Urinary Excretion of Proteolyzed α₁-Antitrypsin: Specificity, Quantitation, and Relation to Therapy Response in Patients with Acute Myeloid Leukemia

Robert Dengler, Andreas Plewan, Ursula Münstermann, Raymonde Busch, Gerhard Eger, and Bertold Emmerich


ABSTRACT

During remission induction chemotherapy, a 41-kDa cleavage product of α₁-antitrypsin (α₁-AT⁴¹) can be found in the urine of patients with acute myeloid leukemia. By using immunoblotting with antibodies against this protein, 27 patients with acute myeloid leukemia were screened for the excretion of this fragment and the amount of α₁-AT⁴¹ compared with treatment response assessed by therapy-induced cyto reduction in the bone marrow and time to reach remission. Patients with acute lymphoblastic leukemia, malignant lymphomas, and solid tumors receiving chemotherapy, patients with nonmalignant diseases like sepsis and kidney dysfunction, and healthy subjects were probed to evaluate the specificity of this phenomenon.

In 74% of the acute myeloid leukemia patients, the truncated inhibitor was detected. Mean concentration of peak excretion was found to be 6.7 μg/mg creatinine (range, 1.1–41 μg/mg). Among the patients treated with induction chemotherapy, those who responded completely (<5% residual marrow blast cells) exhibited significantly higher α₁-AT⁴¹ concentrations than the nonresponders (P < 0.03). Patients who showed a partial response (6–25% residual blasts) excreted intermediate values of the protein. The probability of median time to reach remission was 40 days in patients excreting the truncated inhibitor in measurable amounts compared to 100 days in patients negative for α₁-AT⁴¹ (P < 0.02). The 41-kDa fragment was also found in one of 10 patients with acute lymphoblastic leukemia and in 3 of 18 lymphoma patients but not in those with solid tumors, infections, or kidney disease or in healthy individuals.

INTRODUCTION

Only a few parameters exist to predict the prognosis of individual patients with AML. Besides age and cytog enerics (1), one of the most significant parameters which correlates with survival seems to be the time to reach remission (2, 3). Clinically useful prognostic markers which could predict response to induction therapies would aid in the choice of the most appropriate treatment for an individual patient, e.g., single induction versus double induction or myeloablative versus nonmyeloablative postremission therapy. However, there are hardly any parameters predictive for achievement of remission (4). Morphological tracing of therapy response during the induction period is limited. Blast cell reduction in peripheral blood does not reflect quantitative clearance of the leukemic cell burden (2). In addition, a significant proportion of patients present with leukemic disease. Microscopic counting of residual blasts in the bone marrow does not allow exact quantitation and is subject to interobserver variability. Sequential counting of residual blasts, which has been used for monitoring of cytoreduction (5), is not feasible without compromising the patient inadequately.

In order to find a quantitative marker for treatment response in AML patients which could be detected sequentially, easily, and noninvasively, we have previously screened the urine samples of these patients for proteins and found that during induction chemotherapy, a novel glycoprotein band of 41 kDa appears (6). This molecule was purified to homogeneity and specific antibodies were raised which showed reactivity with α₁-AT, also named α₁ proteinase inhibitor, the major serine proteinase inhibitor (serpin) in humans (6, 7). Amino acid sequence analysis of the isolated protein revealed that indeed α₁-AT was the respective antigen and that proteolytic cleavage had occurred at the N-terminal part as well as within the reactive site loop leading to inactivation of the inhibitor (7). The fragment could not be detected in plasma of AML patients, indicating rapid and quantitative renal clearance.

In this article we used immunoblotting and densitometric quantitation of this α₁-AT⁴¹ in urine samples of patients with AML during remission induction therapy. The amount of excretion of this protein was correlated with cytoreduction as assessed by remission quality in the bone marrow and with time to reach remission in order to investigate its potential value as a response marker in this disease. The urine samples of other patients with hematological diseases, solid tumors, and nonma-

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2 To whom requests for reprints should be addressed, at Medizinische Klinik, Klinikum Innenstadt der Universität München, Ziemssenstrasse 1, D 80336, Munich, Germany.

The abbreviations used are: AML, acute myeloid leukemia; α₁-AT, α₁-antitrypsin; α₁-AT⁴¹, 41-kDa fragment of α₁-AT; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma; TAD, thioguanine; FAB, French-American-British classification.
lignant diseases as well as healthy subjects were screened to investigate the specificity of urinary excretion of α₁-AT.

PATIENTS AND METHODS

Patients. The urine samples of 27 patients diagnosed as AML (23 de novo, 1 secondary AML, and 3 myelodysplastic syndrome) by assessment of peripheral blood and bone marrow according to the FAB classification (8) were investigated (see Table 1). In addition, 10 patients with ALL, 2 with Hodgkin’s disease, 18 with NHL, consisting of 5 centroblastic (cb), 2 diffuse large cell anaplastic, 3 lymphoblastic, 2 follicular (cc-cb), 2 plasmocytoma, 1 immunocytoma, and 3 B-chronic lymphocytic leukemia patients were investigated. Additionally, 13 patients with solid tumors (5 breast cancer, 3 colorectal cancer, 1 non-small cell lung cancer, 1 neuroblastoma, 1 gonadal teratocarcinoma, 1 synovial sarcoma, and 1 rhabdomyosarcoma patient) were analyzed. Patients with nonmalignant diseases were comprised of two patients with nephrotic syndrome, two with systemic lupus erythematosides and renal involvement, two patients with septic infections, and one with Crohn’s disease. Seventeen apparently healthy subjects were also investigated (Fig. 3).

Treatment. Twenty-four AML patients were treated according to the German AML Cooperative Group by an induction course with TAD (9). Patients under 60 years of age received high-dose 1-β-d-arabinofuranosylcytosine plus mitoxantrone as a second course (reinduction), followed by TAD for consolidation and a 3-year course of maintenance chemotherapy until relapse as described (9). Three patients received the MAV (daunorubicin, ara-C, VP-16) regimen (10). ALL patients were treated according to the German BMFT protocol (11). High-grade lymphoma patients received the COP-BLAM regimen (12) while low-grade lymphoma patients were treated with either Cyclophosphamide–vincristine–prednisone, Mitoxantrone–prednimustine, or Fludarabine. Patients with Hodgkin’s disease were given Cyclophosphamide–vincristine–procarbazine–prednisone/Adriamycin–bleomycin–vinblastine–dacarbazine. Breast cancer patients received either Cyclophosphamide–methotrexate–5-fluorouracil or epirubicin. Colorectal cancer patients were under treatment with either 5-fluorouracil plus α-interferon or 5-fluorouracil plus leucovorin. The remaining patients with solid tumors were treated with standard high-dose polychemotherapy regimens (see Ref. 13 and literature cited therein).

Definition of Therapy Response in AML. Cytoreduction was assessed by microscopic evaluation of residual blast cells in bone marrow aspirates on day 14 and before the start of the second course (reinduction), which was usually commenced between day 21 and day 28. According to the Cancer and Leukemia Group B criteria (14), patients were grouped as follows: (a) complete response—aplasia or less than 25% blasts on day 14 and no blasts (M0 marrow) or less than 5% residual blasts (M1 marrow) before the second course; (b) partial response—6–25% blasts (M2 marrow) before reinduction; and (c) inadequate (non)response was defined as 25–50% blasts (M3 marrow) or more than 50% (M4 marrow) on day 14 and/or before the start of the second course.

Remission was defined as (a) complete remission: complete response plus normalization of peripheral counts, i.e., no blasts and >3000/µl granulocytes plus >100,000/µl platelets or (b) partial remission: partial response plus <5% blasts and >1000/µl granulocytes plus >50,000/µl platelets (see also Ref. 2).

Urine Processing. Urine was collected two or three times per week during the first 4 weeks of therapy. To avoid the disadvantages of 24-h urine collection (possible protein degradation during the collection period, time consuming), spontaneous early morning urine was collected. After centrifugation, total protein was quantitated in the supernatant as described by Bradford (15) using a commercial kit (Bio-Rad, Munich, Germany). Creatinine was determined according to standard methods (Boehringer Mannheim) in an automated analyzer. The protein:creatinine ratio was calculated to correct for differences in protein contents in the spot urine samples (16, 17). Aliquots were then stored at -20°C until use. Thawed samples were centrifuged at 8000 × g for 10 min at 4°C and supernatants were concentrated between 2- and 10-fold according to the protein:creatinine ratio by using an 8MC ultrafiltration system (Amicon, Danvers, MA) which excludes proteins smaller than 10 kDa.

Western Blotting and Immunostaining. The proteins were subsequently transferred onto a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore, Bedford, MA) equilibrated with a 4–22% polyacrylamide gradient as described (18). Samples plus buffer (0.15 M Tris-HCl, 2% SDS, and 1% dithiothreitol) were loaded in each pocket and the electrophoresis was run with a maximum current of 1200 V and a power of 50 mA at 30 W for 3 h at 5°C.

Gel Electrohoresis. Proteins were separated by casting ultrathin (0.5-mm) continuous SDS gels with a 4–22% polyacrylamide gradient as described (18). Samples plus buffer (0.15 M Tris-HCl, 2% SDS, and 1% dithiothreitol) were loaded in each pocket and the electrophoresis was run with a maximum current of 1200 V and a power of 50 mA at 30 W for 3 h at 5°C.

Densitometric Quantitation. Quantitative determination of α₁-AT was achieved by laser scanning densitometry. For this purpose, the dried blot membrane was mounted in an ultrascans XL densitometer (Pharmacia-LKB, Freiburg) equipped with a neon-helium laser (633-nm wavelength) and each lane was scanned in the range between 20 and 100 kDa. The area under each peak, representing the integration value of absorbance units and width (mm), was compared to the values obtained by scanning a serial dilution of purified α₁-AT yielding a standard curve. Data were analyzed and processed using the GelScan XL software (Pharmacia). Finally, the values (μg/ml) were adjusted to the urinary creatinine of each sample and expressed as μg α₁-AT/mg urinary creatinine. The total amount of excreted α₁-AT of each patient during induction therapy was estimated by integrating the area under the curve of these values using the Simpson equation (21). A second value was termed “peak excretion,” which represents the α₁-AT concentration from the day of maximum excretion during this period.
Table 1  Patient characteristics and response to treatment of the investigated AML patients

<table>
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<tr>
<th>Laboratory code</th>
<th>Patient Code</th>
<th>Patient Name</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>FAB</th>
<th>Leukocyte count (μl)</th>
<th>Karyotype</th>
<th>LDH (units/liter)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Urinary protein (μg/ml)</th>
<th>Therapy</th>
<th>Response (a)</th>
<th>Status (b)</th>
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\(a\) sAML, secondary AML; t-MDS, trilineage myelodysplasia; R, complete response; PR, partial response; NR, no response; n.d., not done; DAV, daunorubicin, ara-C, VP-16 (etoposide); RAEB-T, refractory anemia with excess of blasts in transformation.

\(b\) Status (as to June 15, 1994): A, alive; B, dead; C, censored; D, lost for follow up; CCR, continuing complete remission; ED, early death; BMT, bone marrow transplant. Serum creatinine and urinary protein concentrations shown are from the day of \(\alpha_1\)-AT\(^{41}\) peak excretion.

RESULTS

Table 1 outlines clinical, hematological, and cytogenetic characteristics and the treatment response of the AML patients investigated. Fig. 1 shows an immunoblot of urine samples from an AML M4 patient sequentially collected during remission induction chemotherapy. In addition to a faint 53-kDa band, which represents intact \(\alpha_1\)-AT, a 41-kDa fragment is the dominating immunoreactive protein. To quantify the concentration of this fragment in urine, densitometric scanning of the immunoblots was used. For each patient and on each blot, a serial dilution of the purified 41-kDa inhibitor fragment (1.9–60 ng/ lane, corresponding to a concentration of 0.6–20 μg/ml) was run, yielding the typical standard curve depicted in Fig. 2. The detection limit of this assay was determined to be approximately 3.75 ng, corresponding to a concentration of 1.25 μg/ml \(\alpha_1\)-AT\(^{41}\). When comparing the whole amount of \(\alpha_1\)-AT\(^{41}\) excreted during therapy, estimated by integration of the area under the concentration/time curve, with the value from the day of peak excretion, a good correlation was found by regression analysis \((r = 0.942)\). Therefore, the peak day concentration is used as the representative value in this article. Fig. 3 shows the concentrations of \(\alpha_1\)-AT\(^{41}\) determined in the urine samples of different patients receiving chemotherapy and patients with nonmalignant diseases as well as healthy subjects. Comparable protein concentrations were applied for each individual analyzed. Of 27 AML patients, 20 (74%) patients were found to excrete the 41-kDa protein in measurable amounts with a mean peak excretion of 6.7 μg/mg creatinine (range, 1.1–41 μg/mg). The days of peak excretion varied within the patients from day 2 to day 29. In three patients the truncated inhibitor was already

![Fig. 1 Immunoblot of urine samples from an AML M4 patient collected during induction chemotherapy with TAD. Urinary proteins were adjusted to the protein:creatinine ratio, separated by SDS-PAGE, Western blotted, and immunostained by the monoclonal anti-\(\alpha_1\)-AT\(^{41}\) antibody. Right, 200 ng each of purified \(\alpha_1\)-AT\(^{41}\) (arrow) and intact \(\alpha_1\)-AT (53 kDa) are shown for comparison.](Image)
found before the start of chemotherapy. In most cases, the protein appears during the first week, steadily rises, and disappears at the end of the third week (Fig. 1). One of 10 patients with ALL and 3 of 18 patients with malignant lymphomas (two cb-NHL and one immunocytoma) also showed this molecule during remission induction chemotherapy (Fig. 3). In contrast, none of the patients with Hodgkin's disease and solid tumors receiving chemotherapy or those with kidney disease, infections, or healthy donors had any detectable amounts of α1-AT^{41}.

The variability of the time course and the amounts of α1-AT^{41} in the AML patients tempted us to compare the excretion of this protein with blast cell reduction in bone marrow induced by remission induction chemotherapy. Therapy response was assessed by evaluation of residual leukemic blast cells in bone marrow aspirates from day 14 and before reinduction as outlined in “Patients and Methods.” In 11 of 12 patients who achieved a complete response, the truncated inhibitor could be detected in urine, with a mean concentration of 9.45 μg/ml on peak days (Fig. 4). In contrast, four of seven patients who showed no adequate cytoreduction (no response) were positive for α1-AT^{41} with a mean concentration of 1.5 μg/ml. This difference is statistically significant ($P < 0.03$, Mann-Whitney U test). Partial response patients had intermediate α1-AT^{41} values (5.0 μg/ml).

In a second step α1-AT^{41} excretion was correlated with time to reach remission. In order to analyze a homogenous group of patients, 19 evaluable de novo AML patients treated according to the TAD regimen (Table 1) were selected. The method of Kaplan and Meier (22) was used for calculation of the cumulative probability of time to enter remission and the Log rank test for evaluation of statistical significance of observed differences (23). Fig. 5 shows that those patients who were found to excrete the 41-kDa protein in measurable amounts achieved remission in a significantly shorter time than those in which the fragment could not be detected. Probability of median time to reach remission was 50 days for all patients while patients positive for α1-AT^{41} showed a median of 40 days and
Those negative for the protein were found to have a median time of 100 days ($P < 0.02$).

By using univariate analyses, we correlated the $\alpha_1$-AT$^{41}$ concentrations to some of the known or currently discussed factors being of possible prognostic importance in AML (1). These were: age, leukocyte count at presentation, lactate dehydrogenase, FAB subtype, and karyotype. As Table 2 shows, none of these parameters correlated significantly with the inhibitor fragment in univariate analyses. A multivariate test was not performed due to the small patient numbers.

Various experiments were performed to clarify how the fragment is generated. We investigated whether the chemotherapy itself plays a role in the generation of $\alpha_1$-AT$^{41}$ first. For this purpose, urine samples of AML patients receiving remission consolidation chemotherapy were probed, which consists of exactly the same doses and timing of cytotoxic drugs (TAD) as in the induction course. None of the urine samples from those AML patients who excreted the protein during induction contained detectable concentrations of $\alpha_1$-AT$^{41}$ under consolidation therapy. The fragment does not appear to be a physiological degradation product of $\alpha_1$-AT or a protein lost by impaired kidney function since neither healthy subjects (even after 100-fold concentration of their urines samples) nor patients with nephrotic syndrome, who were found to excrete 5–20 times more total urinary protein than the AML patients, had any detectable amounts of the 41-kDa truncated inhibitor in their urine samples.

To find out whether proteolysis of $\alpha_1$-AT takes place in the urine during sample collection or assaying, urine samples of the same patients were compared in the presence or absence of a cocktail of the following proteinase inhibitors: EDTA (1 mM), leupeptin (10 $\mu$M), aprotinin (1 $\mu$M), and pepstatin (1 $\mu$M). However, no differences in the formation of immunoreactive bands were observed, either qualitatively or quantitatively (not shown).

### Table 2 Univariate comparison of $\alpha_1$-AT$^{41}$ excretion with other factors of known or suspected prognostic importance in AML

<table>
<thead>
<tr>
<th>Factor</th>
<th>Test</th>
<th>$r^a$</th>
<th>$p^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial leukocytes</td>
<td>Spearman</td>
<td>0.178</td>
<td>ns</td>
</tr>
<tr>
<td>Initial lactate dehydrogenase</td>
<td>Spearman</td>
<td>0.176</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>Spearman</td>
<td>0.055</td>
<td>ns</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td>Mann Whitney U</td>
<td>na$^c$</td>
<td>ns</td>
</tr>
<tr>
<td>FAB subtype</td>
<td>Kruskal-Wallis</td>
<td>U</td>
<td>na</td>
</tr>
</tbody>
</table>

$a^a$ Regression coefficient.  
$b^b$ Significance level.  
$c^c$ Not significant ($P > 0.05$).

### DISCUSSION

During remission induction therapy in AML patients, a novel fragment of $\alpha_1$-AT appears in the urine samples of these patients (6, 7). The kinetics of excretion of this protein could be specifically detected by immunoblotting and at least semiquantitatively monitored using densitometric scanning. In a first step we compared the $\alpha_1$-AT$^{41}$ values with treatment response as assessed by cytoreduction and remission quality to evaluate its potential role as a response marker in AML. The amount of excretion of the cleaved inhibitor varied considerably within the AML population and accounted for up to 5% of total urinary protein at days of peak excretion.

The observed variability was found to be associated with therapy-induced cytoreduction in the bone marrow. Excretion of the truncated inhibitor also correlated with time to reach remission, a finding which one would demand if this protein indeed reflects quantitative blast cell reduction. However, these data stem from a preliminary study and are derived from a small patient population. Therefore, they will have to be controlled in a larger investigation.

Concerning correlation with other prognostic factors, the results from the statistical analyses suggest that $\alpha_1$-AT$^{41}$ is not a covariable of other factors like age, leukocyte count, lactate dehydrogenase, or FAB subtype, as far as this conclusion can be derived from an univariate analysis. Clearly, this has to be controlled by a multivariate approach with larger patient numbers.

The origin of the proteolyzed inhibitor is so far not well understood. The possibility that the fragment is generated in the urine or during processing and assaying is unlikely. We compared urine samples with and without the addition of a cocktail of proteinase inhibitors. However, no difference in the formation of immunoreactive protein bands was observed. In addition, incubation of purified $\alpha_1$-AT with urine samples of healthy donors did not lead to a stable 41-kDa degradation product (not shown). The finding that only in AML patients, but not in any others, the truncated inhibitor was found before therapy and not under consolidation chemotherapy indicates that the generation of $\alpha_1$-AT$^{41}$ is not initiated by the cytotoxic therapy itself. Renal function of the investigated AML patients assessed by serum creatinine, blood urea nitrogen plus urinary protein, and creatinine excretion did not differ from that of the ALL, NHL, and solid tumor patients.
A second and more likely answer concerning the mechanism of $\alpha_1$-AT* generation is that the inhibitor is degraded in the circulation, i.e., in plasma. During spontaneous disintegration and, to a larger extent, during cytotoxic therapy, proteolytic enzymes present in the granules of the myeloid blast cells may be released into the circulation. This would result in a pronounced alteration of the proteinase/inhibitor balance in plasma with subsequent proteolytic inactivation of plasma proteins. This mechanism has been discussed for neutrophil elastase, an enzyme which can inactivate and degrade proteins of the coagulation and fibrinolytic system and their inhibitors (24–27) and might thus contribute to the hemorrhagic complications observed in acute promyelocytic leukemia (28).

Recent in vitro studies have shown that $\alpha_1$-AT is subject to proteolytic inactivation by a variety of proteinases including collagenases and cathepsin $L$ (29, 30, 31) as well as bacterial enzymes (32, 33). Cleavage of the 53-kDa intact inhibitor typically takes place within a short peptide sequence of 10 amino acids around the reactive center Met530 as this region is exposed on the surface of the molecule (29). This reaction leads to an increase in electrophoretic mobility on SDS-PAGE of 4 kDa, corresponding to the liberation of the C-terminal cleavage product (29, 30). However, a 41-kDa fragment has not been described yet. We did not find an association of the appearance of $\alpha_1$-AT* with bacterial or fungal infections or fever of unknown origin in the AML patients studied.

A third possibility stems from the observation that $\alpha_1$-AT is also present in neutrophils and monocytes as well as in normal and leukemic myeloid progenitor cells (34, 35). We could indeed show that a prominent 41-kDa immunoreactive band of $\alpha_1$-AT can be detected in the granule extracts of blast cells from AML patients (7). This finding would indicate that cellular $\alpha_1$-AT could be the precursor of the 41-kDa fragment. At present, we can however not completely rule out the possibility that $\alpha_1$-AT or its 41-kDa fragment could have been taken up by these cells. Therefore, the precise mechanism and the involved proteinase(s) responsible for the generation of $\alpha_1$-AT* remain to be elucidated.

$\alpha_1$-AT* is not absolutely specific for AML as it was also detected, although in relatively small amounts, in the urine of one ALL patient and in three NHL patients. In our opinion, however, this does not reduce the potential value of this molecule. (a) Most currently used tumor markers are not specific for a certain type of cancer or are even found in nonmalignant diseases like infections (36). (b) $\alpha_1$-AT* was not evaluated as a diagnostic tool, but as a possible response parameter in already diagnosed patients. The crucial feature here is the correlation with clinically relevant parameters like therapy response.

We present evidence that a recently characterized novel fragment of proteolytically inactivated $\alpha_1$-AT, which can be quantitated and measured sequentially and noninvasively, is associated with therapy-induced blast cell reduction in AML patients and correlates with time to reach remission, an important prognostic parameter in this disease. The data presented here are the result of a pilot study derived from a limited number of patients. Additional data of more patients have to be collected and analyzed to definitely elucidate the association of $\alpha_1$-AT* with treatment response. In addition, the $\alpha_1$-AT* values will in the future have to be compared with relapse-free and overall survival to find out whether or not this protein can serve as a marker for prognosis in AML patients.

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R Dengler, A Plewan, U Münstermann, et al.


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