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Soluble Urokinase Receptor Levels Correlate with Number of Circulating Tumor Cells in Acute Myeloid Leukemia and Decrease Rapidly during Chemotherapy[1]

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ABSTRACT

The importance of plasminogen activation, mediated by urokinase (uPA) and its receptor (uPAR), is well established in many physiological and pathological processes, such as in cell migration and tumor-cell invasion. Recently, additional functions have been described for uPA and uPAR, particularly in cell adhesion and chemotaxis. The amounts of uPA and uPAR in various tumor types and in the plasma/serum samples of cancer patients have been shown to correlate with survival prognosis, indicating the relevance of these molecules in malignancy. We previously showed that in acute myeloid leukemia, a high level of plasma soluble uPAR (suPAR) at diagnosis correlates with poor response to chemotherapy. However, in this case, as in other cancers, the origin of suPAR is unknown. Therefore, we have now analyzed uPAR in cells, plasma, and urine of patients with acute leukemia ($n = 35$) at 0, 5, 14, 28, and 56 days after start of chemotherapy. In response to cytotoxic treatment, suPAR levels decreased rapidly, and the decreasing plasma suPAR (p-suPAR) levels correlated highly with decreasing numbers of circulating tumor cells, suggesting that the elevated p-suPAR was produced by circulating tumor cells. Moreover, the p-suPAR level appeared to correlate with the amount of uPAR in tumor cell lysates at diagnosis. Our results also show for the first time that in lysates of circulating tumor cells, studied by immunoprecipitation and immunoblotting, uPAR was partly in fragmented form, whereas only full-length uPAR was found in normal leukocytes. We also detected fragmented suPAR in peripheral blood plasma, in urine, and especially in the plasma compartment of bone-marrow aspirates of acute myeloid leukemia patients, in a pattern differing considerably from that found in healthy individuals. Because proteolytic cleavage of uPAR induces a potent chemotactic response *in vitro*, it is possible that these fragments may play a role in the pathophysiology of acute leukemia.

INTRODUCTION

The plasminogen activation cascade has been studied extensively in various physiological and pathological conditions (1), and its importance in processes such as cell migration and tumor invasion is well established. It is thought that uPA[3] acts mainly by activating plasminogen to proteolytically active plasmin when bound to its high-affinity receptor, uPAR (CD 87; Ref. 2). However, in recent years, growing evidence has demonstrated that uPA and especially uPAR are also involved in other processes independent of plasmin formation, *e.g.*, in proliferation, chemotaxis, and cell adhesion (3–6).

uPAR is a glycosylphosphatidylinositol-anchored cell-surface receptor that consists of three homologous domains (2). uPAR binds uPA through its NH_2-terminal domain (D1) and is also able to bind the adhesion protein vitronectin (7), mainly through domains D2D3, although the full-size receptor is required for both of these high-affinity interactions (7–11). uPA, plasmin, and chymotrypsin are able to proteolytically cleave uPAR between domains 1 and 2, releasing the ligand-binding domain (12, 13). *In vitro*, proteolytic cleavage of uPAR with chymotrypsin can substitute for the requirement for uPA binding, and it has been suggested that this or a nearby cleavage is required for uPAR to induce chemotaxis (14). Indeed, the uPAR chemotactic epitopes are located in the vicinity of the uPA-mediated cleavage, *i.e.*, in the linker region between domains D1 and D2 (15).

In recent years, a soluble form of uPAR, suPAR, has been discovered in various human body fluids: in the blood of normal individuals and cancer patients, in ascitic and cystic fluids, and in urine (16–21). In healthy individuals, suPAR levels are quite stable in the blood and urine (20) and are independent of age, sex, or sampling (17, 21, 22). In several pathological conditions, such as paroxysmal nocturnal hemoglobinuria (16), autoimmune diseases (23), and various types of solid tumors, *e.g.*, non-small cell lung cancer (24), breast (17), colorectal (22), prostate (25), and ovarian cancer (21), increased levels of suPAR have been found in plasma and serum. Furthermore, it has been shown that in certain carcinoma patients, enhanced suPAR levels correlate with a worse survival prognosis (21, 22). We previously found increased levels of p-suPAR in patients with acute leukemia. Especially in patients with AML, high p-suPAR levels at diagnosis correlated with poor response to chemotherapy (26).

Neither the source of suPAR in human body fluids nor the mechanism of receptor release from the cell surface has been defined. Both COOH-terminal protease cleavage and a glycosylphosphatidylinositol-specific phospholipase D may catalyze uPAR shedding from the cell surface (18, 27). Interestingly, it has been demonstrated that human suPAR can be found in plasma samples from mice carrying human xenograft tumors (18, 28). In human cancers, a correlation between tumor content of uPAR and plasma/serum suPAR has not been demonstrated. Moreover, no longitudinal studies have been published addressing the behavior of suPAR during treatment of human cancer. We therefore studied uPAR in plasma, urine, and tumor cells of patients with acute leukemia. We now report that the level of p-suPAR correlates both with tumor-cell count and with the content of uPAR in cell lysates. In addition, p-suPAR levels decrease rapidly when the tumor cells are removed from the circulation by chemotherapy. Interestingly, the amount, and especially the expression pattern of fragmented forms of uPAR in urine, plasma, and tumor-cell samples of patients with AML differed markedly from that found in healthy individuals.

MATERIALS AND METHODS

Patient Samples. Blood and urine samples from 47 adult patients with hematological disorders were included in the study. Samples were obtained at the time of diagnostic sampling from patients referred for suspicion of acute leukemia before any cytoreductive treatment. If a patient was diagnosed with acute leukemia ($n = 35$), follow-up samples were collected 5 ($n = 32$), 14 ($n = 32$), 28 ($n = 30$), and 56 days ($n = 22$) after start of chemotherapy

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[3] The abbreviations used are: uPA, urokinase type plasminogen activator; uPAR, uPA receptor; D1, domain 1 of uPAR; D2D3, domains 2+3 of uPAR; suPAR, soluble uPAR; p-suPAR, plasma suPAR; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; u-suPAR, urinary suPAR.

(n_{total} = 151). Some patients were lost during follow-up because of death (n = 8) or discontinuation of chemotherapy (n = 2). The study protocol was approved by the local ethics committee. All samples were collected at the Helsinki University Central Hospital. The WBC count and percentage of blast cells were obtained from simultaneous routine tests. Tumor-cell count in the circulation was determined as percentage of blast cells × WBC count.

AML was the diagnosis in 25 of these patients [French-American-British types M0 (n = 2), M1 (n = 2), M2 (n = 13), M4 (n = 4), M5 (n = 1), and M7 (n = 1), and 2 not classified; median age, 61 years; range, 23–78 years] and ALL was the diagnosis in 8 patients (French-American-British type L2; median age, 47 years; range, 21–72 years). Two other patients had hybrid phenotypes, *i.e.*, both lymphoid and myeloid markers were on the tumor-cell surface. Other hematological disorders included chronic myeloid leukemia (n = 5), chronic lymphocytic leukemia (n = 1), myelodysplastic syndrome (n = 2), and reactive pancytopenia (n = 4). The diagnoses were based on morphological, cytochemical, cytogenetic and cell-surface markers. Blood samples from 40 healthy volunteers served as controls (median age, 33 years; range, 22–62 years).

Blood samples were collected into EDTA tubes and kept on ice before plasma separation. Plasma was separated within 2 h by centrifugation for 30 min at 4°C at 1800 × g and stored frozen in aliquots at −70°C until assay. The mononuclear cell fraction was separated with Ficoll-Hypaque centrifugation. In leukemia patients, most (>82%) of the mononuclear cells were tumor cells (range, 0–96%). Cells were lysed in PBS (pH 7.4) containing 1% Triton X-100 and protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany). The lysates were centrifuged at 14,000 × g for 10 min at 4°C, and the supernatants were stored at −70°C until assay. Total protein was determined by the BCA protein assay kit (Pierce, Rockford, IL). The same amount of protein from each sample was analyzed with the uPAR ELISA and with immunoprecipitation followed by immunoblotting as described below.

Urine samples were centrifuged at 1800 × g for 10 min at 4°C and stored frozen in aliquots at −70°C before assay. Urine samples from 30 healthy controls were treated similarly.

Bone-marrow aspirates were collected from 15 patients with acute leukemia, from 6 patients with other hematological disorders, and from 5 healthy bone-marrow donors. Aspirates were collected into EDTA tubes, and plasma was separated by centrifugation at 1800 × g for 30 min at 4°C and stored frozen in aliquots at −70°C until assay.

Treatment of Patients with Acute Leukemia. Patients with AML were treated with high-dose combination chemotherapy containing idarubicin, cytarabine, thioguanine, mitoxantrone, etoposide, and amsacrine according to the Finnish Leukemia Group protocol. Elderly patients were treated with reduced doses according to established guidelines. Patients with ALL were treated according to Finnish Leukemia Group protocol ALL94, which consists of cycles of high doses of mitoxantrone, cytarabine, etoposide, daunorubicin, vincristine, asparaginase, methotrexate, and dexamethasone.

Assay of Soluble uPAR and uPAR in Cell Lysates. The ELISA for suPAR has been described previously (17). In brief, immunoplates were coated overnight with purified polyclonal antihuman uPAR antibodies. After blocking and washing, the wells were incubated with standard dilutions of purified recombinant suPAR or with 1:10 dilutions of plasma or urine samples. For the cell lysates, protein concentrations were determined, and then a volume equal to 20 μg of protein was put into each well. After antigen binding, the wells were rinsed and then incubated with a mixture of monoclonal antihuman uPAR antibodies (R2, R3, and R5), followed by alkaline phosphatase-conjugated antibodies. A color reaction with *p*-nitrophenyl phosphate substrate was allowed to develop at room temperature. The absorbances were read at 405 nm. The lower detection limit of the assay is 0.03 ng/ml.

The exact amount of uPAR in lysates (ng/mg of protein) was multiplied by the mononuclear cell count in the peripheral blood at that same time to estimate the total uPAR load in the cells in the circulation. Each sample was also tested without specific monoclonal antibodies; the few plasma samples that gave low positive reactions were not included in the analysis. All samples were tested in three separate experiments, with mean, median, and SE values calculated from those values.

Creatinine Measurement. The creatinine concentrations of the urine samples were measured by the Jaffé method according to manufacturer's instructions (Boehringer Mannheim) with a Hitachi 917 analyzer. u-suPAR levels were normalized for the dilution factor using creatinine values as described previously (20); uPAR/creatinine ratios were expressed as ng/ml uPAR divided by mg/dl creatinine.

Immunoprecipitation and Immunoblotting. The methods for immunoprecipitation and immunoblotting have been described elsewhere (20, 29). In brief, urine, plasma, and cell lysate samples were immunoprecipitated with biotinylated R2 and R3 monoclonal antibodies (Finsen Laboratory, Copenhagen, Denmark) prebound to immobilized streptavidin (Boehringer Mannheim). These antibodies recognize different domains of uPAR: R2 reacts with the COOH-terminal domain D3 of uPAR, and R3 with the NH_2-terminal domain D1. Immunoprecipitated proteins were fractionated by 12% SDS-PAGE under nonreducing conditions. The proteins were transferred to nitrocellulose membranes and detected with polyclonal rabbit anti-uPAR IgG, with chemiluminescent visualization of the complexes (SuperSignal Ultra; Pierce).

Statistical Analysis. Results are reported as values for mean ± SE and range. Student's paired and unpaired t tests were used for comparison of the results. The correlation coefficients r and Rho were calculated according to Pearson and Spearman rank correlation tests, respectively. Results were considered significant when $P \leq 0.05$.

RESULTS

p-suPAR Was Elevated in AML Patients and Correlated with Number of Circulating Tumor Cells. Blood samples from 35 patients with acute leukemia were taken at the time of diagnosis, and plasma and mononuclear cells were separated. Twenty-five of 35 patients had AML, 8 had ALL, and 2 had hybrid leukemia. The mean p-suPAR level in acute leukemia patients was significantly higher than that in healthy volunteers (2.42 ± 0.41 ng/ml; range, 0.4–10.5 *versus* 0.82 ± 0.04 ng/ml; range, 0.46–1.56; $P < 0.001$, Student's unpaired t test). Patient data are summarized in Table 1. In leukemia patients, the amount of p-suPAR at diagnosis appeared to correlate significantly with the number of tumor cells in the circulation (Rho = 0.59; $P = 0.002$, Spearman's rank correlation). This was especially clear when only the AML patients were considered (Rho = 0.69; $P = 0.001$; Fig. 1*A*) because cell lysates from ALL patients showed little or no uPAR (Table 1). Indeed, all AML patients with high p-suPAR values (>1.34 ng/ml, based on the mean of the control plus 2 SD) showed correspondingly high numbers of tumor cells.

These results suggest a direct correlation between p-suPAR levels and circulating tumor cells in AML, a hypothesis that was confirmed by two other indicators. A direct comparison between p-suPAR values and the uPAR content in lysates of circulating mononuclear cells (consisting mostly of tumor cells) also revealed a strong correlation (Rho = 0.86; $P = 0.0004$; Fig. 1*B* and Table 1). In addition, the effect of chemotherapy on the decrease in tumor-cell numbers and on p-suPAR levels showed a strong similarity, as demonstrated in Fig. 2 (*top* and *bottom panels*) for one representative patient.

Table 1 *Summary of patient data*

uPAR levels measured with ELISA. U-suPAR values normalized with amount of creatinine in urine samples.

	Patient group		
	AML	ALL	Control
suPAR (ng/ml) in plasma at diagnosis			
Mean	2.6	1.7	0.8
Median	1.7	1.5	0.8
Range	0.4–10.5	1.1–2.6	0.5–1.6
suPAR (ng/mg) in urine at diagnosis			
Mean	4.7	2.6	1.5
Median	3.2	2.4	1.4
Range	0.5–23.1	1.4–4.2	0.8–3.0
uPAR (ng/mg) in cell lysates at diagnosis			
Mean	0.37	0.11	0.34
Median	0.25	0.10	0.33
Range	ND[a]–1.45	ND–0.25	0.2–0.5

[a] ND, not detectable.

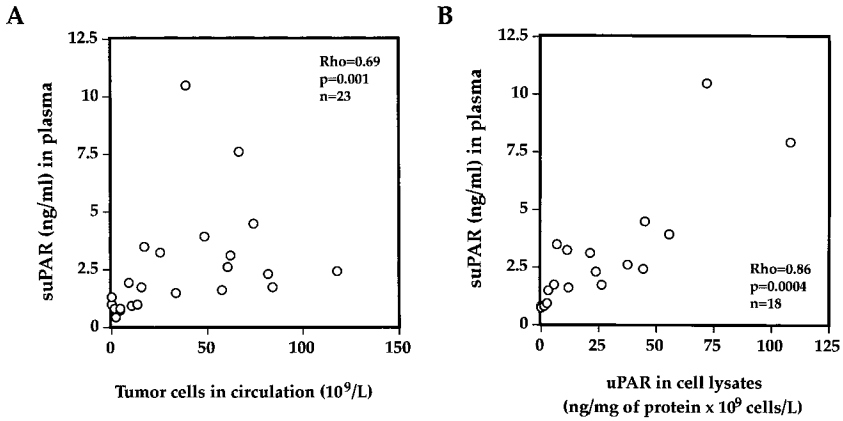

Fig. 1. Correlation between tumor-cell count and p-suPAR (*A*) and between uPAR in tumor cell lysates and in plasma (*B*) in AML patients. (s)uPAR was measured with uPAR ELISA and correlation coefficients (Rho) were analyzed with the Spearman rank correlation test. The exact amount of uPAR in lysates (ng/mg of protein) was multiplied by the mononuclear cell count in the peripheral blood to estimate the total uPAR load in the cells in the circulation (*B*).

p-suPAR Levels Decreased in AML Patients during Chemotherapy. Fig. 3*A* shows p-suPAR values during the follow-up of 21 AML patients at days 0, 5, 14, 28, and 56 after start of chemotherapy. In 18 of 20 patients with AML, p-suPAR levels dropped to the normal range (<1.5 ng/ml) within 2 weeks, correlating with the disappearance of tumor cells from circulation (Fig. 3*A*). The exception was patient A4, who still showed an exceptionally high suPAR level (3.6 ng/ml) after 4 weeks of chemotherapy. A bone marrow aspirate taken 5 weeks after the start of chemotherapy revealed that this particular patient had residual leukemia in the bone marrow (25% blast cells and promonocytes in a normocellular marrow).

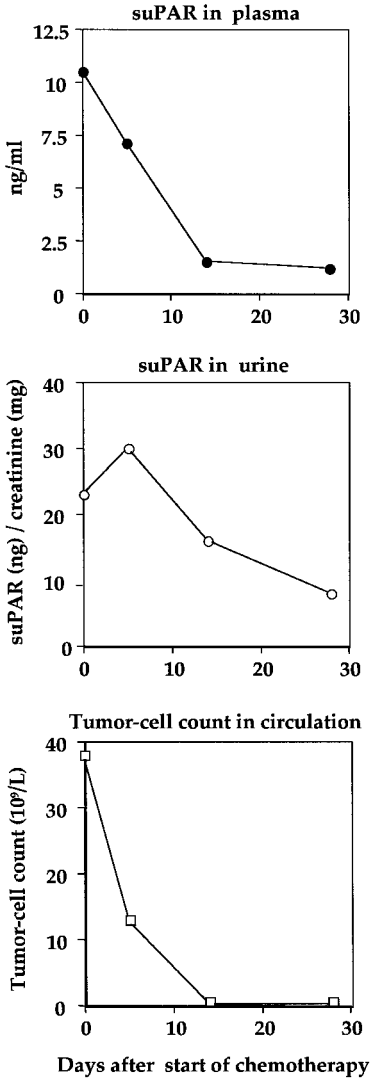

Fig. 2. suPAR in plasma (*top*) and urine (*middle*) samples of one representative AML patient and tumor-cell count in circulation (*bottom*) during chemotherapy. *Top* and *middle panels* show suPAR values measured with ELISA.

The decrease in p-suPAR levels showed a highly significant correlation with decrease in tumor cells in circulation at 2 weeks of treatment (Fig. 4; Rho = 0.81; $P = 0.0004$; $n = 20$). The decrease in p-suPAR after treatment for 2 ($n = 20$) or 4 weeks ($n = 18$) also significantly correlated with the p-suPAR levels at diagnosis in AML patients ($r = 0.94$; $n = 20$ and $r = 0.95$; $n = 18$, respectively; $P < 0.001$). Plasma samples of patients who at diagnosis had a normal p-suPAR level and few tumor cells in the circulation showed no marked decrease in suPAR level during therapy. Overall, these data strongly suggest a positive correlation between circulating tumor cells and p-suPAR.

suPAR Levels in Urine from AML Patients at Diagnosis and during Chemotherapy. Urine samples were collected at the same time points as plasma from leukemia patients. The effect of differences in dilution of the urine on suPAR levels was corrected with the amount of creatinine, as described previously (20). The u-suPAR and p-suPAR levels in leukemia patients at diagnosis were highly correlated (Rho = 0.80; $P < 0.0001$; $n = 31$). In AML patients during chemotherapy, the amount of u-suPAR in most cases paralleled the dynamics of p-suPAR, and suPAR levels decreased to the normal u-suPAR level (Figs. 3*B*; see also Fig. 2 and Fig. 5). However, in 14 of 19 patients with AML, a significant increase in u-suPAR was evident in the samples on day 5 after start of therapy (paired *t* test, $P = 0.01$; Fig. 3*B*; see also Fig. 2).

This increase in u-suPAR during chemotherapy, which was not noticeable in plasma, indicates that urine and plasma suPAR levels are not completely comparable. The u-suPAR peak may be a reflection of a massive tumor cell lysis in the body during the first 5 days of cytotoxic therapy because of the positive correlation between decrease in tumor cell count in 5 days and increase in u-suPAR (Rho = 0.58; $P = 0.02$; $n = 17$). In addition, if urinary levels originate from plasma, one would expect a certain time lag from plasma to urine. In the few ALL patients studied, the level of u-suPAR at diagnosis was similar to that in healthy controls (Table 1).

Two Different Forms of p-suPAR and Tumor-Cell Lysate uPAR in AML Patients. When p-suPAR was studied by immunoprecipitation and immunoblotting, the results were in accordance with the ELISA data, showing a major band of ~55 kDa at the time of diagnosis, which decreased during chemotherapy (Fig. 5). Interestingly, in more than half the plasma samples taken at the time of

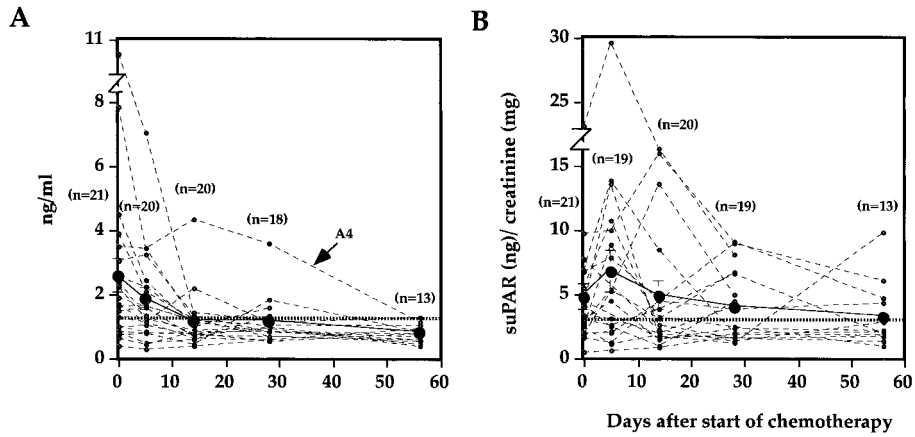

Fig. 3. Plasma (*A*) and urinary (*B*) suPAR in AML patients at diagnosis and on days 5, 14, 28, and 56 after start of chemotherapy. suPAR was measured with uPAR ELISA, and u-suPAR values were normalized for creatinine. *Dashed lines* represent individual patients, and the *solid line* represents the mean value for different patients during chemotherapy (*bars*, SE). The *horizontal dotted line* indicates the mean suPAR value + 2 SD in healthy controls. *A4*, AML patient with a persistent disease in the bone marrow after 4 weeks of chemotherapy; *n*, number of patients studied at each time point.

diagnosis from AML patients, an extra second band was visible, corresponding in size to the D2D3 fragment (35 kDa) of suPAR (Fig. 5 and Fig. 6). This band has never before been observed in human plasma from healthy controls, not in this study or previously (29, 30). The same phenomenon was observed in lysates made from the mononuclear cell fraction, consisting mostly of tumor cells from blood samples from the same patients (Fig. 6). In addition to full-size uPAR, AML patients had fragmented uPAR in their lysates. In contrast, lysates of cells from healthy controls and from AML patients with no tumor cells in circulation contained only full-length uPAR, and the amount was smaller (Fig. 6).

When the plasma compartments of bone-marrow aspirates from healthy bone-marrow donors and from patients with acute and chronic leukemia were also studied with immunoprecipitation and immunoblotting, in all five samples from healthy bone-marrow donors, only the full-length receptor appeared (Fig. 6). Moreover, bone-marrow samples from patients with chronic leukemia or with other hematological disorders ($n = 6$) contained only the full-length receptor. Nine of 12 AML patients had an additional band in their bone-marrow plasma samples corresponding to the size of fragmented uPAR (D2D3; Fig. 6). When peripheral plasma and bone-marrow plasma samples from the same AML patients were analyzed, more suPAR was found in bone-marrow plasma samples. In addition, immunoblotting revealed that bone-marrow plasma contained more fragmented suPAR than peripheral plasma (Fig. 6). This phenomenon may be explained by the higher concentration of tumor cells in the bone marrow, leading to a higher amount of suPAR released from the tumor cell surfaces.

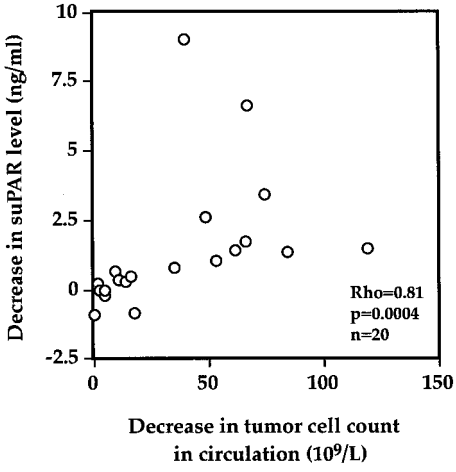

Fig. 4. Correlation between decrease in tumor-cell count and in p-suPAR level during the 2 weeks following start of chemotherapy. Correlation coefficient (Rho) measured with the Spearman rank correlation test.

Change in Fragment Pattern of u-suPAR during Chemotherapy in AML Patients, with a Prominent D1 Band as an Indicator of Circulating Tumor Cells. When urine samples were studied for suPAR with the same immunoblotting technique, results were in good accordance with ELISA results (Fig. 5). Before blotting, urine samples were immunoprecipitated, with sample volumes corresponding to equal amounts of creatinine. In healthy controls and in ALL patients, both the full-length receptor and a fragment corresponding to D2D3 were found in urine, as described previously (20, 29). Patients with AML also showed at diagnosis high amounts of domain D1 in the urine (Fig. 6; see also Fig. 5 and Fig. 7). Indeed, 14 of 15 AML patients with a high number of tumor cells in the circulation showed at diagnosis D1 in the urine (Fig. 6 and Fig. 7). Only one of eight patients with a low number of tumor cells in the circulation had, at diagnosis, detectable amounts of D1 in the urine.

In follow-up urine samples, the amount of suPAR was measured first with ELISA, and subsequently a volume corresponding to the equal amount of suPAR from each sample was taken for immunoprecipitation to compare the pattern of suPAR fragments in different samples from the same patient. In the follow-up samples, the amount of D1 was lower than in the samples before chemotherapy (Fig. 7*A*; see also Fig. 5), and the D2D3 fragment became the most abundant form, just as in the urine samples from healthy controls (Fig. 6). In one patient (Fig. 7*A*, *patient A4*) a large amount of D1 was still present 4 weeks after the start of chemotherapy. A bone marrow aspirate taken at that time revealed that this patient still had tumor cells in the bone marrow. In patients with a low tumor-cell count in the circulation (Fig. 7*B*), the u-suPAR pattern did not show marked changes and resembled the pattern seen in the healthy controls (Fig. 6). Minor bands corresponding to the D1 fragment of uPAR were visible in some patients with a low tumor-cell count (Fig. 7*B*), but the amount was considerably smaller than in patients with high counts (Fig. 7*A*). Small amounts of D1 have also been detected in some healthy individuals (29).

DISCUSSION

This study sheds light on the origin of the excess suPAR found in plasma samples of cancer patients and on the dynamics of suPAR during chemotherapy. In addition, the results show that differences exist in the expression pattern of fragmented forms of uPAR in various body fluids and in mononuclear cells between leukemia patients and healthy controls.

Unlike the case with solid tumors, in leukemia the tumor cells are

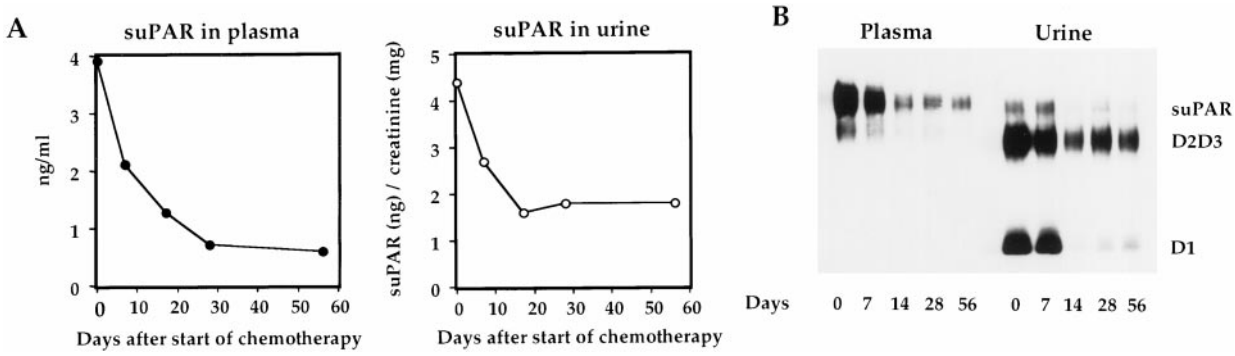

Fig. 5. suPAR in plasma and urine samples of one AML patient with a high number of tumor cells in circulation at diagnosis. Shown are ELISA (*A*) and immunoblotting (*B*) results. Plasma (50 μl) and urine samples (containing 75 μg of creatinine) were used in immunoprecipitation before immunoblotting.

in continuous contact with the circulation, which means that leukemia provides a good model for studying proteins expressed on the tumor cell surface and shed to the plasma. In animal models of human xenografted tumors, it recently was shown that the amount of suPAR in plasma correlates with tumor volume (28). *In vivo*, such correlations have not been demonstrated for human solid cancers, and the question remains as to whether the increased amounts of suPAR found in the plasma of cancer patients (17, 21, 22, 24–26, 30) originate from the tumor cells or, *e.g.*, from the tumor-infiltrating macrophages. Indeed, in various human solid tumors, uPAR is expressed by cancer and/or stromal cells (31), whereas in AML patients, we previously have shown that most of the blast cells express uPAR (26). The results presented here show for the first time that the increased amount of suPAR found in plasma from patients with AML correlates with tumor-cell count in the circulation and with the level of uPAR found in tumor-cell lysates. In addition, preliminary results from our ongoing study in patients with chronic myeloid leukemia in chronic phase demonstrated no correlation between elevated WBC count (range, 82–225 $\times$ 10^9 cells/l) and p-suPAR levels, and all of the p-suPAR levels in this patient population fell within the normal range. It is therefore likely that the excess p-suPAR in AML patients is the result of blast-cell production of uPAR and not elevated total WBC count.

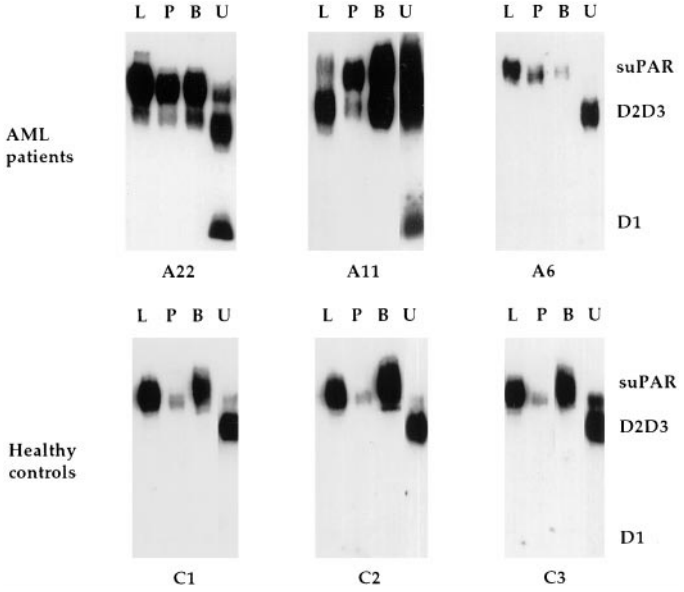

Fig. 6. Immunoblotting of uPAR immunoprecipitated from three different healthy controls (*C1–C3*) and three patients at diagnosis (*A22* and *A11*, AML patients with high tumor-cell count in circulation; *A6*, AML patient with no circulating tumor cells). *L*, cell lysates of mononuclear cells from normal and leukemic blood; *P*, plasma samples (50 μl of plasma); *B*, plasma samples (50 μl) of bone-marrow aspirates; *U*, urine samples (same amount of creatinine, 75 μg, used in immunoprecipitation of each sample).

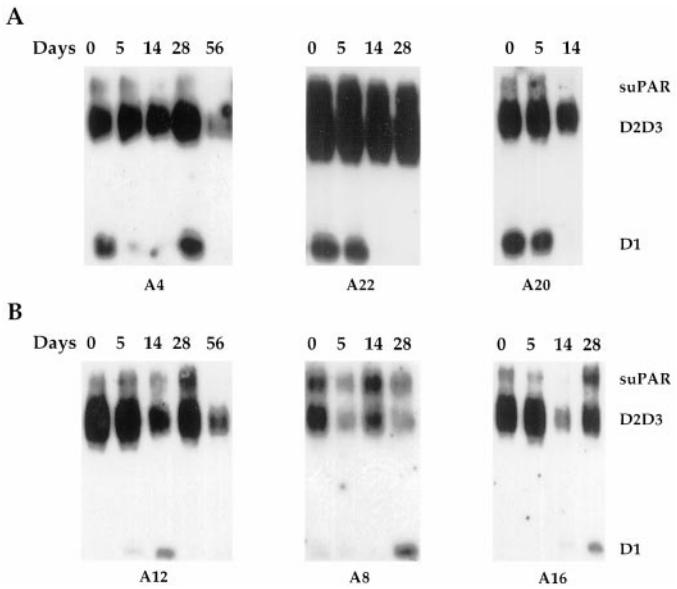

Fig. 7. Immunoprecipitation and immunoblotting of urine samples from AML patients during chemotherapy. Patient samples shown grouped according to high (*A*) or low (*B*) tumor-cell count in circulation at diagnosis. An equal amount of suPAR was taken from each sample to compare changes in domain pattern at different time points (at 0, 5, 14, 28, and 56 days after start of chemotherapy). Patient *A4* (*panel A*) had a persistent disease in the bone marrow after 4 weeks of chemotherapy.

The conventional therapy for solid tumors is surgical removal of the tumor tissue, often followed by chemotherapy. However, the effects of chemotherapy on solid tumors are often slower and more difficult to assess than in leukemia. Although studies have reported correlations between suPAR levels and survival prognosis in untreated cancer patients (22), thus far, no longitudinal studies have evaluated the effect of cytotoxic agents on the disappearance of tumor cells and on p-suPAR level. In this study, we observed that p-suPAR levels decreased dramatically shortly after the start of chemotherapy in AML patients, with the simultaneous disappearance of tumor cells. This further strengthens the view of tumor cells being the source of suPAR.

Although plasma and urinary suPAR levels correlate well with the presence of tumor cells in the circulation, they fail to reveal the persistence of tumor cells in the bone marrow; we found no correlation between the bone-marrow leukemic infiltrate and p-suPAR levels. Indeed, in some patients a decrease in suPAR occurred although the bone marrow aspirate revealed the presence of cancer cells. Possibly, the suPAR produced by these tumor cells was insufficient to

increase the suPAR level above the normal range. However, in one patient with a high p-suPAR level at diagnosis, we saw a transient decrease 2 weeks after the beginning of chemotherapy, followed by an increase at 4 weeks, at the same time that tumor cells reappeared in the circulation.

A recent study observed that serum suPAR levels correlated well with the u-suPAR levels in healthy controls and ovarian cancer patients (20). Our results in leukemia patients were similar; moreover, both u-suPAR and p-suPAR decreased during chemotherapy. However, we found an apparent increase in u-suPAR 5 days after the initiation of chemotherapy. This increase probably reflects the accumulation of suPAR attributable to massive cell death in the body caused by the cytotoxic agents. No such increase was evident in the plasma, possibly because of efficient processing/clearing of suPAR from plasma to urine (see below).

Fragmented cellular uPAR consisting of D2D3 was first described in cultured human monocytoid U937 cells (12) and subsequently in primary cultures of normal and neoplastic thyroid cells (32) and in human xenograft tumors implanted in mice (28, 33) However, there seem to have been no studies describing uPAR fragments in clinical tumor specimens. Our results show that fragmented uPAR (D2D3) is present in the blast cells of patients with acute leukemia but not in blood leukocytes from healthy volunteers, which expressed only small amounts of full-length receptor. Leukemic cells have shown increased uPA activity on the cell surfaces (34), and high amounts of uPA antigen have also been found in plasma samples of leukemia patients (26). On the basis of these findings, an excess of uPA, a possible candidate responsible for uPAR cleavage (12), could explain the observed uPAR fragmentation on the blast cells. The cleavage of uPAR on the tumor cell surface would lead to decreased proteolytic activity because the ligand-binding domain D1 would be released. Truncation of cell-bound uPAR also results in other major changes in uPA and uPAR function. Cleaved uPAR is unable to bind uPA/plasminogen activator inhibitor-1 complexes, will not be internalized, does not have high affinity for vitronectin (9), and may be unable to act as mediator of cell adhesion (35, 36) through binding to integrins. Moreover, uPAR cleavage exposes an extremely potent chemotactic epitope (15), which transforms it into either a soluble or a cell-surface-attached chemokine (3).

These findings lead to interesting considerations regarding the mechanism of clearance of soluble forms of uPAR. First, full-length uPAR is found in cell lysates, in plasma, in bone-marrow plasma, and in urine. However, different fragments of uPAR show different distributions: whereas fragment D2D3 is also found in cells, plasma, and urine, fragment D1 is found only in urine (see Fig. 6). The presence of D2D3 in cells (this work and Refs. 32, 33) and our previous demonstration that cleavage does not occur in urine (29) indicate that D1 is produced by cleavage of uPAR in the cells. The clearance time of D1 must therefore be very short because we have never detected it in cell lysates or in plasmas. Moreover, we were able to detect D2D3 in leukemic plasmas (Fig. 5 and Fig. 6), but were unable to show it in plasmas from healthy individuals (29) or from patients with solid tumors. In these cases, we observed only full-length suPAR. The latter difference is most likely attributable to the differential concentration of circulating cancer cells in leukemia patients *versus* patients with solid cancers. Because D2D3 is observed in tumor tissues, the clearance time of D2D3 from plasma must be shorter than that of full-length suPAR. There appears, therefore, to be a gradient of clearance rates, full-length suPAR having the longest, D2D3 an intermediate, and D1 the shortest half-life.

In conclusion, we show that suPAR correlates with tumor-cell count in the circulation and with the amount of uPAR in circulating cells in AML patients, and that enhanced amounts of uPAR fragments are found in tumor cells and in the various body fluids of leukemia patients. These data also warrant investigation as to whether these fragments have biological relevance and whether the evaluation of uPAR fragments may be a more valuable tool for clinical use than the measurement of total uPAR.

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