value of 5% labeled cells to avoid false-positive results, the intracellular PAI2 antigen expression correlated with the grade of the tumor cells in relation to the presence in the tumor of PAI2 positive cells (macrophagic cells) ($P = 0.05$). However, there was no relationship with the other clinicopathological factors (size and number of tumors, recurrence number, disease-free survival, age or sex). Labeling imprints with both PAI2 and an epithelial marker (keratin) would specifically identify the double positive epithelial cells, thus improving the reliability of the detection of tumor cells and the statistical analysis of these assays.

Our results with uPA-R confirm those of other studies on bladder and other epithelial cancers [17, 21, 23, 32]. Recent results show that uPA-R antigen expression decreases cell adhesion and increases the proteolysis of the extracellular matrix, both of which are associated with tumor cell migration and metastasis, and correlate with a poor prognosis [28, 34]. Therefore, it is possible that the tumor cell receptor for uPA (uPA-R) binds uPA released from either tumor cells or stroma cells, focusing proteolytic action to the surface of tumor cells. The uPA/uPA-R complex converts enzymatically inactive plasminogen into the active serine protease plasmin that degrades proteins of the extracellular matrix, thus facilitating tumor cell invasion and metastasis. However, despite the secretion of high levels of uPA, RT4 and RT112, cell lines did not migrate because they did not express uPA-R antigen. These results show the predominant role for uPA-R at the tumor cell's surface. Indeed, uPA-R antigen expression at the cellular level was found to be associated with hepatocyte growth factor (HGF) levels in the culture supernatant from the ten bladder tumors ($P = 0.016$) and the cell motility of the seven BCCLs ($P = 0.014$) (Tables 2, 4). Urinary and cell extract HGF content has been found to be specifically elevated in patients with bladder carcinomas as compared with normal subjects [19] and HGF levels were especially elevated in bladder carcinomas from high-grade invasive tumors [14]. By demonstrating a relationship between uPA-R antigen expression and both cell migration ability and HGF secretion of bladder tumor cells or the histopathological classification of

resected tumors, our results, together with those mentioned in the introduction, suggest that the overexpression of uPA-R antigen at the cellular level could have a prognostic value. Therefore, uPA-R antigen expression could be associated with the metastatic potential of bladder carcinomas. This could explain why the 60-month overall survival rate of the uPA-R-positive group was significantly lower than that of the uPA-R-negative group.

In conclusion, because histologically similar bladder tumors show marked differences in their development, uPA-R and possibly intracellular PAI2 antigen expression at the cellular level could be of great interest. Analogous to breast cancer [21], uPA-R status could become an additional prognostic factor for the progression of bladder tumors.

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