HELSINKI UNIVERSITY BIOMEDICAL DISSERTATIONS
No. 4

UROKINASE, UROKINASE RECEPTOR, AND ICAMs
IN HUMAN LEUKEMIA

SATU MUSTJOKI

DEPARTMENT OF VIROLOGY
HAARTMAN INSTITUTE
UNIVERSITY OF HELSINKI
FINLAND

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public criticism in the Small Lecture Hall of the Meilahti Theoretical Institutes, Haartmaninkatu 3, on January 19th, 2001, at 12 noon.

HELSEINKI 2001
Supervised by:  Professor Antti Vaheri
Department of Virology
Haartman Institute
University of Helsinki
Helsinki, Finland

Reviewed by:  Professor Jorma Keski-Oja
Departments of Pathology and Virology
Cancer Biology Laboratory
Haartman Institute
University of Helsinki
Helsinki, Finland

Professor Tapani Ruutu
Department of Medicine
Division of Hematology
Helsinki University Central Hospital
Helsinki, Finland

Opponent:  Professor Keld Danø
Finsen Laboratory
Rigshospitalet
Copenhagen, Denmark

ISSN:  1457-8433
ISBN:  951-45-9684-6 (Printed version)
http://ethesis.helsinki.fi
To my parents
## TABLE OF CONTENTS

### ORIGINAL PUBLICATIONS 7

### ABBREVIATIONS 8

### SUMMARY 9

### INTRODUCTION 11

### REVIEW OF THE LITERATURE 12

#### NORMAL HEMATOPOIESIS AND ITS CONTROL 12

#### LEUKEMIA 13

- Classification 13
- Clinical findings 14
- Acute promyelocytic leukemia 14

#### PLASMINOGEN ACTIVATION SYSTEM IN GENERAL 15

#### PLASMINOGEN AND PLASMIN 15

#### PLASMINOGEN ACTIVATORS 16

- Urokinase-type plasminogen activator (uPA) 16
- Tissue-type plasminogen activator (tPA) 17

#### INHIBITORS OF PLASMINOGEN ACTIVATORS AND OF PLASMIN 18

- PAI-1 19
- PAI-2 20
- $\alpha_2$-antiplasmin 20
- $\alpha_2$-macroglobulin 21

#### UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR) 21

- Structure and general function of uPAR 21
- Soluble uPAR 22
- uPAR and signal transduction 23
- Association of uPAR with vitronectin and integrins: functions in cell adhesion and migration 24
- uPAR in chemotaxis 25

#### REGULATION OF PLASMINOGEN ACTIVATION CASCADE 27

- Regulation by hormones 27
- Regulation by cytokines 27
- Regulation by growth factors 28

#### PLASMINOGEN ACTIVATION SYSTEM IN CANCER 29

#### PLASMINOGEN ACTIVATION IN LEUKEMIA 32

- Components of the PA system in normal blood cells 32
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components of the PA system in leukemia</td>
<td>32</td>
</tr>
<tr>
<td>INTERCELLULAR ADHESION MOLECULES (ICAMs)</td>
<td>34</td>
</tr>
<tr>
<td>Structure and function of ICAMs</td>
<td>34</td>
</tr>
<tr>
<td>ICAMs and leukemia</td>
<td>35</td>
</tr>
<tr>
<td>AIMS AND OUTLINE OF THE PRESENT STUDY</td>
<td>37</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>38</td>
</tr>
<tr>
<td>Patient samples</td>
<td>38</td>
</tr>
<tr>
<td>Cell culturing</td>
<td>39</td>
</tr>
<tr>
<td>Antibodies and molecular probes</td>
<td>40</td>
</tr>
<tr>
<td>Other reagents</td>
<td>40</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assays and other microwell plate assays</td>
<td>41</td>
</tr>
<tr>
<td>Other immunological methods</td>
<td>41</td>
</tr>
<tr>
<td>RNA extraction and Northern analysis</td>
<td>42</td>
</tr>
<tr>
<td>Zymography</td>
<td>42</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>42</td>
</tr>
<tr>
<td>RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>Enhanced plasminogen activation and plasmin formation by retinoids and interferons in acute promyelocytic leukemia cells</td>
<td>43</td>
</tr>
<tr>
<td>Extravasation of blast cells and promyelocytic leukemia cells in organotypic model for vessel wall: impact of various adhesion molecules</td>
<td>45</td>
</tr>
<tr>
<td>Soluble and cellular uPAR and uPA in acute leukemia patients at diagnosis: implications for diagnosis and prognosis</td>
<td>48</td>
</tr>
<tr>
<td>Tumor-cell production of uPAR and uPAR fragments in acute leukemia patients during chemotherapy</td>
<td>54</td>
</tr>
<tr>
<td>Release of soluble uPAR (suPAR) by endothelial cells and peripheral blood mononuclear cells: effect of cell interactions on suPAR release</td>
<td>58</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>61</td>
</tr>
<tr>
<td>Impact of plasminogen activation in leukemia</td>
<td>61</td>
</tr>
<tr>
<td>Invasion of leukemia cells</td>
<td>64</td>
</tr>
<tr>
<td>uPAR- a multifunctional protein. Biological function in vivo in cancer patients?</td>
<td>66</td>
</tr>
<tr>
<td>PERSPECTIVES</td>
<td>69</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>70</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>72</td>
</tr>
</tbody>
</table>
ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.


ABBREVIATIONS

\( \alpha_2 \)M  \( \alpha_2 \)-macroglobulin
ALL  acute lymphoid leukemia
AML  acute myeloid leukemia
APL  acute promyelocytic leukemia
ATF  aminoterminal fragment (of uPA)
ATRA  all-trans retinoic acid
BSA  bovine serum albumin
CD  cluster of differentiation
CLL  chronic lymphocytic leukemia
CML  chronic myeloid leukemia
CNS  central nervous system
CRP  C-reactive protein
D1  domain 1 of uPAR
D2D3  domains 2-3 of uPAR
DBSA  Dulbecco's balanced salt solution containing BSA
DEX  dexamethasone
DIC  disseminated intravascular coagulation
ECM  extracellular matrix
ELISA  enzyme-linked immunosorbent assay
FAB  French-American-British (classification)
FACS  fluorescence-activated cell sorter
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
GAPDH  glyceraldehyde-3-phosphate-dehydrogenase
GPI  glycosyl-phosphatidylinositol
HMEC-1  human microvascular endothelial cells
HUVEC  human umbilical vein endothelial cells
ICAM  intercellular adhesion molecule
Ig  immunoglobulin
IL  interleukin
IFN  interferon
ITP  idiopathic thrombocytopenia
MDS  myelodysplastic syndrome
MMP  matrix metalloproteinase
PA  plasminogen activator
PAGE  polyacrylamide gel electrophoresis
PAI  plasminogen activator inhibitor
plg  plasminogen
PBS  phosphate-buffered saline
PMA  phorbol 12-myristate 13-acetate
PBMC  peripheral blood mononuclear cell
RA  retinoic acid
RAR  retinoic acid receptor
SDS  sodium dodecyl sulphate
SD  standard deviation
SE  standard error
SSC  standard saline citrate
suPAR  soluble uPAR
TGF  transforming growth factor
TNF  tumor necrosis factor
tPA  tissue-type plasminogen activator
uPA  urokinase-type plasminogen activator
uPAR  uPA receptor
WBC  white blood cell
SUMMARY

Adhesion and invasion are important processes both for normal and malignant cells. However, malignant cells usually differ from normal cells in their capability to produce large amounts of proteolytic enzymes, which they use in cell invasion to degrade extracellular matrix proteins. The plasminogen activation cascade is known to be one of the most important extracellular proteolytic mechanisms in adherent tumors, whereas its role in leukemia have remained unclear. It has been thought that the traditional pericellular proteolytic roles of plasminogen activators are unimportant in leukemia, since these cells are growing in "suspension" in the circulation, and they are not invading in the same manner as do adherent tumor cells. It is very likely, however, that the plasminogen activation cascade may play an important role in leukemia as well, because in some circumstances leukemia cells are also invading extramedullary organs and even the central nervous system. In addition, novel non-proteolytic roles for the plasminogen activation system have recently been discovered. We therefore chose to evaluate the roles of the plasminogen activation (PA) system and especially the role of the urokinase receptor (uPAR) in leukemia and to analyze the invasion mechanisms of leukemia cells.

The first goal of the project was to characterize the significance of plasminogen activation in acute promyelocytic leukemia (APL) in cell culture (I). We found that plasmin is formed on the surface of APL cells, and retinoic acid (RA), which is used in the treatment of APL, stimulated transiently plasmin generation. Interferons potentiated RA in its effects on plasminogen activation, and dexamethasone, which relieves the side-effects of RA therapy, totally suppressed the effect of RA on plasminogen activation. These results suggest that plasmin with a potent protease activity can interfere with the normal fibrinolytic balance and be part of a cascade leading to the excessive bleeding problems encountered with APL patients. Increase in uPAR mRNA expression caused by RA fits in well with the results of those clinical studies in which infiltration of maturing myeloid cells into various organs has occurred in some APL patients with retinoic acid syndrome.

The extravasation of APL cells was characterized in a newly established three-dimensional organotypic model for vessel wall in vitro (II). We found that RA induced rapid extravasation of APL cells, and that ICAM-1 antibodies could totally inhibit this extravasation process. In addition to APL extravasation, we also examined the extravasation of normal mononuclear cells and blast cells ex vivo from leukemia patients and evaluated the roles of certain adhesion molecules (ICAM-1, -2, -3, CD11a, and CD18) in these processes (II). Our results show that extravasation of both normal mononuclear cells and leukemia cells could be inhibited with ICAM-1 antibodies, whereas antibodies against the other adhesion molecules had no significant effects. Purified soluble ICAM-1 (sICAM-1) had no effect on this extravasation. Furthermore,
we determined the presence of soluble adhesion molecules (sICAM-1 and sICAM-2) in plasma samples of acute leukemia patients at diagnosis and found that although the levels were increased, they showed no correlation with the numbers of tumor cells in circulation or with the chemosensitivity of blast cells.

Because our in vitro results in APL showed that the PA system and especially the levels of uPA/uPAR varied during RA treatment (I), we decided to analyze the presence of these components in vivo in leukemia patient samples (III, IV). We examined blood samples from patients with newly diagnosed acute leukemia in order to define whether the components of the PA system can be used as tools in leukemia diagnosis and prognosis. Our main findings were that uPAR correlated with the type of leukemia, since only patients with acute myeloid leukemia (AML) and with hybrid leukemia expressed uPAR on their blast cell surface. Blast cells from acute lymphoid leukemia (ALL) patients were negative for uPAR (III). Further, soluble uPAR (suPAR) was significantly elevated in the plasma of patients with acute leukemia, and in AML patients, high levels of suPAR correlated with poor prognosis (III).

Thus far, no longitudinal studies have addressed the behavior of uPAR during treatment, and the source of the excess suPAR in cancer patients remains undetermined. We therefore continued our project in acute leukemia and measured uPAR in the plasma, urine, and tumor cells of patients with acute leukemia during chemotherapy (IV). Our results showed for the first time that the amount of plasma suPAR correlated both with tumor-cell count and with the content of uPAR in cell lysates. We also found that plasma suPAR levels decreased rapidly when tumor cells were removed from the circulation with chemotherapy, suggesting that circulating tumor cells are the source of the suPAR in plasma and in urine samples. Furthermore, we observed that increased amounts of uPAR fragments could be found in the body fluids of acute leukemia patients. Since 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.

We studied next the release of suPAR from various cell types in culture conditions, to understand the cellular origin of the plasma suPAR in healthy individuals (V). Our results showed that suPAR can be found in the culture medium of blood mononuclear cells and endothelial cells. Furthermore, we noticed that enhanced amounts of suPAR were released from the cell surface when either blood mononuclear cells or thrombocytes were cultured in contact with endothelial cells, whereas co-culture without cell-cell contact failed to enhance suPAR release. We also observed that suPAR fragments were present in co-culture growth media. These results suggest that normal plasma suPAR is produced by endothelial and blood mononuclear cells and that its release is enhanced when cells are in physical contact with each other, implying that receptor shedding is a possible regulatory mechanism in cell adhesion.
INTRODUCTION

The plasminogen activation (PA) system comprises several activators, inhibitors, and receptors, all of which have strictly controlled roles (see Fig. 1). Under physiological conditions, activation of plasminogen to proteolytically active enzyme plasmin takes part in many processes, such as in fibrinolysis, cell migration, wound healing, trophoblast invasion, angiogenesis, tissue destruction, and activation of growth factors and of other proteolytic enzymes. Many of these roles of the PA system in normal physiology are equally important in cancer. Cancer cells need to adhere to basement membranes and subsequently detach from them to be able to move on in the extracellular matrix (ECM). They need to degrade various proteins and maintain their own growth by activating various growth factors and enzymes.

In leukemia, the production of normal hematopoietic cells is disrupted, and in the bone marrow, undifferentiated leukemic cells overgrow normal differentiating blood cells. Gradually, these malignant cells burst from the bone marrow into the circulation and in some cases into other organs as well (e.g., skin, gums, and the central nervous system). Although the importance of the PA system, and particularly the essential role of urokinase-type plasminogen activator (uPA), is well established in solid tumors, the significance of pericellular proteolysis is less clear in hematopoietic malignancies. Therefore, in this work, we have chosen to study the importance of the PA system and of various adhesion molecules in leukemia.

**Fig. 1. Overview of uPA-mediated plasminogen activation system.** Plasminogen is a proenzyme that needs to be activated to be able to degrade extracellular matrix proteins. In human beings, there exist two main plasminogen activators: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). In addition to the activators are also two inhibitors, PAI-1 and –2.

uPAR is a specific cell-surface receptor for uPA. Binding of uPA to uPAR localizes plasminogen activation at the cell surface. In addition to its function in proteolytic cascades, uPAR is also associated with several important adhesion proteins such as vitronectin and integrins and plays an important role in cell migration and adhesion.
NORMAL HEMATOPOIESIS AND ITS CONTROL

The life-span of normal blood cells is limited, and these cells are constantly produced in the bone marrow. It has been estimated that approximately 0.5-1x10^{12} new blood cells are formed every day in a healthy adult (Gale and Butturini, 1992). Furthermore, in response to various stimuli (hypoxia, infection, bleeding) the production of certain blood cell types is multiplied. The real hematopoietic stem cell has not been characterized, but recent reports suggest that blood cells and endothelial cells may have a common progenitor cell, called the hemangioblast (Choi et al., 1998). In clinical use, CD34-positive cells are regarded as hematopoietic stem cells, although it is likely that only a small fraction of these cells are true hematopoietic stem cells. However, these cells are able to differentiate into mature blood cells and to repopulate the bone marrow. They are used as stem-cell support in connection with high-dose chemotherapy for various cancers. Pluripotent stem cells give rise to myeloid and lymphoid precursor cells, which in turn can differentiate into mature granulocytes, erythrocytes, monocytes, platelets, and T- and B-cells (see Fig. 2). Several humoral factors have been characterized which are able to regulate the growth and differentiation of hematopoietic precursor cells. Various interleukins, erythropoietin, trombopoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF) can induce the proliferation of hematopoietic cells (Groopman et al., 1989; Metcalf 1991; Ogawa 1993). In contrast, interferons, transforming growth factor-β (TGF-β), and macrophage inflammatory protein-1 reduce the production of hematopoietic cells (Fortunel et al. 2000). In addition, retinoic acid and interferons can induce differentiation of various leukemia cell types (Warrel et al., 1991).

![Fig. 2. Hematopoiesis](image-url)
LEUKEMIA

Classification

In leukemia, the control of the growth of leukemic cells is disrupted, and gradually, these cells overgrow normal differentiating blood cells. These immature cells subsequently burst into the circulation and also into other organs. Leukemias can be roughly divided into acute and chronic leukemias based on their degree of cell-maturation, and myeloid and lymphoid leukemias based on the lineage of differentiation of these malignant cells. In chronic leukemias, malignant cells usually resemble mature blood cells and/or their precursors, but in acute leukemias the differentiation of blood cells ceases at much earlier, primitive stages. In acute leukemias, the French-American-British (FAB) classification is widely used (Bennett et al., 1976), although a new classification system of hematological malignancies has also been described (Harris et al., 1999). According to the FAB classification, acute myeloid leukemias (AML) are divided into eight different subclasses (M0-M7), whereas in acute lymphoid leukemias (ALL) there are three subclasses (L1-L3). However, in ALL, the FAB classification has very limited clinical value; instead, the immunophenotypic classification is usually used. In adults, AML is the most common (80%) acute leukemia. In contrast, in children, most acute leukemias are of lymphoid origin. Yet, acute leukemia is, overall, quite a rare cancer compared to some solid tumors. In Finland, the annual incidence of adult acute leukemia is about 150 new cases (3/10^5 inhabitants), compared to about 3000 breast cancers (60/10^5 inhabitants) (Dickman et al., 1999).

The etiology of acute leukemia is largely unknown. No evidence exists of its inheritability, although in some families it seems that the risk of developing leukemia is higher than in others (Cartwright and Staines, 1992). Some chemicals (such as benzene and ethylene oxide), irradiation, and cytotoxic agents are known risk factors (Cartwright and Staines, 1992), because these can cause gene alterations and chromosomal instability that can block normal blood cell differentiation. In various types of leukemias, several characteristic chromosomal abnormalities occur, and some of these abnormalities can be used as markers for diagnosis, such as the 15;17 translocation in acute promyelocytic leukemia (Larson et al., 1984; Grignani et al., 1994). Furthermore, some chromosomal abnormalities are prognostic markers, such as the Philadelphia chromosome (t9;22) in ALL.

Patients with acute leukemia are usually treated with combination chemotherapy, which consists of several cycles of high-dose cytotoxic agents. After the intensive induction and consolidation phase, patients with ALL usually receive low-dose maintenance therapy. In addition to chemotherapy, a common treatment is stem-cell transplantation. Treatment results have considerably improved during the last two decades, and at present, approximately 30 to 40% of adult patients under the age of 65 years become long-term survivors. In childhood acute leukemia the situation is even more optimistic: of every four children with acute leukemia, three
are cured. Without treatment, median survival time of acute leukemia patients is only 2 to 3 months.

**Clinical findings**

Most of the signs and symptoms of acute leukemia relate to cytopenias caused by deficient production of normal blood cells. The shortage of thrombocytes can cause petechias and bleeding diathesis, and the shortage of normal leukocytes can predispose patients to infections. Fatigue and fever are relatively common. In addition, some of the symptoms are caused by leukemia cells able to infiltrate various organs, such as the skin and gums. Furthermore, infiltration of blast cells into the central nervous system is a feared and often fatal complication. In addition to these symptoms, the intensive treatment of acute leukemia causes numerous other symptoms. On the other hand, patients with chronic leukemia are often asymptomatic for a long time, with their abnormal blood leukocytosis found only by accident during routine laboratory tests. At more advanced stages, problems caused by the large mass of abnormal cells, infections, and also immunological problems may appear. But overall, the general condition of chronic leukemia patients is often better than that of patients with acute leukemia.

**Acute promyelocytic leukemia (APL)**

Acute promyelocytic leukemia is a rare subtype of AML (FAB-type: M3), which accounts for 10% of acute myeloid leukemias (Warrell et al., 1993). It is characterized by a reciprocal 15;17 chromosomal translocation (Larson et al., 1984; Grignani et al., 1994). The translocation breakpoint on chromosome 17 resides within the gene encoding the retinoic acid receptor (RAR-α). This fuses to the PML gene in chromosome 15 and results in production of a fusion protein that blocks the normal myeloid differentiation (Grignani et al., 1993). APL patients more often have coagulation and bleeding problems than do patients with other types of acute leukemia. This hemorrhagic diathesis is thought to result either from disseminated intravascular coagulation or from abnormal fibrinolysis (Tallman and Kwaan, 1992).

APL patients are treated with all-trans-retinoic acid (ATRA) in addition to normal chemotherapy (Castaigne et al., 1990; Warrell et al., 1991; Fenaux, 1993; Tallman et al., 1997; Warrell, 1997). ATRA induces differentiation of leukemic cells into mature granulocytes in vitro and in vivo, and it also reduces APL patient’s bleeding tendency (Dombret et al., 1993). It is often a better-tolerated treatment than normal chemotherapy, but about one-third of the patients encounter severe side-effects from RA treatment, called the retinoid acid syndrome (RA syndrome) (Frankel et al., 1992). The patients with RA syndrome experience fever, bleeding problems, and leukocytosis. Dexamethasone treatment and discontinuation of ATRA administration usually relieve these side-effects, but the mortality is higher among these patients.
than among patients without the syndrome (De Botton et al., 1998). APL patients, overall, show better survival than do other AML patients (Grignani et al., 1994).

PLASMINOGEN ACTIVATION SYSTEM IN GENERAL

PLASMINOGEN AND PLASMIN

Plasminogen is a proenzyme that can be converted to active plasmin, capable of cleaving many extracellular proteins (Pöllänen et al., 1991; Collen, 1999). The gene for human plasminogen is located in the long arm of chromosome 6 (Lijnen and Collen, 1995). The 92 kDa native form of plasminogen, produced mainly by liver cells, is abundantly present in most extracellular fluids and in plasma at a 2.0 µM concentration (Lijnen and Collen, 1995). In its A-chain, plasminogen has 5 homologous kringle domains (Ponting et al., 1992), which have high affinity for lysine, and they mediate the binding of plasminogen to fibrin and to the cell surface. Plasminogen-type kringles are also present in other proteins, such as in hepatocyte growth factor (HGF) and in prothrombin, as well as in tPA and uPA (Ponting et al., 1992). In addition to kringle domains, plasminogen has a highly active serine protease domain in its B-chain in the C-terminal end (Ponting et al., 1992). The cleavage of the peptide bond between Arg-560 and Val-561 converts the one-chain proenzyme plasminogen to the two-chain structure of plasmin. The plasminogen activators, tPA and uPA, mediate this specific cleavage process (Danø et al., 1985; Pöllänen et al., 1991) (see Fig.1 and Table 1).

Plasmin is a trypsin-like serine protease with wide substrate specificity. In addition to fibrin degradation, plasmin can cleave several ECM proteins, such as fibronectin, thrombospondin, and laminin. Plasmin can also indirectly participate in ECM degradation by activating other proteases (latent procollagenases and other metalloproteinases) (Werb et al., 1977; Pöllänen et al., 1991). Knockout studies have revealed that plasminogen is not essential in the embryonic development of mice, since plg-/- mice are born with a normal appearance and survive to adulthood, although they develop spontaneous fibrin lesions due to impaired thrombolysis (Ploplis et al., 1995; Rømer et al., 1996). In addition to fibrin formation, plg-/- mice show high mortality, spontaneous gastrointestinal lesions, and impaired wound healing. Interestingly, studies with plg and fibrinogen double-knockout mice showed that pathology associated with plasminogen deficiency was eliminated with the simultaneous fibrinogen deficiency (Bugge et al., 1996), suggesting that the most important role of plasminogen is fibrinolysis.

The reason for the unexpected normal embryonic development of plg-/- mice may be that there is a functional overlap between plasmin and other extracellular proteinases, which may compensate for the loss of plasmin-mediated proteolysis. This view is strengthened by recent
studies that show that simultaneous inhibition of matrix metalloproteases in plg-/- mice completely arrests wound healing (Lund et al., 1999). In contrast, those normal plg-/- mice without any inhibition of MMPs show delayed wound healing, but eventually wound closure is achieved.

**PLASMINOGEN ACTIVATORS**

**Urokinase-type plasminogen activator (uPA)**

In addition to tissue-type plasminogen activator, uPA is the main activator of plasminogen in humans. In bacteria, there are also other plasminogen activators such as streptokinase and staphylokinase (Rijken, 1995). The name urokinase points to urine, from whence uPA was first purified. In addition to urine, uPA is also present in plasma (Wun et al., 1982b), but in a relatively low concentration of 40 pM with a half-life of 5 min (Collen and Lijnen, 1991). The gene for human uPA is located in the long arm of chromosome 10 (Rajput et al., 1985; Tripputi et al., 1985) (see Table 1).

The 54 kDa protein is produced as a single-chain proenzyme (pro-uPA) (Wun et al., 1982a) and is converted to the two-chain active form by plasmin, which in turn results in activation of plasminogen to plasmin (Pöllänen et al., 1991). This conversion of inactive proenzyme to the fully active enzyme is an important regulatory step in the uPA-mediated plasminogen activation cascade. A large variety of cells can produce uPA in our body: blood leukocytes and also endothelial cells, smooth muscle cells, epithelial cells, fibroblasts (Pöllänen et al., 1991; Camoin et al., 1998; Collen, 1999). In contrast to these adherent cells, all of which produce proenzyme the form of uPA, leukemia cells have an exceptional ability to produce active uPA (see page 33) (Stephens et al., 1988; Tapiovaara et al., 1991; Stephens et al., 1992; Tapiovaara et al., 1996).

uPA comprises several domains (Danø et al., 1985). The growth factor domain is responsible for the binding of uPA to its cellular receptor uPAR and has a homology to epidermal growth factor. The kringle domain, with a homology to plasminogen, promotes binding to other proteins, such as laminin (Stephens et al., 1992). The active site of uPA is located in the C-terminal end in the catalytic domain, which has a homology to other serine proteinases. In addition to plasmin, some other serine proteinases (such as trypsin and kallikrein) and cathepsin B can also activate pro-uPA (Kobayashi et al., 1991; List et al., 2000).

uPA has a specific receptor on cell surfaces, uPAR (CD87) (Behrendt and Stephens, 1998). It binds with high affinity both single- and two-chain forms of uPA as well as uPA in complexes with inhibitors. Binding of uPA to its receptor enhances and localizes plasminogen activation on the cell surface. While tPA is thought to be the main plasminogen activator in fibrinolysis, uPA is thought to be the main activator of plasminogen in cell migration and cell invasion (Pöllänen et al., 1991). Many studies in various cancers have shown that uPA is expressed in increased
amounts by the tumor cells and especially at the leading front of migrating cells (Danø et al., 1994) (See Fig. 1).

Since homozygous deficiencies of uPA or tPA have not been observed in humans, it had been expected that inactivation of these genes in mice would lead to death during embryonic life. However, just like plg-knockout mice, uPA knockouts appear normal at birth, suggesting that uPA is not required for normal embryonic development (Carmeliet et al., 1994). Some of these mice suffer from rectal prolapse and develop spontaneous ulceration and fibrin deposits more often than do wild-type mice, but still had a normal life-span. Studies with tumors showed that melanocytic neoplasms do not progress to malignant melanomas in uPA-deficient mice, suggesting that uPA may be important in tumor progression (Shapiro et al., 1996). However, the effects of uPA deficiency in these knockout mice were overall surprisingly small in the areas to which uPA activity has traditionally been related. But knockout studies revealed some new functions for uPA: uPA-/- mice failed to recruit inflammatory cells in response to inoculation with pathogenic bacteria and developed uncontrollable lethal infection (Gyetko et al., 1996; Beck et al., 1999). This suggests that uPA might play a role in inflammation and chemotaxis, as well. Later, it has also been shown that in uPA knockout mice, T-cell activation and proliferation in response to T-cell receptor-mediated signaling is impaired (Gyetko et al., 1999). This may explain their impaired inflammatory response.

**Tissue-type plasminogen activator (tPA)**

TPA is thought to be the main activator of plasminogen in fibrinolysis (Danø et al., 1985). It has 40% homology with uPA, and it is coded by a different gene located in the long arm of chromosome 8, yielding a protein of 68 kDa (Rajput et al., 1985) (see Table 1). The growth factor domain, kringle domain, and catalytic domain of tPA are similar to those of uPA, but in addition, tPA also has a second kringle domain and an amino-terminal finger domain. This difference in domain structure also has functional consequences, since the second kringle domain, and partially the finger domain of tPA, are essential for its affinity to fibrin. When bound to fibrin, the tPA catalytic activity is markedly enhanced, and it activates plasminogen efficiently, resulting in dissolution of fibrin clots. This specific ability of tPA to locally activate plasminogen on the surface of fibrin has led to its successful use as one thrombolytic therapy in myocardial infarction and in ischemic stroke (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

In the circulation, tPA is found at a concentration of 70 pM with a half-life of 4 min (Lijnen and Collen, 1995). It is also produced as a single-chain proenzyme, but differing from uPA, both the single-chain and two-chain forms are active. Endothelial cells, keratinocytes, and some leukocytes express and release tPA, but no specific high-affinity cell-surface receptor for tPA has been characterized (Redlitz and Plow, 1995). Although uPA is related more closely to the
pericellular proteolysis in malignancy, tPA has been shown to be the main plasminogen activator produced by melanoma and neuroblastoma cells (Neuman et al., 1989; Bizik et al., 1993).

In addition to the function of tPA in fibrinolysis, new, possibly non-proteolytic roles for tPA have been found in neural cell development and remodeling (Carroll et al., 1994). Neurons and microglial cells have been shown to synthesize tPA in most areas of the developing murine brain, and tPA is induced during cerebellar motor learning (Seeds et al., 1996). Furthermore, enhanced tPA activity has been related to multiple sclerosis (MS) (Akenami et al., 1996; Akenami et al., 1997), and it has been suggested that in MS, tPA can participate in neuronal demyelination (Akenami et al., 2000). The first knockout studies revealed rather little of the function of tPA, since these mice were normal at birth, had no macroscopic or histological abnormalities up to the age of 14 months, and had a normal life span (Carmeliet et al., 1994). Only the lysis of pulmonary plasma clots was significantly reduced. It is, however, possible that studies of neuronal function in tPA knockouts reveal additional and more drastic changes, since it has already been shown that tPA-/- mice are resistant to kainate-induced neurotoxicity (Tsirka et al., 1995) and that neuronal migration in tPA knockouts is retarded (Seeds et al., 1999).

Table 1. Components of the plasminogen activation system

<table>
<thead>
<tr>
<th>Component</th>
<th>Chromosomal location</th>
<th>Mol. weight kDa</th>
<th>Amino acids</th>
<th>Plasma conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen</td>
<td>6q26-27</td>
<td>92</td>
<td>791</td>
<td>200 mg/L</td>
</tr>
<tr>
<td>uPA</td>
<td>10q24</td>
<td>54</td>
<td>411</td>
<td>0.002 mg/L</td>
</tr>
<tr>
<td>tPA</td>
<td>8q12</td>
<td>68</td>
<td>530</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td>uPAR</td>
<td>19q13</td>
<td>55</td>
<td>283</td>
<td>0.001 mg/L</td>
</tr>
<tr>
<td>PAI-1</td>
<td>7q21-22</td>
<td>52</td>
<td>379</td>
<td>0.050 mg/L</td>
</tr>
<tr>
<td>PAI-2</td>
<td>18q21</td>
<td>46/60-70</td>
<td>393</td>
<td>&lt;0.005 mg/L</td>
</tr>
</tbody>
</table>

See related reviews (Lijnen and Collen, 1995; Rijken, 1995; Collen, 1999).

INHIBITORS OF PLASMINOGEN ACTIVATORS AND OF PLASMIN

Plasminogen activator inhibitors 1 and 2 (PAI-1 and -2) are the main inhibitors of uPA and tPA (Andreasen et al., 1990), although protease nexin 1 and PAI-3 are also known to affect plasminogen activators to some extent. PAI-1 and -2 are also quite specific for uPA and tPA and react only very slowly with other serine proteases.
Plasminogen activator inhibitor 1 (PAI-1)

The production of PAI-1 was originally described in endothelial cells, and therefore was initially designated as endothelial cell type PA-inhibitor (Hekman and Loskutoff, 1985; Sprengers and Kluft, 1987). However, many other cell types produce PAI-1 as well (e.g., fibroblasts, smooth-muscle cells, and many tumor cells) (Sprengers and Kluft, 1987; Andreasen et al., 1990). PAI-1 is a single-chain 52 kDa glycoprotein and is present in plasma at a 200 pM concentration, although the concentrations vary greatly in different individuals and depending on time of day. Part of the plasma PAI-1 is thought to be released by platelets. The gene for PAI-1 is located in the long arm of chromosome 7 (Rijken, 1995) (see Fig.1 and Table 1).

PAI-1 reacts both with single- and two-chain forms of tPA (Andreasen et al., 1990). This leads to the formation of irreversible enzyme complexes with no proteolytic activity. It has been thought that PAI-1 reacts only with the two-chain form of uPA, but some evidence also exists that PAI-1 may form reversible complexes with the single-chain type of uPA as well (Manchanda and Schwartz, 1995). It can also bind receptor-bound uPA, but fibrin-bound tPA has been shown to react much more slowly with PAI-1 than does free tPA (Rijken, 1995). The main function of PAI-1 in circulation has been thought to be the regulation of tPA activity, and by this function, it proves an important regulator of fibrinolysis. The results obtained from various thrombotic disorders strengthen this view: patients with coronary heart disease and myocardial infarction show elevated plasma PAI-1 levels (Juhan-Vague and Alessi, 1993).

PAI-1-/- knockout mice are viable as are the other PA-system knockout mice (Carmeliet et al., 1993). They resemble tPA-knockout mice in the sense that no macroscopic or histological abnormalities are observable in their organs. However, there are some studies showing that PAI-1 deficiency in humans leads to excessive bleeding disorders (Diéval et al., 1991). Recent results on tumorigenesis in PAI-1 knockout mice have shown that PAI-1 deficiency prevents cancer cell invasion and tumor angiogenesis (Bajou et al., 1998). This fits in well with the clinical observation showing that high PAI-1 level is a poor prognostic marker of various cancers.

In the blood, most of the circulating PAI-1 is bound to vitronectin. In addition, vitronectin in the ECM has been shown to co-localize with PAI-1. The somatomedin B domain in vitronectin is responsible for PAI-1 binding (Deng et al., 1996); uPAR binds to this same domain of vitronectin and competes with PAI-1 binding. It has been suggested that PAI-1 plays a central regulatory role in uPAR/vitronectin-mediated cell adhesion, since an excess of PAI-1 will compete with and replace uPAR from its association with vitronectin, and in turn, an excess of uPA will neutralize PAI-1 and again promote cell adhesion (Loskutoff et al., 1999).

PAI-1 binding to vitronectin prevents integrin binding to vitronectin as well. Active PAI-1 can block vitronectin binding to vitronectin receptor αvβ3 and in this manner, inhibit cell migration (Stefansson and Lawrence, 1996). However, when uPA binds to PAI-1, it abolishes the ability of PAI-1 to bind to vitronectin and restores cell migration by allowing vitronectin again to bind
Review of the Literature

integrins (Loskutoff et al., 1999). This suggests that the function of uPA at the leading edge of migratory tumor cells is not only to enhance plasminogen activation and pericellular proteolysis, but also to reinforce cell migration by blocking PAI-1 binding to vitronectin. However, because the synthesis of PAI-1 is rapidly stimulated by a variety of growth factors and cytokines, it is likely that PAI-1 binding and release from vitronectin, or from the extracellular matrix in general, occurs in a stepwise manner, and therefore, the pro- and contra-adhesive signals are following each other in a continuous cascade. Taken together, through this vitronectin-binding ability, PAI-1 is an important regulator of cell adhesion and migration.

**Plasminogen activator inhibitor 2 (PAI-2)**

PAI-2, earlier called placental-type plasminogen activator inhibitor, as it was originally purified from human placenta, is a 46 kDa protein expressed by monocytes, keratinocytes, and placental syncytiotrophoblasts (Sprengers and Kluft, 1987; Andreasen et al., 1990). It also has another higher molecular weight protein form (60-70 kDa), which is found extracellularly and has a higher degree of glycosylation. The normal PAI-2 plasma levels are very low (<70 pM) or even undetectable, but during pregnancy the levels increase (Rijken, 1995). The PAI-2 gene has been mapped to chromosome 18 (Rijken, 1995) (see Table 1).

PAI-2 mainly inhibits uPA (Baker et al., 1990), as it has been shown to react considerably more slowly with tPA than with uPA. In vivo functions of PAI-2 are less well known than those of PAI-1. Bacterial endotoxins induce PAI-2 expression in monocytes, suggesting that PAI-2 may be involved in inflammatory processes (Belin, 1993). Furthermore, PAI-2 locally inhibits cancer cell degradation of basement membranes (Baker et al., 1990). However, PAI-2 knockout mice have not been described in the literature, maybe because no major important roles for PAI-2 have thus far, been found.

**α₂-antiplasmin**

α₂-antiplasmin is the main inhibitor of plasmin in the plasma. The plasma concentration of α₂-antiplasmin is high (1 μM) (Lijnen and Collen, 1995), and it is able to react rapidly with plasmin. It has been calculated that the half-life of free plasmin in the blood is only 0.1 sec (Collen, 1999). The gene for α₂-antiplasmin has been located in the short arm of chromosome 18, and the protein produced has a molecular weight of 70 kDa (Lijnen and Collen, 1995).

In vivo, α₂-antiplasmin deficiency has been related to serious bleeding complications (Collen, 1999). Homozygous deficiency has been shown to be associated with severe hemorrhagic diathesis, whereas only mild bleeding problems are seen with the heterozygotic phenotype. In addition, enhanced fibrinolysis in disseminated intravascular coagulation in APL patients is possibly caused by acquired α₂-antiplasmin deficiency (see page 34) (Dombret et al., 1993; 1995).
\( \alpha_2 \)-macroglobulin

In addition to \( \alpha_2 \)-antiplasmin, \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M) is also an important inhibitor of plasmin in plasma (Borth, 1992). However, it is rather unspecific for plasmin, as it is able to inhibit almost any type of proteinases. \( \alpha_2 \)M is a high molecular-weight (725 kDa) glycoprotein, and its plasma concentration is 3 \( \mu \)M. The gene for \( \alpha_2 \)M is located in the short arm of chromosome 12 (Collen and Lijnen, 1991).

\( \alpha_2 \)M is produced by a large variety of cells: fibroblasts, monocyte-macrophages, hepatocytes, astrocytes (Borth, 1992). Some tumor cells, e.g., melanoma and sarcoma cell lines, have also been observed to secrete this protein (Bizik et al., 1986). \( \alpha_2 \)M is not only a broad-spectrum protease inhibitor. It functions also as a carrier protein for several growth factors, such as transforming growth factor \( \beta \) (TGF-\( \beta \)) and platelet-derived growth factor (PDGF) (Borth, 1992).

Cellular receptor for \( \alpha_2 \)M (\( \alpha_2 \)MR) is identical with a low-density lipoprotein receptor-related protein (LRP). This receptor is a multi-functional protein also capable of binding and internalizing uPA-uPAR-PAI-1 complexes. In addition, it has been defined as a monocyte differentiation marker, because it appears at an early stage of monocyte differentiation and is not expressed by other types of blood cells (Borth, 1992).

UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR)

Structure and general function of uPAR

A high affinity and specificity cell-surface receptor for uPA, uPAR, was first discovered in monocytes (Vassalli et al., 1985) and in monocytoid U937 cells (Stoppelli et al., 1985; Vassalli et al., 1985), and since then it has been characterized in a variety of cell types such as granulocytes, endothelial cells, keratinocytes, fibroblasts, and smooth-muscle cells (Blasi et al., 1994; Danø et al., 1994; Behrendt and Stephens, 1998). uPAR binds to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991) (Fig. 3) and thus has no intracellular portion. The heavily glycosylated protein consists of three homologous domains (D1-D3), which have different binding properties for various ligands. The amino-terminal domain D1 is responsible for the binding of uPA (Behrendt et al., 1990; Behrendt et al., 1991), and the other two, D2 + carboxyterminal domain D3, are important in vitronectin binding (Wei et al., 1994). However, the full-size receptor is required for high-affinity interactions with both of these ligands (Ploug et al., 1994; Wei et al., 1994; Behrendt et al., 1996; Riittinen et al., 1996; Høyer-Hansen et al., 1997a; Sidenius and Blasi, 2000). The uPAR gene, encoding a 55 kDa protein, is located in the long arm of chromosome 19 (Collen and Lijnen, 1991) (see Figs. 1, 3 and Table 1).
uPAR binds both single- and two-chain forms of uPA, as well as uPA complexed with PAI-1. uPA/PAI-1 complexes will be internalized rapidly (Olson et al., 1992) when bound to the receptor, but free uPA remains attached to the cell surface. This internalization facilitates recycling of the receptor, since the receptor is internalized together with the activator-inhibitor complex, and subsequently, free receptor is recycled to the cell surface (Nykjaer et al., 1997). α₅MR/LRP is involved in this endocytosis process.

At first, uPAR was thought to occur only in its full-size form on the cell surface, but later, also a cleaved form (D2D3) lacking the amino-terminal uPA-binding domain D1 was discovered in vitro in U937 cells (Høyer-Hansen et al., 1992) and also in vivo in transplanted murine tumors (Solberg et al., 1994). Several enzymes (uPA, plasmin, and chymotrypsin) can cleave the receptor at the linker region between domains 1 and 2 (Behrendt et al., 1991; Høyer-Hansen et al., 1992; Høyer-Hansen et al., 1997b). The receptor cleavage by uPA occurs quite slowly in solution, whereas on the cell surface the cleavage is highly accelerated (Høyer-Hansen et al., 1997b). All of this suggests that the receptor cleavage is a negative feedback mechanism for plasminogen activation on the cell surface. Both phosphatidylinositol-specific phospholipase C and D are able to catalyze the cleavage of the whole receptor from the cell surface in vitro (Ploug et al., 1991; Wilhelm et al., 1999), but the physiological enzyme functioning in vivo remains unknown (see Figs. 3 and 4).

The primary function of uPAR originally appeared to be the localization of uPA and the whole PA system onto specific areas on the cell surface. Through this function, uPAR participates in localized proteolysis in wound healing, ovulation, angiogenesis, and cancer invasion (Danø et al., 1994). Unexpectedly, the first knockout studies found no abnormalities in uPAR-deficient mice: the mice were viable, healthy, and fertile (Dewerchin et al., 1996). Later on, new, non-proteolytic functions for uPAR have been discovered, meaning that the function of uPAR is no longer limited solely to the localization of plasminogen activation on the cell surface (Danø et al., 1994; Blasi, 1997; Chapman, 1997; Blasi, 1999a; Blasi, 1999b; Preissner et al., 2000; Waltz et al., 2000); it has other functions as well (see functions of uPAR below).

**Soluble uPAR (suPAR)**

The soluble form of the uPA receptor (suPAR) was first discovered in plasma from patients with paroxysmal nocturnal hemoglobinuria (Ploug et al., 1992) and from ascitic fluid of ovarian cancer patients (Pedersen et al., 1993). No uPA-ligand was bound to it, and it was also devoid of the GPI anchor. Later, suPAR was also found in plasma from healthy individuals (Rønne et al., 1995; Stephens et al., 1997), and the levels were quite stable at a median concentration of 1.2 ng/ml (range 0.8-1.7 ng/ml), with no correlation between age and suPAR (Stephens et al., 1997). In addition to plasma/serum, suPAR also exists in cystic fluid of carcinoma patients (Wahlberg et al., 1998) and more recently in their and in control urine samples (Sier et al., 1999). Such
urine samples have been shown to contain uPAR fragments D2D3 and D1 in addition to the full-length receptor (Sier et al., 1999; Sidenius et al., 2000), whereas only the full-length form of uPAR is present in plasma samples (Sidenius et al., 2000). However, the physiological mechanisms involved in the shedding of fragments or the entire receptor from the cell surface have remained unknown (see Fig. 3).

In several different types of malignancies, elevated levels of serum/plasma suPAR have been detected. For example, patients with non-small-cell lung cancer (Pappot et al., 1997), and cancer of the breast (Stephens et al., 1997), colon (Stephens et al., 1999), ovary (Sier et al., 1998), and prostate (Miyake et al., 1999), and with head and neck squamous cell carcinoma (Schmidt and Hoppe, 1999) have significantly increased plasma suPAR levels. Furthermore, it has recently been shown that suPAR levels correlate with survival prognosis, with those ovarian and colon cancer patients showing high suPAR levels having a worse survival prognosis (Sier et al., 1998; Stephens et al., 1999). The origin of suPAR in cancer patients' plasma has been unknown (Brännner et al., 1999), and only recently was it demonstrated that mice carrying transplanted human xenograft tumors have human suPAR in their plasma (Holst-Hansen et al., 1999), suggesting that suPAR may be derived from tumor cells. The functions and importance of the soluble receptors in vivo in body fluids are also largely unknown, although in vitro experiments have shown that suPAR fragments may be involved in chemotaxis (Resnati et al., 1996; Fazioli et al., 1997) (see below).

**uPAR and signal transduction**

There have been mechanical problems in understanding how uPAR can activate intracellular signal transduction pathways, because it has no transmembrane or intracellular domain. One possibility is that the activation of uPA, when bound to the receptor, induces the activation of latent growth factors (like TGF-β and pro-HGF), which in turn can activate signal transduction pathways (Besser et al., 1996; Koshelnick et al., 1999). However, in chemotaxis, current results...
suggest that no proteolytic activity of uPA is needed to induce the activation of p56/p59 hck kinases (Resnati et al., 1996). Furthermore, synthetic uPAR peptides, which carry a specific epitope (see uPAR in chemotaxis, below), are able to induce chemotaxis in THP-1 cells and activate the same tyrosine kinases (Fazioli et al., 1997). In addition, uPAR appears to be physically associated with a number of other intracellular proteins, which participate in signal transduction pathways (such as G-proteins, STAT proteins, and protein kinase C) (Koshelnick et al., 1997; Dumler et al., 1998; Ossowski and Aguirre-Ghiso, 2000; Preissner et al., 2000). It has therefore been suggested that a special transmembrane adaptor molecule exists, which can mediate the signals between extracellular uPAR and intracellular kinase pathways (Resnati et al., 1996; Blasi, 1999b). However, such an adaptor molecule has not been identified as yet, and it is also possible that several of them exist, since not all signals induced by uPA-uPAR interaction are uniform. The transmembrane glycoprotein 130 and β1-integrins are possible adaptor-molecule candidates, because both these molecules are able to form complexes with uPAR and activate intracellular signaling pathways (Aguirre-Ghiso et al., 1999; Koshelnick et al., 1999; Yebra et al., 1999). In any case, the conformational change in uPAR appears to be the prerequisite for inducing a signaling pathway (Ossowski and Aguirre-Ghiso, 2000).

**Association of uPAR with vitronectin and integrins: functions in cell adhesion and migration**

The first reports of uPAR association with vitronectin and integrins came only a few years after the discovery of uPAR. Bohuslav et al. (1995) showed that in human monocytes, uPAR is one part of a receptor complex also including β2-integrins LFA-1 and Mac-1 and several protein tyrosine kinases. This fact suggested functional cooperation between these molecules. Later, Xue et al. (1997) observed that uPAR associates with β1 and β3 integrins also in cells of non-hematopoietic origin, namely in fibrosarcoma cells. Furthermore, uPAR was able to form stable complexes with integrins, which promoted cell-adhesion to vitronectin (Wei et al., 1996). Recently, it was observed that uPAR interacts directly with integrins. The interaction site of uPAR in Mac-1 was found to be in the CD11b subunit, and one peptide from this area disrupted the association of uPAR with specific β1 and β2 integrins, and thus broadly impaired integrin function (Simon et al., 2000).

uPAR interaction with integrins is also important in leukocyte migration. First, Bianchi et al. (1996) showed that the stimulus likely to be associated with interaction between T-cells and endothelial cells or ECM (co-clustering of the antigen receptor complex and β1 or β2 integrins) induced the expression of uPAR in T-lymphocytes. Secondly, in uPAR-deficient mice, the β2-integrin-dependent recruitment of leukocytes to the inflamed area is markedly reduced, and also the removal of uPAR from the leukocyte surface blocks the β2-integrin-mediated leukocyte adhesion to endothelium in vitro (May et al., 1998; Preissner et al., 2000).
The uPAR/vitronectin/PAI-1 interaction is a complex procedure that regulates cell adhesion (Blasi, 1997). It has been demonstrated that vitronectin binding to uPAR induces specific uPAR-dependent cell adhesion (Carriero et al., 1999). But as PAI-1 and uPAR have a common binding site in vitronectin in the somatomedin B domain, they compete with each other (Deng et al., 1996). An excess of PAI-1 will replace uPAR from vitronectin and detach cells from vitronectin and inhibit cell migration, whereas an excess of uPA will neutralize PAI-1 and release adhesion-promoting vitronectin, and therefore give the "go" signal for uPAR-vitronectin-dependent cell migration (Deng et al., 1996). These findings can explain the clinical observations that high tumor PAI-1 level is a poor prognostic marker (Schmitt et al., 1997), because PAI-1-mediated release of cells from vitronectin can promote dissemination of these cells to distant sites, i.e., promote metastasis formation.

In addition to these non-proteolytic mechanisms such as uPAR binding to various integrins as well as to vitronectin cell migration can also be promoted by uPAR-mediated proteolysis. Especially in tumor cells, uPAR is localized at the leading edge of migrating cells. Therefore, by binding uPA, uPAR locally enhances plasminogen activation and the formation of plasmin, which in turn, can degrade ECM components. However, as other ligands (vitronectin, integrins, PAI-1) are also present at the same sites, it is likely that the proteolytic and non-proteolytic cell migration-promoting mechanisms of uPAR function in a tightly coordinated manner in vivo.

### uPAR in chemotaxis

Gyetko et al. (1994) have found that monocyte chemotaxis in vitro is dependent on uPAR. First, when monocytes were exposed to a chemotactic gradient, uPAR localized strongly to the leading front of migrating cells. Secondly, monoclonal antibodies against uPAR inhibited the monocyte chemotaxis induced by FMLP in Boyden chambers. Moreover, they noticed that the function of uPAR was basically independent of active uPA, as inactivation of the catalytic activity of uPA had no marked effect on monocyte chemotaxis (Gyetko et al., 1994). Recently the same authors reported that in response to *Pseudomonas aeruginosa* infection, neutrophil recruitment is reduced in uPAR knockout mice, but in uPA knockouts no difference occurred in recruitment (Gyetko et al., 2000). In addition, their results suggested that recruitment of neutrophils requires both β2-integrins and uPAR. Earlier they reported that uPA-deficient mice have an impaired inflammatory response to pulmonary *Cryptococcus neoformans* infection (Gyetko et al., 1996). However, uPAR was still functionally involved, as only the recruitment and migration of uPAR-expressing leukocytes was affected in uPA -/- mice and not the recruitment of B-cells (those not expressing uPAR) (Gyetko et al., 1996). These results suggested that uPA may play a role through uPAR in chemotaxis. At the same time, Resnati et al. (1996) found that physiological concentrations of uPA as well as of the aminoterminal fragment of uPA (ATF), which is capable of binding to the receptor but has no proteolytic activity, induced chemotaxis in monocytoïd
THP-1 cells. However, the monoclonal antibody that blocks the binding of ATF to uPAR inhibited the chemotactic activity of ATF. In addition, purified suPAR is capable of inducing chemotaxis in the same cells, but the activity of suPAR in chemotaxis requires the cleavage of the purified protein by chymotrypsin, at the same site where uPA can cleave uPAR. This observation gave further support to the view that the interaction of uPA/uPAR is important in chemotaxis, a view strengthened by the following experiments: Fazioli et al. (1997) tested the chemotactic effect of several uPAR variants expressing different fragments of uPAR and found that the chemotactic activity lies in the protease-sensitive region between domains 1 and 2, i.e., in the same region efficiently cleaved by physiological concentrations of uPA. Furthermore, only those synthetic peptides containing the sequence 88-92 of the uPAR linker region had chemotactic activity, whereas peptides containing other parts of uPAR had no chemotactic activity (see Fig. 4). In addition, in smooth-muscle cells, the cleaved form of suPAR and a peptide covering the area between domains 1 and 2 induced chemotaxis and cytoskeleton reorganization, which was accompanied by relocalization of uPAR, vitronectin receptor, and src tyrosine kinase to the leading edge of migrating cells (Degryse et al., 1999). It therefore seems that uPA cleavage is required for the conformational change in uPAR that uncovers its potent chemotactic epitope. Although these are results only from in vitro experiments, it is tempting to think that the uPAR fragments now found in cancer patients' urine and ascitic fluid samples may have biological functions in vivo (Wahlberg et al., 1998; Sier et al., 1999). However, the detailed amino-acid sequence of these in vivo fragments needs to be determined first, and the chemotactic activity of the purified fragments obtained from ex vivo patient samples needs to be tested.

**Fig. 4. Linker region of urokinase receptor.** This diagram presents a protease-sensitive region of uPAR between domains 1 and 2. Chemotactic activity of uPAR has been reported to be located between aminoacids 88 and 92 (Fazioli et al., 1996)
REGULATION OF PLASMINOGEN ACTIVATION CASCADE

The plasminogen activation system is regulated at many different levels. As described above, several inhibitors (e.g., PAI-1 and -2) regulate the activity of plasminogen activators and in that manner the whole activity of the PA cascade. This activity is also regulated by controlled secretion of various components of the cascade, by conversion of proenzymes to active enzymes, and by degradation or recycling of the components (e.g., recycling of the urokinase receptor). In addition to this regulation at the protein level, the regulation naturally occurs also at the transcriptional and translational level.

Regulation by hormones

In various cell types (both adherent and hematopoietic cells), glucocorticoids have been reported to suppress uPA synthesis (Danø et al, 1985). The enhancer area in the uPA gene is negatively regulated by dexamethasone. However, the mechanism is probably indirect, because the glucocorticoid receptor fails to bind directly to the enhancer region (Besser et al., 1996). In addition to their suppressive effects on uPA synthesis, glucocorticoids also seem to suppress uPA activity. This is, in part, mediated by inhibitors, because dexamethasone, for example, can increase PAI-1 expression in various cell types. The effect of glucocorticoids on tPA activity depends on cell type. In some cell types, they seem to have no effect on tPA (e.g., in melanoma cell lines), but in primary cultures of acute myeloid leukemia cells, glucocorticoids can either stimulate or suppress tPA, depending on the patient from whom the cells came (Wilson et al, 1983a).

In addition to glucocorticoids, many other steroid hormones can also affect plasminogen activator synthesis and activity: for instance, estrogen can increase uPA activity in rat uterus and in human breast carcinoma cells, and androgens can induce plasminogen activator activity in breast carcinoma cells. Other hormones as well, such as calcitonin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), are able to either down- or up-regulate the plasminogen activation cascade depending on cell type (Danø et al, 1985).

Regulation by cytokines

Inflammatory cells and endothelial cells produce various cytokines, such as interleukins (ILs), tumor necrosis factor-α (TNF-α), and interferons. In addition, these same cells (monocyte-macrophages, activated lymphocytes, and endothelial cells) are able to produce uPA. Furthermore, in vitro studies have demonstrated that all these cytokines are able to increase uPA activity (Hovi et al., 1981; van Hinsbergh et al., 1990; Gyetko et al., 1993). In monocytes, too, the granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to increase uPA activity (Hart et al. 1991). In addition to uPA, IL-1 and TNF-α can also induce the
production of PAI-1 and –2, and similarly, in monocytoid cells, uPAR is upregulated by TNF-α and interferon-γ (Lu et al., 1988). Furthermore, normal lymphocytes show no uPAR expression on the cell surface, but various interleukins can induce uPAR expression in these cells (Nykjær et al., 1994). In human myeloid cells, cytokines such as TNF-α prime myeloid cells for uPA-mediated signals leading to cell adhesion (Waltz et al., 1993).

**Regulation by growth factors**

The plasminogen activation system interacts with growth factors in many ways. On the one hand, uPA and/or plasmin can activate various latent growth factors (Taipale and Keski-Oja, 1997; Taipale et al., 1998) such as transforming growth factor-beta (TGF-β) and hepatocyte growth factor (HGF), and on the other hand, these same growth factors can regulate the expression of various components of the PA system (Laiho and Keski-Oja, 1989; Rifkin et al., 1999).

Growth factors have high-affinity receptors on cell surfaces. By binding to the extracellular part of the receptor, growth factors trigger intracellular signals via various kinase pathways. But TGF-β, for example, is released from cells in an inactive form unable to bind the receptor. The inactive latent form consists of TGF-β in complex with its binding protein (LTBP), and this interaction inhibits the ligand-receptor interaction (Taipale et al., 1998). Plasmin, together with other proteases, is able to release the TGF-β molecule from its propeptide protein and disrupt the small latent TGF-β complex (Lyons et al., 1988). Therefore, it is able to play an important role in TGF-β activation.

In normal physiology, TGF-β is a growth modulator which enhances ECM production and suppresses ECM proteolysis. In various cell types, TGF-β induces PAI-1 expression and suppresses the expression of both uPA and tPA (Laiho et al., 1987; Laiho and Keski-Oja, 1989). In addition, TGF-β enhances uPAR synthesis in various cell types, such as lung carcinoma cells (Lund et al., 1991) and retinal pigment epithelial cells (Sirén et al., 1999). In malignancy, TGF-β may act either as a tumor suppressor by inhibiting cell growth and degradation of ECM, or as an oncogene because of its angiogenic effects. In addition, TGF-β is able to induce many other growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which in turn also have an effect on various components of the PA system. Both bFGF and HGF can enhance PAI-1 expression (Wojta et al., 1999). In endothelial cells, in addition to increased PAI-1 expression, bFGF increases uPA activity (Mandriota and Pepper, 1997). Furthermore, uPA and plasmin release VEGF and other growth factors from ECM (Taipale and Keski-Oja, 1997) and in this manner promote angiogenesis. In endothelial cells, the binding of VEGF-B to its receptor has also been shown to lead to increased expression of uPA and PAI-1 (Olofsson et al., 1998), suggesting that VEGF-B may play a role in extracellular proteolysis and cell adhesion. Taken
Review of the Literature

together, these reports confirm that several growth factors either directly induce or suppress the
synthesis of plasminogen activators or indirectly affect their activity by inducing or repressing
the expression of plasminogen activator inhibitors. In cancer, this regulation can be disrupted;
the synthesis and activation of various components of PA system may then occur in an
uncontrolled manner.

PLASMINOGEN ACTIVATION SYSTEM IN CANCER

The traditional view of the PA system in cancer was that tumor cells produce enhanced
amounts of plasminogen activators, mainly uPA, which in turn activate plasminogen to plasmin.
Plasmin then catalyzes the degradation of ECM proteins and in this manner, facilitates cancer
cell invasion into the tissues (Mignatti and Rifkin, 1993; Bell, 1996) (see Fig. 5). However,
because novel functions are being found for different components of the PA system, also the
traditional view has been broadening (Andreasen et al., 2000). Although uPA has been
suggested to be the main plasminogen activator in cancer—in melanoma and neuroblastoma—tPA
may play a role (Neuman et al., 1989; Bizik et al., 1993). Furthermore, that endothelial cells
produce a lot of tPA can be related to vasculature formation in cancer.

Both activators and inhibitors of the PA system as well as uPAR are widely expressed by
various tumor cell lines (Kwaan et al., 1991; Andreasen et al., 1997). Also in vivo, primary
tumors and metastases both express these same components in enhanced amounts (Bianchi et
al., 1994). In addition to tumor cells, also stromal cells and tumor-infiltrating macrophages can
produce several components of the PA system (Pyke et al., 1993). Indeed, it has been proposed
that tumor cells act in concert with stromal cells in cancer invasion (Johnsen et al., 1998). In this
manner, the synthesis of proteolytic factors can be distributed between neoplastic and non-
neoplastic cell types, and it is possible that tumor cells recruit and control stromal cells in order
to produce these factors (Danø et al., 1994; Danø et al., 1999). This view is strengthened by
results from in vivo studies; for example, in breast carcinoma, uPAR is expressed more often in
tumor-associated macrophages in the areas of infiltrating cancer cells than in the actual tumor
cells (Pyke et al., 1993).

Studies in the early eighties were already showing that the inhibition of uPA impaired tumor-
cell invasion in experimental tumor models (Ossowski and Reich, 1983). In addition,
enhancement of uPA expression increases the in vitro invasiveness of tumor cells. Systemic
analysis of tumor tissues and plasma samples of cancer patients confirms further the importance
of the PA system in cancer invasion (Andreasen et al., 1997). It has been demonstrated that the
expression of uPA is associated with unfavorable prognosis in several cancer types, such as
breast and colon carcinoma (Ganesh et al., 1994; Andreasen et al., 2000). Furthermore, uPA
correlates with the histologic grade and with the metastatic potential of the carcinoma. In addition to uPA, also high tumor antigen PAI-1 and uPAR levels and decreased PAI-2 levels are associated with poor disease outcome (Schmitt et al., 1997; Stephens et al., 1998). However, as the measurements of PA components in the tumor tissue are often quite laborious and also difficult to standardize (Stephens et al., 1998), attempts have been made to correlate the levels of uPA, uPAR, and PAI-1 in the circulation with prognosis. Circulating uPAR levels in cancer patients have already been shown to associate with poor survival prognosis in colon carcinoma and ovarian carcinoma patients (Sier et al., 1998; Stephens et al., 1999).

In addition to tumor cell invasion, the PA system may also participate in other cancer-related processes, such as in hemostatic disorders often suffered by cancer patients (Carroll and Binder, 1999) and in angiogenesis (Carmeliet and Collen, 1998; Reijerkerk et al., 2000). Indeed, reports have described severe bleeding disorders in cancer patients with normal platelet counts and other fibrinolytic markers, but active uPA and plasmin generation in their circulation and production of uPA by their tumor cells (Bennett et al., 1997). In angiogenesis, the uPA-mediated PA system has also been shown to play an important role. uPA and plasmin can activate latent growth factors (like HGF, TGF-β), which in turn can regulate the formation of new vessels (Carmeliet and Collen, 1998) (Fig. 5). Furthermore, plasmin-activated matrix metalloproteinases stimulate endothelial cell proliferation and migration (Werb et al., 1999). Vascular endothelial cell growth factor (VEGF) upregulates uPA/uPAR expression in endothelial cells, and plasmin can release VEGF from the ECM (Carmeliet and Collen, 1998). Moreover, angiotatin, which has potent antiangiogenic activity, is a section of plasminogen, consisting of its kringle 1 to 4 (Reijerkerk et al., 2000).

In cancer cell invasion, the PA system may also function in concert with other proteinases. Many matrix metalloproteinases have also been related to tumor-cell invasion by their degradation of ECM proteins (Blobel, 2000; Curran and Murray, 2000), and in MMP-2 deficient mice, tumor progression, for example, is significantly decreased (Itoh et al., 1998). Plasmin’s activation of several metalloproteinases can facilitate cancer cell invasion through the other proteinases (Fig. 5). Furthermore, as shown in wound-healing experiments in mice, simultaneous inhibition of plasminogen and of a variety of matrix metalloproteinases totally arrested wound healing, suggesting a functional overlap between these two systems which may also have an effect on the tissue remodeling processes in cancer (Lund et al., 1999).

Because in vitro experiments have shown that cancer cell invasion can be quite efficiently inhibited by antibodies against uPA or uPAR (Crowley et al., 1993; Reijerkerk et al., 2000), many attempts have been made to develop for cancer patients similarly acting drugs for anti-invasive therapy. The classic old proteinase inhibitors inhibit both uPA and tPA and may, in this way, disturb fibrinolytic balance. Therefore, a need exists for selective uPA inhibitors or for inhibitors inhibiting the binding of uPA to its receptor (Tressler et al., 1999). The uPA active-site
inhibitor B-428 inhibits breast carcinoma invasion and metastasis in the in vitro matrigel model, as well as in mice (Xing et al., 1997; Alonso et al., 1998). Furthermore, molecules inhibiting uPA binding to the receptor (e.g., peptides antagonizing uPA-uPAR interaction) have been demonstrated to inhibit angiogenesis and tumor metastasis formation in mice (Min et al., 1996; Guo et al., 2000; Mishima et al., 2000). Although these experiments are encouraging, it remains to be seen whether these molecules may be useful in clinical tumor therapy in the future.

Fig. 5. Proteinases in cancer cell invasion. Cancer cell invasion is a complex process involving several enzymes, growth factors, and matrix and membrane proteins. Invasive cells can secrete both matrix metalloproteinases (MMPs) and serine proteases (plasmin), which also interact with each other. Cancer cells can also recruit stromal cells to produce these enzymes. Growth factors are able to induce the production of these enzymes and their inhibitors, but in addition, plasmin, for example, controls the activation of certain growth factors. Furthermore, several inhibitors (PAI-1, TIMP) control the activity of enzymes, and PAI-1 also mediates cell adhesion and migration pathways together with integrins, vitronectin, and uPAR.
PLASMINOGEN ACTIVATION IN LEUKEMIA

Components of the PA system in normal blood cells
Several types of leukocytes express various components of the PA system. For example, uPAR and uPA are expressed by monocytes, macrophages, neutrophils, and myeloid precursors, but not usually by lymphocytes (Wilson et al., 1983b; Miles and Plow, 1987; Wilson et al., 1992; Plesner et al., 1994b; Jardi et al., 1996; Plesner et al., 1997; Mustjoki et al., 1999). However, T-cells from HIV-positive patients express uPAR (Nykjaer et al., 1994), and interleukin or PMA stimulation can also induce uPAR expression in normal lymphocytes (Nykjaer et al., 1994). In addition, it has been recently reported that antibodies against uPAR inhibit dendritic cell-induced T-cell activation, suggesting that uPAR may also play a role in antigen-presenting cell/T-cell interaction (Woodhead et al., 2000). In thrombocytes and erythrocytes no uPAR has been found, but our previous studies demonstrated that megakaryocytes express uPAR on the cell surface in the bone marrow. Both tPA and uPA are expressed by various types of leukocytes. Wilson and Francis (1987) have observed that the type of plasminogen activator produced by leukocytes is a differentiation-linked property. In normal bone-marrow cells, uPA was secreted by the more differentiated cells, whereas the more primitive cells released tPA. tPA protein and mRNA have also been characterized in bone marrow megakaryocytes (Jeanneau and Sultan, 1988; Brisson-Jeanneau et al., 1990). Plasminogen activator inhibitors, PAI-1 and PAI-2, are also produced by blood cells. PAI-2 is mostly expressed by monocytes but not by other leukocytes, whereas PAI-1 is released by thrombocytes, and in addition, in normal bone marrow, PAI-1 mRNA has been found in megakaryocytes (Alessi et al., 1994).

An active fibrinolytic system exists in normal human bone marrow (McWilliam et al., 1996). In contrast to plasma, normal bone marrow has higher levels of active tPA, despite the inhibitors PAI-1 and PAI-2. In addition, the levels of uPA, α2-antiplasmin, and tPA-PAI-1 complexes are low, favoring fibrinolysis in the bone marrow (McWilliam et al., 1996). This tPA activity may be necessary for removing fibrin deposits and ensuring sufficient blood flow through the bone marrow.

Components of the PA system in leukemia
Similarly to normal leukocytes, leukemic cells were also found to produce plasminogen activators into their growth medium (Wilson et al., 1983b; Tapiovaara et al., 1996). Leukemic cells from AML patients produce quite different amounts of plasminogen activators into the growth medium, but the amount produced has no correlation with the clinical severity of the disease (Wilson et al. 1983b). Leukemic cells are able to produce both uPA and tPA, whereas normal granulocytes produce only uPA. Another interesting finding was that patients whose
cells produce only tPA fail to respond to combination chemotherapy (Wilson et al., 1983b). As later experiments showed that in healthy individuals more primitive leukocytes produce tPA, and mature leukocytes produce uPA, the authors postulated that this may be the reason for the tPA producers’ poor response, as poor responses are more often seen in patients whose cells have early-progenitor-type features (Wilson and Francis, 1987). Moreover, both plasminogen activators and their inhibitors occur in higher amounts in leukemic cell homogenates from AML patients than from ALL patients (Wada et al., 1993), and plasma uPA levels are also increased in AML patients (Garcia Frade et al., 1992).

Studies describing the expression of uPAR on the leukemia cell surface have given somewhat discrepant results. Plesner et al. (1994b) found uPAR to be expressed in bone marrow leukemic cells in 12 of their 21 AML patients with FAB classes M2, M4, and M5, but not with FAB class M1. No uPAR was found in lymphoblastic leukemia cells. A few years later, Jardi et al. (1996) found uPAR to be expressed only on the surfaces of the most mature acute myeloid leukemias, M3 and M5 leukemia, but in these groups the pattern was also heterogeneous. At the same time as our observations (Mustjoki et al. 1999), Lanza et al. (1998) showed that leukemia cells from all common AML FAB-groups (M0-M5) can have uPAR on their cell surfaces. Furthermore, they described an AML subgroup (high uPAR positivity, chromosome 11 abnormalities, and M5 FAB morphology) characterized by a more aggressive clinical course. uPAR mRNA production of leukemic blast cells has been shown to be limited to FAB-classes M4 to M5 (Scherrer et al., 1999). In sum, all these studies agree that leukemic cells in lymphoid malignancies do not express uPAR. Moreover, uPAR expression is related to malignancies of myeloid origin, but among them the pattern is heterogeneous.

In vitro experiments with leukemia cell lines have also revealed that leukemia cells have an enhanced capacity to activate the pro-uPA they produce, and mainly the active form of uPA is found on the cell surface and in their growth medium; this differs from adherent tumor cells, which produce only inactive pro-uPA (Stephens et al., 1988; Tapiovaara et al., 1991; Stephens et al., 1992). This suggests that leukemic cells have an active proteolytic potential on their cell surfaces. These results are also applicable in vivo. Tapiovaara et al. (1993) found active uPA in the mononuclear cells of peripheral blood and bone marrow in patients with acute leukemia, but not in control subjects or patients with chronic leukemia. No correlation appeared between the lineage of leukemia and PA activity. However, Scherrer et al. (1999) found recently that leukemic cells only from AML patients have uPA mRNA and produce active uPA protein, whereas cells from ALL patients lack this ability. Active uPA has also been found in the cerebrospinal fluid of acute leukemia patients (Akenami et al., 1996). In addition, in acute myeloid leukemia marrow, the active form of uPA occurs, in contrast to normal bone marrow, which shows mostly tPA activity (McWilliam et al., 1998). This uPA activity was suggested to contribute to the severe hemorrhage occurring in AML patients (see below).
Plasmin formation plays an important role in normal blood coagulation and fibrinolysis. Plasmin inhibitors in plasma (α₂-macroglobulin and α₂-antiplasmin) strictly control the plasmin formation. In leukemia patients, the fibrinolytic balance is often disturbed, and patients have severe bleeding diathesis. This bleeding diathesis is explained not only by thrombocytopenia, since even with reasonable thrombocyte counts leukemia patients can experience severe bleeding. In addition to thrombocytopenia, excessive fibrinolysis has been proposed as causing some of these problems. This view has been strengthened by the finding that patients with bleeding disorders have had free uPA in their plasma (Bennett et al., 1989). In addition, APL patients with disseminated intravascular coagulation (DIC) have shown reduced plasma levels of PAI-1 and increased levels of plasmin-α₂-antiplasmin complexes and hypofibrinogenemia, suggesting that the actual problem in DIC is excessive fibrinolysis rather than excessive coagulation (Sakata et al., 1991; Fukao et al., 1992; Dombret et al., 1993; Dombret et al., 1995).

INTERCELLULAR ADHESION MOLECULES (ICAMs)

Structure and function of ICAMs
ICAMs belong to the immunoglobulin (Ig) superfamily. Humans have at least five of these glycoproteins (ICAM-1 through -5), and they consist of various numbers of Ig domains: ICAM-1 (CD54) and -3 (CD50) have five Ig domains, ICAM-2 (CD102) and -4 (Landsteiner-Wiener antigen) have four, and ICAM-5 (telencephalin) nine Ig domains (Carlos and Harlan, 1994; Gahmberg, 1997; Gahmberg et al., 1997). In addition to Ig domains, all ICAMs also have a single membrane-spanning domain and a short cytoplasmic portion. ICAM-1, -2, and -3 are expressed by leukocytes, whereas ICAM-4 is found in erythrocytes and ICAM-5 in brain gray matter (Gahmberg, 1997; Gahmberg et al., 1997). In addition, endothelial cells express ICAM-1 and -2.

ICAM 1 to 3 are important mediators of leukocyte adhesion; they act as ligands for the leukocyte-specific β2-integrins (CD11/CD18). LFA-1 (CD11a/CD18) has been shown to bind all these three different ICAMs, while Mac-1 (CD11b/CD18) binds only ICAM-1 and -2. In addition, CD11d/CD18 can bind ICAM-3. The binding sites for the β2-integrins are located in the ICAMs’ aminoterminal domain. Only ICAM-1 binds Mac-1 with its third domain (Gahmberg, 1997; Gahmberg et al., 1997).

In addition to cell-surface forms, also circulating forms of these leukocyte ICAMs have been described. Soluble ICAM-1 (sICAM-1) was first found in normal human serum and in increased amounts in patients with leukocyte adhesion deficiency (Rothlein et al., 1991). Later, sICAM-1 levels have been shown to be increased in several conditions, such as inflammation and cancer (Banks et al., 1993; Gearing and Newman, 1993; Nash et al., 1996; Bloom et al.,
The soluble molecule contains most of the extracellular portion of ICAM-1, and it is also able to bind LFA-1 with high affinity (60 nM) (Rothlein et al., 1991; Woska et al., 1998). However, the function of sICAM-1 in plasma is unknown. Similarly to sICAM-1, soluble ICAM-3 (sICAM-3) was first found in human serum (Martin et al., 1995; Pino-Otín et al., 1995) and in increased amounts in various pathological conditions: in chronic inflammatory diseases (rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus) and malignancies (Martin et al., 1995; Pino-Otín et al., 1995; Littler et al., 1997). Recently, it was shown that sICAM-3 can also bind LFA-1, but with much lower affinity than sICAM-1 (Woska et al., 1998). Furthermore, sICAM-3 and sICAM-1 were demonstrated to compete with each other in LFA-1 binding. As in the case of sICAM-1, the biological function of sICAM-3 is unknown.

In response to inflammatory stimuli, leukocytes start to extravasate from the blood stream through the endothelial cell layer and basement membrane into the extracellular matrix to the site of inflammation (Carlos and Harlan, 1994). In this process, they first roll along the endothelial cell layer. This rolling has been shown to be mediated by selectins and their olicosaccharide ligands. After rolling, leukocytes attach firmly to the endothelial cell layer. Adherence is mediated by β2-integrins, which become activated and begin to bind (Carlos and Harlan, 1994; Gahmberg, 1997; Gahmberg et al., 1997). After this attachment, leukocytes need to penetrate through endothelial-cell layers by squeezing themselves through intercellular junctions. Some evidence exists that integrins are involved in this transmigration (Smith et al., 1988; Smith et al., 1989), but the regulation of this process and the molecules participating in it are not yet known in detail. Supporting the theory of integrin involvement, it has been shown that neutrophils from patients with leukocyte adhesion deficiency syndrome (an inherited defect in β2-integrins) are unable to move across the endothelial cell layer (Smith et al., 1988). In addition, ICAM-1 and LFA-1 are important in monocyte transmigration, as antibodies to these molecules inhibit the transmigration of monocytes through the endothelial-cell layer (Mentzer et al., 1987; Te Velde et al., 1987; Arnaout et al., 1988; Luscinskas et al., 1994; Takahashi et al., 1994; Kling et al., 1995; Meerschaert and Furie, 1995). It has also been shown that PECAM-1 (CD31) is involved in neutrophil and monocyte diapedesis through endothelial cells (Carlos and Harlan, 1994). However, further studies are still needed to clarify the exact mechanisms of leukocyte transmigration through endothelial cells and basement membranes.

**ICAMs and leukemia**

As ICAMs are widely expressed and have important functions in normal leukocytes, it has been thought that they play an important role in leukemia cells as well. The actual functions of ICAMs in leukemia cells are not known in detail, but it has been suggested that they may participate in releasing blast cells from the bone marrow into the circulation, in homing blasts to
various organs, and in aiding blast cells to communicate with each other. The expression of adhesion molecules on the blast cell surface varies between leukemia types and also between patients with the same type of leukemia. ICAM-1, CD11a, and CD18 are usually expressed both in AML and in ALL blasts, but some patients in these groups have no expression at all on their blast cells (Maio et al., 1990; Archimbaud et al., 1992; Reuss-Borst et al., 1992; Liesveld et al., 1993; Raspadori et al., 1993; Reuss-Borst et al., 1995; Hirano et al., 1996; Kawada et al., 1996; Mielcarek et al., 1997; Bruserud and Ulvestad, 1999; De Waele et al., 1999). In contrast, CD11b is more commonly expressed in AML blasts than in ALL blasts (Reuss-Borst et al., 1995). In B-cell chronic lymphocytic leukemia (B-CLL) ICAM-1, -2, and -3 are frequently expressed (Molica et al., 1996; Vincent et al., 1996; Csanaky et al., 1997; Behr et al., 1998; Angelopoulou et al., 1999). There have been attempts to find a correlation between blast cell expression of adhesion molecules and clinical behavior of the disease, but the results have been quite discrepant. Some studies report no correlation between blast cell expression of ICAMs and clinical behavior (Archimbaud et al., 1992; Kawada et al., 1996). However, Mielcarek et al. (1997) showed in childhood ALL a positive correlation between low ICAM-1 expression and high peripheral leukocyte count and central nervous system involvement. ICAM-1 seemed to be a favorable prognostic factor, but not independent. In adult AML, LFA-1 expression has been shown to correlate with splenomegaly and resistance to chemotherapy and therefore to shorter survival (Kawada et al., 1996).

To our knowledge, the circulating form only of ICAM-1 has been studied in leukemia thus far, and is increased in childhood ALL and also in adult AML and ALL (Pui et al., 1993; Sudhoff et al., 1996). No correlation exists between the white blood cell count and sICAM-1 levels, sICAM-1 level the correlation with the clinical behavior of leukemia is weak (Pui et al., 1993; Sudhoff et al., 1996). However, in childhood Hodgkin's disease and adult B-CLL, sICAM-1 levels correlate with disease stage and also with frequency of relapse (Pui et al., 1993; Christiansen et al., 1994). In remission, the levels were normal in childhood malignancies. In sum, the number of studies is still small, and more information is needed to clarify the overall prognostic impact of adhesion molecules in leukemia.
AIMS AND OUTLINE OF THE PRESENT STUDY

The active processes of pericellular proteolysis and cell adhesion and invasion are central in tumor invasion. The importance of the plasminogen activation system and especially the roles of uPA and uPAR are well established in solid tumors, but in hematopoietic malignancies these were less clear.

In promyelocytic leukemia, Tapiovaara et al. (1994) found that retinoid acid (RA), which is used as a differentiation therapy for APL patients, transiently increases uPA activity in promyelocytic cells. However, the effects of RA on the plasmin formation were still unknown. Furthermore, some reports suggested that RA together with interferons could be a more efficient differentiation therapy in cancer. Therefore, we aimed to study the effects of RA together with interferons on the activation of plasminogen and on the formation of plasmin in APL cells in culture. RA treatment may result in a severe complication called retinoic acid syndrome characterized by fever, leukocytosis, and pulmonary infiltrations. APL patients with this syndrome have a shorter survival than do patients without the syndrome (De Botton et al., 1998). However, the actual pathophysiological mechanisms behind the syndrome are unclear. Therefore, we chose to evaluate the effects of RA on the invasion capacity of APL cells in three-dimensional culture. Because the invasion mechanisms of leukemia cells are, overall, quite poorly understood, we also chose to evaluate the blast cell extravasation in the same organotypic model, and the involvement of various adhesion molecules.

The presence of the soluble urokinase receptor in plasma was first described by Ploug et al. (1992). In 1997, Stephens et al. reported that some cancer patients show increased serum suPAR levels. In leukemia this had not been studied, and the characterization of the other components of the PA system in ex vivo leukemia cells had also been inconclusive. Therefore, we chose to evaluate uPA and uPAR expression on the blast cell surface and in the plasma samples of leukemia patients at diagnosis, and to study their correlation with the type and clinical behavior of their leukemias. Because results from these studies suggested that AML patients with high suPAR levels at diagnosis had a poor response to chemotherapy, we aimed to further analyze the behavior of suPAR during chemotherapy, and to examine whether it could serve as a marker for relapse in leukemia. Furthermore, we chose to study the origin and cleavage of suPAR in leukemia patients, because uPAR fragments are able to induce a chemotactic effect in vitro (Resnati et al., 1996, Fazioli et al., 1997). In addition, the cellular source of suPAR in plasma samples of healthy individuals was still unclear. Therefore, we also studied the release of suPAR by various cell types, and the influence of cell-cell contact on such release.
MATERIALS AND METHODS

Patient samples

Blood samples

Blood samples were obtained at the time of diagnostic sampling from patients referred with a suspicion of acute leukemia before any cytoreductive treatment. Altogether, samples from 135 adult patients with acute leukemia and 55 patients with other hematological disorders were enrolled into this study. In addition, from 58 of these 135 acute leukemia patients, follow-up samples were obtained during chemotherapy at 5, 14, 28, and 56 days after start of therapy. Samples from 40 healthy volunteers served as controls. All samples were collected at the Helsinki University Central Hospital during the years 1996 to 2000. The study protocol was approved by the local ethics committee.

Blood samples were drawn into EDTA tubes and kept on ice before plasma separation. Plasma was separated within 2 hours by centrifugation for 30 min at 4°C at 1800 x 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 of the mononuclear cells were tumor cells. The white blood cell (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 x white blood-cell count.

Urine samples

Urine samples were collected during the chemotherapy at days 0, 5, 14, 28, and 56 days after start of therapy from 58 patients with acute leukemia. Samples were centrifuged at 4°C at 1800 x g for 10 min and stored frozen in aliquots at -70°C before assay. Urine samples from 30 healthy controls were treated similarly.

Creatinine content of the urine samples was measured by the Jaffé method according to manufacturer's instructions (Boehringer Mannheim) with a Hitachi 917 analyzer. Urinary suPAR levels were normalized for the dilution factor based on creatinine values as earlier described (Sier et al., 1999). Ratios of uPAR/creatinine were expressed as ng/ml uPAR divided by mg/dl creatinine.

Bone marrow samples

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 for 30 min at 4°C at 1800 x g and stored frozen in aliquots at -70°C.

Patient diagnoses

The diagnoses were based on morphological, cytochemical, cytogenetic, and cell-surface markers. Acute myeloid leukemia (AML) was diagnosed in 92 of these 135 acute leukemia patients [French-American-British (FAB) classes: M0: 8, M1: 13, M2: 31, M3: 5, M4: 17, M5: 9, M7: one, NC (not classified): 2, and 6 secondary leukemias] and acute lymphoid leukemia (ALL) in 35 patients (immunophenotypic classes: cALL: 25, T-ALL: 6, pre-pre-B-ALL: 3 and one Burkitt lymphoma). Nine patients had a hybrid phenotype: both lymphoid and myeloid markers were found on the cell surface. In addition, 55 patients with other hematological disorders were included in the study: 18 patients with chronic myeloid leukemia (CML), 2 with chronic lymphocytic leukemia, 4 with myeloma, 2 with idiopathic thrombocytopenia (ITP), 5 with aplastic anemia, 8 with myelodysplastic syndrome (MDS), 2 with hairy-cell leukemia, one with lymphoma, one with heavy-chain disease, one with chloroma, one with low-grade B-cell disorder, one with monocytosis NUD, one with myelofibrosis, and 8 with reactive cytopenia (2 drug-induced, 4 due to infection, one due to systemic lupus erythematosus, and one developed ALL later).

Treatment of patients with acute leukemia

Patients with AML were treated with high-dose combination chemotherapy containing idarubicin, cytarabine, thioguanine, mitoxantrone, etoposide, and amnacrine according to the Finnish Leukemia Group protocol (Elonen et al., 1997). Elderly patients were treated with reduced doses according to established guidelines. Patients with ALL were treated according to the Finnish Leukemia Group protocol ALL94 consisting of cycles of high doses of mitoxantrone, cytarabine, etoposide, daunorubicin, vincristine, asparaginase, methotrexate, teniposide, doxorubicin, cyclophosphamide, mercaptopurine, carbustine, prednisone, and dexamethasone (Elonen et al., 1998).

Response to therapy

The observation period for these patients varied from 6 months to 4 years. All were treated with ≥three cycles of chemotherapy, unless they died. Their response to therapy was evaluated according to routine clinical practice. AML patients were divided into two categories: A) patients with good response to chemotherapy: complete remission with one to two cycles of chemotherapy, and no relapse within 6 months, B) patients with poor
response to chemotherapy: residual disease after two cycles of chemotherapy or relapsed during therapy within 6 months.

Cell culturing

General cell cultures

Cell culturing was performed in a humidified 5% CO₂ atmosphere at +37°C. Cells were grown in RPMI-1640 in the presence of 10% fetal bovine serum (FBS) (GibcoBRL, Karlsruhe, Germany) containing 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, unless otherwise indicated. The promyelocytic NB4 cell line was kindly provided by Dr. Michel Lanotte (INSERM, Paris, France). It was derived from the bone marrow of an acute promyelocytic leukemia (APL) patient in relapse, and carries the t(15;17) translocation characteristic of APL cells (Lanotte et al., 1991). Other non-adherent hematopoietic cell lines used were U937, Jurkat, KG1, RC2A, THP-1, HL60, and Molt from the American Type Culture Collection (ATCC) (Rockville, MD). Human microvascular endothelial cells (HMEC-1) (Ades et al., 1992) were a kind gift from Dr. T.J. Lawley (Emory University School of Medicine, Atlanta, GA). Human umbilical vein endothelial cells (HUVEC) were isolated and grown in endothelial cell growth medium (PromoCell, Heidelberg, Germany).

In addition to normal culture plates, cells were also co-cultured in Transwell chambers (Costar, Corning, NY) in order to have a shared growth medium without cell-cell contacts.

Organotypic model for vessel wall

Collagen type I was purified from rat tail tendons as earlier described (Elsdale and Bard, 1972). Collagen gels were prepared by diluting purified collagen in RPMI medium containing 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, to achieve 0.6 mg/ml collagen in the final concentration. This solution, 0.4 ml per well, was pipetted into 48-well cell culture cluster plates (Costar, Cambridge, MA), and gels were allowed to form for 30 min at +37°C. Endothelial cells (80 000 cells in 0.4 ml of growth medium) were seeded on the collagen gels and left to form monolayers overnight at +37°C. Next morning, the growth medium was removed, and freshly isolated blood mononuclear cells or leukemia cells were added (0.2 million cells/0.4 ml of growth medium) on top of the endothelial cells. Monoclonal antibodies (20 µg/ml) or purified proteins were either included in the growth medium at the same time as mononuclear cells were added, or endothelial cells were preincubated with the antibodies for 30 min at +37°C, before addition of the mononuclear cells. Adherence and invasion of blood cells through the endothelial cell layer into the collagen matrix was monitored by light microscopy. After 1 to 3 days of culture, the collagen rafts were carefully detached from the wells and fixed with 10% formaldehyde. After fixation, the rafts were processed as normal tissue samples, and before casting in paraffin, they were cut vertically to reveal the cross section of the collagen rafts. Sections of 4 to 6 µm were cut from the paraffin blocks and stained with hematoxylin and eosin (HE) for histological evaluation.

Invasion of the blood cells was studied in HE-stained sections under light microscopy. The number of invaded cells in the collagen matrix per x 20 high-power field was counted in four different areas of each section and from three different sections of each sample. The extent of invasion was determined as percentage of invaded cells compared to sections of samples treated with control antibody.

Cell lysates

Cells were lysed in phosphate-buffered saline (PBS), pH 7.4, containing 1% Triton X-100 and protease inhibitors (Complete™; Boehringer Mannheim, Mannheim, Germany). The lysates were centrifuged at 4°C at 14 000 x g for 10 min and the supernatants stored at -70°C until assay. Total protein was determined by the BCA protein assay kit (Pierce, Rockford, IL).

Elution of uPA and plasmin from the cell surface

To determine the amount of cell-bound uPA activity, the cells were first washed twice with Dulbecco's balanced salt solution containing 0.2% BSA (DBSA) and then eluted with acid glycine (20 mM glycine, 100 mM NaCl, pH 3.0) (Stoppelli et al., 1986). The acid glycine fraction was neutralized with 1 M Tris-HCl, pH 8.0. To determine the capacity to activate exogenously added plasminogen to plasmin, the cells were suspended in plasminogen-depleted growth medium, and human plasminogen (50 µg/ml) was added (Tapiovaara et al., 1991). Plasmin generation was allowed to proceed for 30 min at 37°C, after which cells were washed twice with DBSA. Cells without incubation with plasminogen served as negative controls. Cell-bound plasmin was eluted with 10 mM tranexamic acid (Kabi Pharmacia, Stockholm, Sweden) in DBSA (200 µl per 10⁶ cells) for 10 minutes at room temperature. The eluates were then ready for enzymatic assays.
Materials and Methods

Antibodies and molecular probes

Antibodies against different members of the PA system

The following antibodies were used: monoclonal antibodies against human uPAR R2, R3, R4, and R5 recognizing different domains of uPAR molecule (R2 and R4 react with the carboxyterminal domain D3 of uPAR, and R3 and R5 with the aminoterminal domain D1), these kindly provided by Dr. Gunilla Høyer-Hansen, Finsen Laboratory, Copenhagen, Denmark; polyclonal rabbit anti-human uPAR (Finsen Laboratory), monoclonal anti-human uPAR (American Diagnostica, Greenwich, CT; #3936), polyclonal rabbit anti-human uPA (American Diagnostica, #389), monoclonal anti-human uPA clone 12 (Finsen Laboratory), and polyclonal goat anti-human tPA (American Diagnostica; #387).

Antibodies against adhesion molecules

The monoclonal antibodies were UHP cocktail (ICAM-1) (Tiisala et al., 1994), LB2 (ICAM-1) (Patarroyo et al., 1987), TS 1/22 (CD11a) (Sanchez-Madrid et al., 1982), 7E4 (CD18) (Nortamo et al., 1988), BR7 (ICAM-2) (Xie et al., 1995), CBRCIC2/2 (ICAM-2) (Bender Medsystems, Vienna, Austria), and BR1 (ICAM-3).

Leukocyte and control antibodies

The monoclonal antibodies were: anti-C D68-recognizing monocyte-macrophages (KP1, DAKO, Glostrup, Denmark), anti-CD3-recognizing T lymphocytes (DAKO), X63 (mouse IgG) (ATCC), anti-human cytokeratin (DAKO, #M0630), anti-human biotin (DAKO, #M0743), and normal mouse or rabbit IgG (R&D systems, Abingdon, UK; #MAB002/#AB105C).

Molecular probes

The cDNA clone for uPA (fragment BamHI) was obtained from ATCC. The cDNA clones for the uPAR (fragment Sphl Sacl) (Roldan et al., 1990), PAI-1 (fragment BamHI) (Andreasen et al., 1986), and GAPDH (fragment PstI) (Fort et al., 1985) were kindly provided by Dr. Jens Eriksen and Dr. Leif Lund (Finsen Laboratory). The probes were labeled with digoxigenin (DIG) coupled to dUTP by a DIG random primed DNA labeling kit (Boehringer Mannheim; cat. no. 1175 033) according to the manufacturer's instructions.

Other reagents

Effectors

13-trans retinoic acid (Tretinoin) was kindly provided by F. Hoffman-La Roche Ltd, Basel, Switzerland. The stock solutions were dissolved in ethanol, and the concentration used in cell cultures was 1 µM. Interferons α and γ were a kind gift from Drs. Kari Cantell and Ilkka Julkunen of the National Public Health Institute, Helsinki, Finland (Sareneva et al., 1994, 1995). They were dissolved in RPMI-1640, and the concentration used in cell cultures was 1000 IU/ml. Dexamethasone (Sigma, St. Louis, MO; #D8893) was dissolved in ethanol, and the concentration used was 100 nM. Phorbol 12-myristate 13-acetate (PMA) was also dissolved in ethanol and used at a concentration of 50 nM.

Plasminogen

Plasminogen was purified from precleared citrated plasma according to Deutsch and Mertz (1970). In brief, plasma was run through a Lysine-Sepharose column in the presence of several protease inhibitors to inhibit the activation of plasminogen to plasmin. Lysine-bound plasminogen was eluted from the column with the aminocaproic acid, after which the fractions with high protein concentration were re-run through the column and eluted similarly with the aminocaproic acid. Finally, eluted fractions were dialyzed, and protein concentration was determined with the BCA protein assay kit; the purity of the protein was determined by running the samples in 8% SDS-PAGE.

Collagen type I

Collagen type I was purified from rat-tail tendons according to an established method (Elsdale and Bard, 1972). In brief, tendons were dissolved in 10 mM HCl and the concentration used was measured with the BCA protein assay kit (Pierce, Rockford, IL) and was usually 1-1.5 mg/ml.

Production and purification of sICAM-1

A soluble form of ICAM-1 (sICAM-1) was purified as described by Hedman et al. (Hedman et al., 1992). In short, sICAM-1 protein was produced by CHO cells, which were transfected with a fusion cDNA coding the extracellular part of ICAM-1 and a part of IgG heavy chain. Transfected cells produced the fusion protein into the growth medium, and from the growth medium, it was purified on a protein Sepharose A column, which bind
Materials and Methods

the Fc part of the protein. Bound protein was eluted with 0.1 M glycine, pH 3.0, from the columns. Eluted fractions were concentrated, and the buffer was changed to PBS by means of Centricon-30 tubes (Millipore, Bedford, MA). The purity of the fusion protein fraction was evaluated by 8% SDS-PAGE. Protein concentration was determined by the BCA protein assay kit (Pierce).

Enzyme-linked immunosorbent assays (ELISAs) and other microwell plate assays

Assay of soluble uPAR and uPAR in cell lysates

The ELISA for suPAR has been described in detail (Stephens et al. 1997). In brief, immunoplates were coated overnight with purified polyclonal anti-human uPAR antibodies. The wells were blocked with SuperBlock solution (Pierce), and after this blocking, the wells were rinsed with PBS-Tween 20. After washing, the wells were incubated with standard dilutions of purified recombinant suPAR or with 1:10 dilutions of plasma and urine samples made in a sample dilution buffer (7 mM KH2PO4, 40 mM Na2HPO4, pH 7.4, 0.1 M NaCl, 2 mM KCl, 10 g/L BSA, and 1 g/L Tween 20). From the cell lysates, protein concentrations were first determined, and then a volume equal to 20 µg of protein was placed in each well. After antigen binding, the wells were rinsed, and then plates were incubated with a mixture of three monoclonal anti-human uPAR antibodies (R2, R3, and R5), followed by a rabbit antiamouse immunoglobulins-alkaline phosphatase conjugate. After washing, a p-nitrophenyl phosphate substrate solution was added, and a color reaction was allowed to develop at room temperature. The absorbances were read in a Multiscan MCC/340 plate-reader (Labsystems, Helsinki, Finland) at 405 nm. Each sample was tested also without specific monoclonal antibodies, and a few plasma samples gave a low positive reaction. These samples were not included in the analysis and in the tables are marked as reactive (R). Interassay variation for the samples was <10%, and the lower detection limit of this assay was 0.03 ng/ml.

Immunocapture assay for plasminogen activators

uPA and tPA activities were assayed by a microimmunocapture assay (Stephens et al., 1987). It measures inactive proenzymes by activating them to active two-chain forms. It does not measure plasminogen activators in complexes with inhibitors. Briefly, samples were bound in duplicate or triplicate to microwell plates coated with 10 µg/ml of rabbit IgG to human uPA (American Diagnostica, Greenwich, CT; #389) or goat IgG to human tPA (American Diagnostica; #387). After binding, the wells were washed with PBS + 0.05% Tween 20, and then human plasminogen was added. Plasmin formed by antibody-bound activators was measured by its thioesterase activity on Z-lysine thiobenzyl ester (Peninsula Laboratories, Belmont, CA). The absorbances were read in a Labsystems Multiscan MCC/340 plate-reader at 405 nm. Standard dilutions of purified human urokinase (American Diagnostica; #124) and human tPA (American Diagnostica; # 116) served for quantitation of enzymatic activity (0 to 1.0 IU/ml). The absorbance curve was linear between the values 0.1 to 1.0. The lower detection limit of the assay for uPA and tPA is 0.02-0.04 IU/ml.

Assay of eluted plasmin

Eluted plasmin samples (50 µl) were pipetted in duplicate in microwell plates, and plasmin was measured by its thioesterase activity as described above for the immunocapture assay. Standard dilutions of human plasmin (Kabi Diagnostica) were used for quantitation of enzymatic activity.

Assay of uPA antigen

Plasma samples were assayed by an ELISA method according to the manufacturer's instructions (Monozyme, Horsholm, Denmark). This determines active and inactive (uPA-PAI-1-complexes) forms of uPA antigen. The lower detection limit of the ELISA kit is 25 pg/ml of uPA.

Analysis of sICAM-1 and -2

Soluble ICAMs were assayed from plasma samples by ELISA methods. The human sICAM-1 immunoassay kit came from BioSource International (Camarillo, CA), and the human sICAM-2 ELISA kit from Bender MedSystems. The lower detection limits of the ELISA kits were 0.04 ng/ml of sICAM-1 and 0.46 U/ml of sICAM-2.

Other immunological methods

Immunostaining of cell surface uPA, uPAR, and adhesion molecules by fluorescence-activated cell sorter (FACS)

For FACS analysis, the cell samples (1x10^6 cells/ml) were collected, and the cell suspension was centrifuged for 10 min at 1000 x g. The cells were washed with PBS containing 1% of FBS (PBS-FBS) and resuspended after centrifugation in 50 µl of primary monoclonal or polyclonal antibody (conc. 10-50 µg/ml) (antibodies described above). The cells were incubated in the presence of IgG for 30 min on ice, after which they were washed
twice with PBS-FBS and resuspended in 100 µl of fluorescein isothiocyanate (FITC) -conjugated rabbit anti-mouse IgG or swine anti-rabbit IgG (Dako, Copenhagen, Denmark) diluted 1:50 in dilution buffer. After 30 min on ice, the cells were washed twice with PBS-FBS and resuspended in 300 µl of PBS on ice; 10 000 cells were analyzed with a FACScan IV (Becton Dickinson, Mountain View, CA). In the case of patient samples, blast cell populations were detected based on their forward- and sidescatter properties, and only that population was included in the analysis.

**Immunoprecipitation and immunoblotting**

Urine, plasma, and cell lysate samples were immunoprecipitated with biotinylated R2 and R3 monoclonal antibodies (Finsen Laboratory) prebound to immobilized streptavidin (Boehringer Mannheim) (Sidenius et al., 2000). These antibodies recognize different domains of uPAR: R2 reacts with the carboxyterminal domain D3 of uPAR, and R3 with the aminoterminal domain D1. Immunoprecipitated proteins were fractionated by 12% SDS-PAGE under non-reducing 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).

**Immunohistochemistry**

A Ventana automated slidestainer for immunohistochemistry (Ventana Medical Systems, Tucson, AZ) was used for staining the 4 µm paraffin sections. Detection was based on an avidin-peroxidase method using biotinylated secondary antibodies. Counterstaining was done with hematoxylin, and bluing reagent (0.1M lithium carbonate in 0.5 M sodium carbonate) served as the post-counterstain.

**Immunofluorescence**

Cells were grown on glass coverslips in multi-well culture plates. After culturing, the plates were transferred onto ice, and the coverslips were washed twice with DBSA. After the washing, primary antibodies were added to the coverslips and incubated for 30 min on ice, after which the cells were washed again. Before secondary antibody addition, cells were fixed with ice-cold methanol for 15 min at -20°C followed by thorough washing and then incubated with secondary FITC-conjugated antibodies (Myöhänen et al., 1993). At the end, cells were washed again, and coverslips were mounted on object classes.

**RNA extraction and Northern analysis**

RNA extraction was done according to the method of Chomczynski and Sacchi (1987): 1x10^7 cells were lysed in solution D containing guanidium thiocyanate, sodium citrate, sarcosyl, and 2-mercaptoethanol, and the RNA phase was separated with a phenol and chloroform-isoamyl alcohol solution. The RNA isolated was quantitated with a spectrophotometer, and those samples containing the same amount of mRNA (20 µg) were size fractionated in 1% agarose gels and transferred to a Hybond N nylon membrane (Amersham, Buckinghamshire, England). Prehybridizations and hybridizations were performed in a solution containing formamide, standard saline citrate (SSC), N-lauroyl sarcosine, sodium dodecyl sulphate (SDS), and blocking solution (Boehringer Mannheim). The hybridization was carried out overnight, and after that the filters were washed twice at +42°C with 0.5 x SSC and 0.5 x SDS and then twice at +68°C. The detection was done by use of alkaline phosphatase conjugated anti-digoxigenin (DIG) antibodies. Luminescent reactions from filters were exposed to X-ray films. The X-ray films were scanned, and the relative intensities were determined in comparison with the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) signal from the same filter.

**Zymography**

Plasma, growth media, and cell lysate samples were electrophoresed on 10% polyacrylamide SDS-PAGE gels, which were supplemented with plasminogen (10 µg/ml) and dry nonfat milk as a source of casein (Granelli-Piperno and Reich, 1978). After electrophoresis, the gels were washed twice with 2.5% Triton X-100 and incubated in 10 mM CaCl2, 50 mM Tris (pH 7.5) for 24 to 72 hours at 37°C. The gels were then stained with Coomassie blue, after which clear bands were seen in the areas of proteolytic activity against the blue background staining. Purified human uPA and tPA (American Diagnostica) served as positive controls.

**Statistical analysis**

The following tests were used for comparison of the results: Student's unpaired, paired, and one-group t-tests, the Mann-Whitney U-test, and Chi-square test. The correlation coefficients r and Rho were calculated according to Pearson and Spearman Rank Correlation tests. Results were considered significant when p<0.05.
RESULTS

Enhanced plasminogen activation and plasmin formation by retinoids and interferons in acute promyelocytic leukemia cells (I)

Patients with acute promyelocytic leukemia (APL) are treated with retinoic acid (RA) in addition to conventional chemotherapy. RA induces the differentiation of promyelocytic leukemia cells in vitro and in vivo, and it also reduces the bleeding tendency characteristic of APL patients. Severe side-effects (leukocytosis, pulmonary infiltrations, and respiratory distress syndrome) sometimes caused by the RA treatment are called the retinoic acid syndrome. These side-effects are relieved by dexamethasone administration, but occasionally the syndrome has a fatal outcome. The plasminogen activation system and plasmin formation play an important role in fibrinolysis, and it has also been suggested that plasminogen activation can affect the fibrinolytic balance in leukemia patients. Tapiovaara et al. (1994) showed earlier that RA treatment induces a transient uPA increase in promyelocytic NB4 cells. However, the actual formation of plasmin by promyelocytic leukemia cells had not been studied, and also the RA’s effect on it was unknown. Furthermore, other in vitro studies have suggested that interferons and retinoids may have combined effect on cell differentiation. Therefore, we chose to analyze the activation of plasminogen to plasmin in promyelocytic cells and the effects of retinoids, interferons, and dexamethasone on the plasminogen activation cascade. We used the promyelocytic NB4 cell line as a model for acute promyelocytic leukemia and treated NB4 cells with RA to induce differentiation. At the various time points (12, 24, and 48 hours) we collected samples of growth medium and cell surface eluates and extracted mRNA from these cells. Components of the PA system (uPA, uPAR, and PAI-1) were analyzed with specific ELISAs, FACS analysis, and Northern blotting.

RA enhances transient uPA production and plasmin formation

As earlier observed (Tapiovaara et al., 1994), we, too, noticed that RA induced transient uPA production in promyelocytic NB4 cells. The elevation was evident in mRNA level at 10 hours, and at 24 hours the uPA mRNA level had already fallen to its basal level. On the cell surface and in the growth medium, uPA protein levels rose for to 24 hours, this increase being more pronounced in secreted uPA than in cell-bound uPA. Thereafter, uPA protein levels started to fall again, and after 48 hours of treatment, less uPA was seen on the surface of RA-treated cells that on control-treated cells. Our results also indicated that uPA induction led to the activation of plasminogen on the NB4 cell surface. The generation of plasmin followed closely the induction of uPA, with the highest cell-surface plasmin levels detected at 24 hours. After 48 hours of RA treatment, plasmin levels fell below their basal level.
Combined effects of RA and interferons or dexamethasone on the plasminogen activation cascade

In contrast to RA, in NB4 cells, neither interferon α nor γ had any effect on the plasminogen activation cascade. Yet, when either of these two interferons was combined with RA, more pronounced induction of uPA appeared both in the mRNA level and in protein level. In addition, a similar effect was seen in plasmin formation on the cell surface. This suggests that in promyelocytic cells, RA and interferons have a synergistic effect on plasminogen activation.

We also studied the effect of dexamethasone on the PA system in promyelocytic cells. When used alone, dexamethasone had no significant effect on the production of uPA or on plasmin formation. When dexamethasone was added together with RA, it suppressed the effect of RA, and neither cell surface induction of uPA nor induction of plasmin formation was any longer observed.

Increase in levels of uPAR and PAI-1 after uPA induction

Because uPAR and PAI-1 are closely related to cell adhesion and migration, it was of interest to study the effects of RA and interferons on uPAR and PAI-1 expression. In contrast to uPA mRNA levels, for which a transient increase with RA treatment was evident at 10 hours, uPAR and PAI-1 mRNA levels increased more markedly only after 21 hours of RA treatment, and these levels continued to rise for up to 46 hours. The interferons potentiated especially the effect of RA on the induction of PAI-1 expression, and a slight increase was also observable in uPAR expression. Alone, interferons had no marked effect on uPAR or PAI-1 expression.

It is noteworthy that expression of uPAR and PAI-1 was still significantly elevated after 48 hours of RA treatment. Later, we observed that RA treatment induces rapid extravasation of promyelocytic cells (see page 46). It is thus possible that the enhanced expression of uPAR and PAI-1 is associated with these cells’ invasiveness. We have also studied the effect of RA treatment on the release of uPAR into the culture medium and noticed that concomitant with the increased uPAR expression in these cells, the amount of suPAR also increased significantly in the culture medium (Fig. 6).

Fig. 6. suPAR in the growth medium of promyelocytic leukemia (NB4) cells
Extravasation of blast cells and promyelocytic leukemia cells in organotypic model for vessel wall: impact of various adhesion molecules (II)

Leukemia cells have a strong tendency to invade from the blood stream into various organs, but the mechanisms behind leukemia cell extravasation are largely unknown. Since ICAMs are important mediators in normal leukocyte adhesion, we chose to examine their roles in leukemia cell invasion.

We developed a novel three-dimensional organotypic model for vessel wall, in which we studied the extravasation of normal mononuclear cells, blast cells from acute leukemia patients, and promyelocytic leukemia cells. This model consisted of an underlying matrix lattice, made of collagen type I purified from rat tail tendons. On the top of the collagen gel, we seeded endothelial cells and allowed them to form a confluent cell layer (see Fig. 7). Leukemic or peripheral mononuclear cells were loaded in suspension on the lattice. Extravasation and infiltration of cells was followed by light microscopy. After 1 to 2 days, lattices were cast in paraffin blocks, and sections were cut and stained with hematoxylin and eosin (HE) to visualize the invasion of the cells.

**Fig. 7. Organotypic model for extravasation**

Rapid invasion of healthy mononuclear cells and leukemic cells: inhibitory effect of ICAM-1 antibodies on extravasation

Mononuclear cells from healthy individuals invaded through endothelial cell layers into the underlying collagen matrix in only a few hours. Immunohistochemical staining with monocyte and lymphocyte antibodies confirmed that monocyte-macrophages and lymphocytes were both
able to invade through endothelial cells. Moreover, isolated blast cells from AML and ALL patients had the capacity to extravasate in a couple of hours, and after 24 hours, most of the blast cells were detected in the collagen matrix under the endothelial cells (see Fig. 8).

In order to evaluate the roles of different adhesion molecules (ICAM-1, -2, -3, CD11a, and CD18) on the extravasation process, antibodies against these adhesion molecules were added to the mononuclear cell suspension, or endothelial cells were preincubated with them. Our results showed that extravasation both of normal mononuclear cells and of leukemia cells could be totally inhibited with ICAM-1 antibodies. In healthy individuals, ICAM-1 antibodies inhibited almost 90% of the invasion (13±2% of cells invaded as compared to cells treated with control antibody). In leukemia patients, ICAM-1 antibody prevented >80% of the extravasation of blast cells in five of six AML patients and one of two ALL patients. Antibodies to other adhesion molecules (CD11a, CD18, ICAM-2, and ICAM-3) had some, but not significant effects on the extravasation.

Because earlier in vitro experiments have shown that soluble ICAM-1 can bind to its counter receptor LFA-1, we produced and purified soluble ICAM-1 protein from transfected CHO cells in order to interfere with integrin/ICAM-1 binding and in that manner influence extravasation of mononuclear cells. However, at the concentrations used (10-40 µg/ml), sICAM-1 protein had no effect on the extravasation (105±9% of cells extravasated compared to control treated cells). Yet, when ICAM-1 antibodies were preincubated with the purified sICAM-1 protein, the antibodies lost their ability to block the extravasation of mononuclear cells.

**Fig. 8. Invasion of leukemia cells in an organotypic culture**

HE staining of the collagen lattice. Leukemia cells from an AML patient (monocytic type) were allowed to invade for 24 hours in an organotypic culture. Bold arrows indicate endothelial cell layer and thin arrows invaded leukemia cells.

**Extravasation of acute promyelocytic leukemia cells: dramatic effect of retinoic acid**

Our earlier results showed that RA treatment induced uPAR and PAI-1 expression in promyelocytic leukemia cells, and because both of these molecules are associated with the adhesion and migration capacity of the cells, we decided to test the invasion of promyelocytic cells in the organotypic model, and the effect of RA on the extravasation process.
First, we treated promyelocytic NB4 cells, which carry the same translocation as seen in vivo in APL cells, for 48 hours with RA. At the same time as the differentiation was induced, promyelocytic cells began to attach and invade through the endothelial cells. Without RA treatment, no extravasation of cells was evident. We also obtained promyelocytic leukemia cells ex vivo from an APL patient in relapse. Notably, these cells also invaded rapidly after RA treatment, and no extravasation was seen without the treatment.

We then studied cell-surface expression of adhesion molecules by FACS analysis before and after RA treatment. Only ICAM-3 was detectable on the surface of control-treated cells. However, after 48 hours of RA treatment, ICAM-1, CD11c, and CD18 were also strongly expressed; CD11a and ICAM-2 were not induced. Since these molecules were induced by RA treatment, we studied on the extravasation the effect of antibodies against these adhesion molecules. ICAM-1 antibodies were, in fact, also able to inhibit the extravasation of RA-treated NB4 cells (13±2% of cells invaded compared to the case for cells treated with control antibodies). Antibodies against other adhesion molecules (CD11a, CD18, ICAM-2, and ICAM-3) showed no significant effect. Similarly, purified sICAM-1 protein alone showed no effect on extravasation, but when it was preincubated with ICAM-1 antibodies, sICAM-1 protein inhibited the effect of the antibodies.

**Elevated levels of sICAMs in leukemia patients’ plasma samples at diagnosis: divergent effects of chemotherapy on sICAM-1 vs. sICAM-2**

We also examined the presence of soluble adhesion molecules (sICAM-1 and sICAM-2) in plasma samples of acute leukemia patients at diagnosis and their possible correlation with the clinical characteristics of the disease. Both sICAM-1 and sICAM-2 levels were increased in acute leukemia patients. In AML patients (n=45), sICAM-1 levels were 327±21 ng/ml and in ALL patients (n=25), 372±54 ng/ml, compared to 148±14 ng/ml in healthy controls. Plasma sICAM-2 levels were significantly higher in ALL patients (625±110 IU/ml) than in AML patients (297±42 IU/ml) or healthy individuals (171±12 IU/ml). No correlation between sICAM-1 levels and WBC or tumor-cell count appeared in ALL patients (r=-0.03 and r=-0.04), and also in AML patients the correlations were weak (r=0.45 and r=0.40). Similarly, sICAM-2 levels in AML and ALL patients correlated only weakly with the WBC count (r=0.45 and r=0.47, respectively). Furthermore, no correlation existed between FAB classes and sICAM levels (Fig. 9). To study the possible association between sICAM levels and therapy response, AML patients were divided into two groups according to their responses to chemotherapy. Neither mean sICAM-1 nor sICAM-2 levels differed between the well-responding group (sICAM-1 303 ng/ml, sICAM-2 155 IU/ml) and poorly-responding group (316 ng/ml and 150 IU/ml, respectively). In ALL, most of the patients achieved complete remission with one to two cycles of chemotherapy, but their levels, however, varied considerably.
Follow-up samples were collected at 5, 14, and 28 days after start of chemotherapy to examine the effect of treatment on sICAM levels. Only sICAM-2 levels fell to the normal plasma levels within 2 weeks, simultaneously with the disappearance of tumor cells from circulation. sICAM-1 levels remained elevated during one month of chemotherapy, although at that time-point, tumor cells were no longer detected in the circulation.

**Fig. 9. sICAM-1 in plasma in AML patients**

In plasma samples collected from AML patients at diagnosis, sICAM-1 levels were measured with ICAM-1 ELISA. Patients divided into groups according to FAB classes (M0-M5; NC, not classified).

**Soluble and cellular uPAR and uPA in acute leukemia patients at diagnosis: implications for diagnosis and prognosis (III)**

The expression of uPA and uPAR and their correlation with prognosis have been examined in a large variety of adherent tumors (Andreasen et al., 2000), but in leukemia these are less well characterized. In addition, in recent years a soluble form of uPAR (suPAR) was discovered in human body fluids, and preliminary studies in breast and colon cancer have suggested that suPAR may serve as a marker for prognosis and tumor recurrence in cancer patients (Stephens et al., 1997). We therefore characterized the levels of uPA and uPAR in the plasma and on the cell surface of patients with acute leukemia to examine whether any correlation existed between components of the plasminogen activation system and/or prognosis of leukemia.

A total of 110 blood samples from 80 adult patients with hematological disorders were taken at the time of diagnosis (before any cytoreductive therapy). In addition, blood samples from 21 healthy volunteers were analyzed. Acute leukemia was diagnosed in 53 of these 80 patients: 36 patients had AML, 13 ALL, and 4 a hybrid phenotype (Table 2). The other 27 hematological disorders included lymphoma, multiple myeloma, aplastic anemia, heavy-chain disease, idiopathic thrombocytopenia, myelodysplastic syndrome, reactive pancytopenia, hairy-cell leukemia, and chronic myeloid leukemia (CML).
EDTA plasma was collected and mononuclear cells were separated by Ficoll-Hypaque centrifugation. Cell surface antigens for uPA and uPAR were assayed with FACS analysis. From plasma, tPA and uPA activity and uPA and uPAR antigens were measured with ELISAs.

**uPA and uPAR expressed on the blast cell surface of AML patients**

In healthy controls, expression of uPA and uPAR was restricted to monocytes, and lymphocytes lacked any detectable amounts of these on their surfaces. We found in 28 of 29 patients with AML uPAR on their leukemic cell surfaces, whereas most patients with ALL were negative for uPAR (7/9) (see Table 2 and Fig. 10). In addition, two of three patients with a hybrid phenotype had uPAR on the cell surface. In AML patients, uPAR was expressed in all FAB subtypes, with the highest expression in subclasses M3, M4, and M5. The expression pattern of uPA resembled the pattern seen with uPAR: it was expressed by myeloid blasts and not by lymphoid blasts. However, usually a smaller percentage of cells was uPA- than uPAR-positive.

We also studied with immunohistochemistry the uPAR expression in bone marrow biopsies of acute leukemia patients and of healthy individuals and found that uPAR is abundantly expressed in myeloid blasts in the bone marrow. In healthy individuals, uPAR expression was restricted to granulocytes, monocytes, and megakaryocytes; lymphoid and erythroid precursors stained negative for uPAR.

**Fig. 10. uPA and uPAR on leukemia cell surfaces.** uPA and uPAR characterized by FACS analysis on the blast cell surfaces of patients with acute leukemias. Dark curves show staining with uPAR and uPA antibodies, and white curves with control antibodies.
Table 2A. uPAR and uPA in plasma samples and on the blast cell surfaces of patients with AML

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>FAB type</th>
<th>Age</th>
<th>WBC 10^9/L</th>
<th>Blasts (%) of WBC</th>
<th>suPAR (ng/ml)</th>
<th>uPA (ng/ml)</th>
<th>uPA (IU/ml)</th>
<th>uPAR-positive cells of blast cells (%)</th>
<th>uPA-positive cells of blast cells (%)</th>
<th>Response to therapy/ comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>secondary</td>
<td>66</td>
<td>3.5 / 74</td>
<td>2.37</td>
<td>0.50</td>
<td>0.162</td>
<td>27</td>
<td>ND</td>
<td>ND</td>
<td>RD (2)*</td>
</tr>
<tr>
<td>2</td>
<td>M2</td>
<td>50</td>
<td>2.9 / 2</td>
<td>3.01</td>
<td>0.75</td>
<td>0.216</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
</tr>
<tr>
<td>3</td>
<td>M3</td>
<td>41</td>
<td>108.7 / 95</td>
<td>2.54</td>
<td>0.55</td>
<td>0.116</td>
<td>82</td>
<td>31</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M4</td>
<td>16</td>
<td>3.2 / 25</td>
<td>1.24</td>
<td>0.65</td>
<td>0.104</td>
<td>94</td>
<td>63</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M2</td>
<td>47</td>
<td>32.0 / 67</td>
<td>1.63</td>
<td>0.42</td>
<td>0.079</td>
<td>89</td>
<td>31</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M3</td>
<td>70</td>
<td>6.7 / 92</td>
<td>r</td>
<td>0.53</td>
<td>0.094</td>
<td>98</td>
<td>25</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M1</td>
<td>71</td>
<td>112.1 / 95</td>
<td>5.75</td>
<td>0.95</td>
<td>0.115</td>
<td>40</td>
<td>6</td>
<td>RD (3)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M2</td>
<td>32</td>
<td>13.2 / 74</td>
<td>0.73</td>
<td>0.50</td>
<td>0.151</td>
<td>73</td>
<td>39</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>secondary</td>
<td>58</td>
<td>10.2 / 63</td>
<td>2.94</td>
<td>0.32</td>
<td>0.080</td>
<td>14</td>
<td>1</td>
<td>RD (1)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M3</td>
<td>72</td>
<td>1.1 / 0</td>
<td>2.39</td>
<td>0.41</td>
<td>0.113</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>secondary</td>
<td>52</td>
<td>3.3 / 0</td>
<td>1.16</td>
<td>0.17</td>
<td>0.050</td>
<td>ND</td>
<td>ND</td>
<td>RD (2)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>M4</td>
<td>72</td>
<td>95.4 / 72</td>
<td>5.83</td>
<td>1.06</td>
<td>0.147</td>
<td>93</td>
<td>63</td>
<td>RD (3)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M2</td>
<td>59</td>
<td>0.6 / 0</td>
<td>0.81</td>
<td>0.31</td>
<td>0.051</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M5</td>
<td>49</td>
<td>21.0 / 60</td>
<td>2.28</td>
<td>0.43</td>
<td>0.111</td>
<td>97</td>
<td>28</td>
<td>CR (2)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>M1</td>
<td>86</td>
<td>170.0 / 98</td>
<td>11.28</td>
<td>2.37</td>
<td>0.516</td>
<td>0</td>
<td>4</td>
<td>died, no therapy</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>M2</td>
<td>19</td>
<td>36.0 / 86</td>
<td>0.99</td>
<td>0.36</td>
<td>0.083</td>
<td>31</td>
<td>ND</td>
<td>RD (4)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>NC</td>
<td>24</td>
<td>19.4 / 88</td>
<td>1.39</td>
<td>0.28</td>
<td>0.087</td>
<td>90</td>
<td>6</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>M2</td>
<td>72</td>
<td>4.2 / 20</td>
<td>r</td>
<td>0.62</td>
<td>0.109</td>
<td>38</td>
<td>5</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>M4</td>
<td>58</td>
<td>2.0 / 15</td>
<td>1.67</td>
<td>0.34</td>
<td>0.055</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>secondary</td>
<td>67</td>
<td>5.4 / 5</td>
<td>1.39</td>
<td>0.11</td>
<td>0.062</td>
<td>24</td>
<td>17</td>
<td>RD (3)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>M2</td>
<td>73</td>
<td>5.6 / 34</td>
<td>1.21</td>
<td>0.09</td>
<td>0.065</td>
<td>17</td>
<td>16</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>M2</td>
<td>50</td>
<td>9.0 / 52</td>
<td>1.88</td>
<td>0.31</td>
<td>0.071</td>
<td>97</td>
<td>3</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>M0</td>
<td>56</td>
<td>25.0 / 30</td>
<td>5.84</td>
<td>1.12</td>
<td>0.208</td>
<td>62</td>
<td>7</td>
<td>RD (4)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>M1</td>
<td>75</td>
<td>173.0 / 100</td>
<td>3.67</td>
<td>0.89</td>
<td>0.173</td>
<td>96</td>
<td>63</td>
<td>CR (1), relapse in 5 months</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>NC</td>
<td>64</td>
<td>1.9 / 10</td>
<td>0.68</td>
<td>0.32</td>
<td>0.065</td>
<td>56</td>
<td>26</td>
<td>CR (2)</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>M4</td>
<td>53</td>
<td>86.0 / 20</td>
<td>5.06</td>
<td>0.90</td>
<td>0.104</td>
<td>32</td>
<td>12</td>
<td>RD (1)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>M2</td>
<td>60</td>
<td>1.7 / 0</td>
<td>r</td>
<td>0.28</td>
<td>0.056</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>M2</td>
<td>78</td>
<td>16.9 / 83</td>
<td>0.44</td>
<td>0.065</td>
<td>88</td>
<td>79</td>
<td>lost to follow-up</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>M2</td>
<td>58</td>
<td>0.5 / 0</td>
<td>1.58</td>
<td>0.39</td>
<td>0.075</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>M2</td>
<td>72</td>
<td>11.7 / 72</td>
<td>2.74</td>
<td>0.34</td>
<td>0.065</td>
<td>92</td>
<td>7</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>M5</td>
<td>52</td>
<td>76.0 / 86</td>
<td>6.04</td>
<td>0.37</td>
<td>0.077</td>
<td>96</td>
<td>96</td>
<td>CR (1), BMT after the second cycle of chemotherapy</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>M0</td>
<td>78</td>
<td>9.5 / 63</td>
<td>2.63</td>
<td>0.48</td>
<td>0.087</td>
<td>93</td>
<td>87</td>
<td>RD (1)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>M2</td>
<td>72</td>
<td>4.8 / 32</td>
<td>1.70</td>
<td>0.33</td>
<td>0.053</td>
<td>35</td>
<td>6</td>
<td>died, no therapy</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>M5</td>
<td>42</td>
<td>22.3 / 87</td>
<td>0.88</td>
<td>0.36</td>
<td>0.036</td>
<td>71</td>
<td>80</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>M4</td>
<td>41</td>
<td>88.4 / 96</td>
<td>8.80</td>
<td>1.11</td>
<td>0.028</td>
<td>92</td>
<td>85</td>
<td>CR (1), BMT after the second cycle of chemotherapy</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>M1</td>
<td>62</td>
<td>2.9 / 37</td>
<td>1.33</td>
<td>0.42</td>
<td>0.043</td>
<td>72</td>
<td>4</td>
<td>CR (1)</td>
<td></td>
</tr>
</tbody>
</table>

Mean AML 57 33.0 / 51 2.92 0.55 0.105 65 33
Median 59 9.9 / 62 2.08 0.42 0.082 73 25
Range 16-86 0.5-173 / 0-100 0.68-11.28 0.09-2.37 0.028-0.516 0-98 1-96
### Table 2B. uPAR and uPA in plasma samples and on the blast cell surfaces of patients with ALL and hybrid leukemia

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Immuno-phenotype</th>
<th>Age</th>
<th>WBC $10^9$/L</th>
<th>Blasts (%) of WBC</th>
<th>suPAR ng/ml</th>
<th>uPA (ng/ml)</th>
<th>uPA (IU/ml)</th>
<th>uPAR-positive cells of blast cells (%)</th>
<th>uPA-positive cells of blast cells (%)</th>
<th>Response to therapy/ comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cALL</td>
<td>33</td>
<td>25.2 / 83</td>
<td>3.49</td>
<td>0.75</td>
<td>0.172</td>
<td>3</td>
<td>3</td>
<td>CR (1)*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>cALL</td>
<td>74</td>
<td>4.9 / 40</td>
<td>r</td>
<td>0.42</td>
<td>0.082</td>
<td>2</td>
<td>2</td>
<td>lost to follow-up</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>cALL</td>
<td>48</td>
<td>4.4 / 18</td>
<td>1.48</td>
<td>0.51</td>
<td>0.092</td>
<td>4</td>
<td>6</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>cALL</td>
<td>29</td>
<td>40.7 / 84</td>
<td>2.26</td>
<td>0.74</td>
<td>0.152</td>
<td>3</td>
<td>4</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>cALL</td>
<td>27</td>
<td>23.6 / 45</td>
<td>2.66</td>
<td>0.72</td>
<td>0.171</td>
<td>20</td>
<td>12</td>
<td>CR (2)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>cALL</td>
<td>79</td>
<td>1.2 / 0</td>
<td>1.25</td>
<td>0.33</td>
<td>0.091</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>cALL</td>
<td>61</td>
<td>17.0 / 24</td>
<td>1.55</td>
<td>0.47</td>
<td>0.100</td>
<td>2</td>
<td>1</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>cALL</td>
<td>53</td>
<td>9.3 / 40</td>
<td>1.68</td>
<td>0.25</td>
<td>0.098</td>
<td>3</td>
<td>2</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>T-ALL</td>
<td>40</td>
<td>4.9 / 0</td>
<td>0.79</td>
<td>0.25</td>
<td>0.049</td>
<td>ND</td>
<td>ND</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>cALL</td>
<td>62</td>
<td>12.8 / 53</td>
<td>3.52</td>
<td>1.27</td>
<td>0.212</td>
<td>ND</td>
<td>ND</td>
<td>RD (2)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>cALL</td>
<td>23</td>
<td>1.2 / 0</td>
<td>0.80</td>
<td>0.30</td>
<td>0.052</td>
<td>ND</td>
<td>ND</td>
<td>CR (2)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>cALL</td>
<td>67</td>
<td>21.4 / 90</td>
<td>3.14</td>
<td>1.37</td>
<td>0.287</td>
<td>2</td>
<td>3</td>
<td>CR (1)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>cALL</td>
<td>17</td>
<td>2.1 / 25</td>
<td>2.72</td>
<td>0.70</td>
<td>0.063</td>
<td>10</td>
<td>2</td>
<td>CR (2)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>ALL</td>
<td>47</td>
<td>13.0 / 39</td>
<td>2.11</td>
<td>0.62</td>
<td>0.125</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>48</td>
<td>9.3 / 40</td>
<td>1.97</td>
<td>0.51</td>
<td>0.098</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>17-79</td>
<td>1.2-40.7 / 0-90</td>
<td>0.79</td>
<td>0.25</td>
<td>0.049</td>
<td>1.2-20</td>
<td>1-12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 A, B, and C.** uPA and uPAR in plasma and on cell surfaces in patients and healthy controls. Plasma samples were tested for uPAR and uPA antigens and for uPA activity; results given as ng/ml for antigen assays and IU/ml for activity assay. Values are means from three separate experiments. Cell-surface uPAR and uPA were analyzed with FACS; results given as a percentage of positive blast cells. WBC, white blood cells; CR (1), (2), complete remission with one/two cycle(s) of chemotherapy; RD (1), (2), (3), residual disease after one/two/three cycles of chemotherapy; ND, not defined; NC, not classifiable; r, reactive (see Materials and Methods, page 41); cALL, common ALL; T-ALL, T-cell ALL; BMT, bone-marrow transplantation; ITP, idiopathic thrombocytopenia; SLE, systemic lupus erythematosus; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia.
### Table 2C. uPAR and uPA in plasma samples of patients with other hematological disorders and controls

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>WBC $10^9/L$</th>
<th>suPAR (ng/ml)</th>
<th>uPA (ng/ml)</th>
<th>uPA (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low-grade B-cell disorder</td>
<td>34</td>
<td>64.9</td>
<td>1.40</td>
<td>ND</td>
<td>0.150</td>
</tr>
<tr>
<td>2</td>
<td>Heavy-chain disease</td>
<td>55</td>
<td>3.1</td>
<td>1.46</td>
<td>0.62</td>
<td>0.182</td>
</tr>
<tr>
<td>3</td>
<td>Aplastic anemia</td>
<td>33</td>
<td>2.0</td>
<td>0.70</td>
<td>0.37</td>
<td>0.116</td>
</tr>
<tr>
<td>4</td>
<td>CML, at diagnosis</td>
<td>59</td>
<td>90.6</td>
<td>4.62</td>
<td>1.16</td>
<td>0.272</td>
</tr>
<tr>
<td>5</td>
<td>CML, during IFN treatment</td>
<td>25</td>
<td>3.0</td>
<td>1.51</td>
<td>12.05</td>
<td>0.080</td>
</tr>
<tr>
<td>6</td>
<td>CML, during IFN treatment</td>
<td>38</td>
<td>4.0</td>
<td>1.83</td>
<td>1.74</td>
<td>0.168</td>
</tr>
<tr>
<td>7</td>
<td>Myeloma</td>
<td>50</td>
<td>2.1</td>
<td>1.45</td>
<td>0.48</td>
<td>0.110</td>
</tr>
<tr>
<td>8</td>
<td>Pancytopenia (drug-induced)</td>
<td>51</td>
<td>0.7</td>
<td>1.89</td>
<td>0.16</td>
<td>0.043</td>
</tr>
<tr>
<td>9</td>
<td>Aplastic anemia</td>
<td>26</td>
<td>1.5</td>
<td>0.52</td>
<td>0.32</td>
<td>0.080</td>
</tr>
<tr>
<td>10</td>
<td>Pancytopenia (due to SLE)</td>
<td>55</td>
<td>1.0</td>
<td>1.87</td>
<td>0.25</td>
<td>0.074</td>
</tr>
<tr>
<td>11</td>
<td>Aplastic anemia</td>
<td>26</td>
<td>1.9</td>
<td>0.55</td>
<td>0.27</td>
<td>0.065</td>
</tr>
<tr>
<td>12</td>
<td>ITP, SLE, thrombi</td>
<td>17</td>
<td>1.8</td>
<td>5.45</td>
<td>0.42</td>
<td>0.116</td>
</tr>
<tr>
<td>13</td>
<td>MDS</td>
<td>53</td>
<td>19.7</td>
<td>2.62</td>
<td>0.37</td>
<td>0.123</td>
</tr>
<tr>
<td>14</td>
<td>ITP</td>
<td>26</td>
<td>1.0</td>
<td>1.16</td>
<td>0.14</td>
<td>0.069</td>
</tr>
<tr>
<td>15</td>
<td>Cytopenia (due to infection)</td>
<td>22</td>
<td>7.7</td>
<td>2.81</td>
<td>0.60</td>
<td>0.110</td>
</tr>
<tr>
<td>16</td>
<td>MDS</td>
<td>33</td>
<td>2.3</td>
<td>1.03</td>
<td>0.28</td>
<td>0.079</td>
</tr>
<tr>
<td>17</td>
<td>Cytopenia (due to infection)</td>
<td>51</td>
<td>3.2</td>
<td>$r$</td>
<td>0.37</td>
<td>0.083</td>
</tr>
<tr>
<td>18</td>
<td>Hairy-cell leukemia</td>
<td>53</td>
<td>2.5</td>
<td>0.64</td>
<td>0.41</td>
<td>0.070</td>
</tr>
<tr>
<td>19</td>
<td>Monocytosis NUD</td>
<td>63</td>
<td>15.8</td>
<td>1.04</td>
<td>0.15</td>
<td>0.038</td>
</tr>
<tr>
<td>20</td>
<td>MDS</td>
<td>27</td>
<td>2.1</td>
<td>0.17</td>
<td>0.30</td>
<td>0.054</td>
</tr>
<tr>
<td>21</td>
<td>MDS (later, AML)</td>
<td>68</td>
<td>1.8</td>
<td>3.05</td>
<td>0.56</td>
<td>0.073</td>
</tr>
<tr>
<td>22</td>
<td>CML</td>
<td>61</td>
<td>60.2</td>
<td>2.58</td>
<td>0.43</td>
<td>0.085</td>
</tr>
<tr>
<td>23</td>
<td>Hairy cell leukemia</td>
<td>34</td>
<td>30.0</td>
<td>1.35</td>
<td>0.34</td>
<td>0.064</td>
</tr>
<tr>
<td>24</td>
<td>Myelofibrosis</td>
<td>31</td>
<td>9.7</td>
<td>1.37</td>
<td>0.20</td>
<td>0.042</td>
</tr>
<tr>
<td>25</td>
<td>Follicular lymphoma</td>
<td>57</td>
<td>24.9</td>
<td>0.84</td>
<td>1.22</td>
<td>0.057</td>
</tr>
<tr>
<td>26</td>
<td>Cytopenia NUD (later, ALL)</td>
<td>63</td>
<td>0.3</td>
<td>2.08</td>
<td>0.60</td>
<td>0.056</td>
</tr>
<tr>
<td>27</td>
<td>Cytopenia (drug-induced)</td>
<td>74</td>
<td>1.1</td>
<td>0.71</td>
<td>0.46</td>
<td>0.039</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>17-74</td>
<td>0.3-</td>
<td>0.17-</td>
<td>0.14-</td>
<td>0.038-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90.6</td>
<td>5.45</td>
<td>12.05</td>
<td>0.272</td>
</tr>
<tr>
<td>1-21</td>
<td>Healthy controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>Control</td>
<td>39</td>
<td>1.14</td>
<td>0.32</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>39</td>
<td>1.11</td>
<td>0.32</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>24-61</td>
<td>0.79-</td>
<td>0.14-</td>
<td>0.070-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.72</td>
<td>0.60</td>
<td>0.153</td>
<td></td>
</tr>
</tbody>
</table>

**Increased plasma suPAR levels in AML patients: correlation with prognosis**

Patients with AML and ALL had significantly higher levels of suPAR in their plasma than did healthy controls (2.92±0.44 ng/ml in AML, p<0.005; 2.11±0.29 ng/ml in ALL, p<0.005 vs. 1.14±0.06 ng/ml in healthy individuals). The highest plasma suPAR value, 47.4 ng/ml, was for a patient with a hybrid phenotype. Blast cells of that patient also expressed uPAR. Values for
suPAR showed no correlation with serum C-reactive protein levels, D-dimer values, or WBC count.

Of 32 AML patients, 10 had high plasma suPAR levels (>2.9 ng/ml, mean level in AML patients), 8 of these 10 patients actually failed to respond to chemotherapy or relapsed within a short time (5 months after start of therapy) (see Fig. 11). Furthermore, in only 2 of 18 well-responding patients was the plasma suPAR level above the mean. These two patients (31 and 35 in Table 2A) had the myelomonocytic type of AML and were treated with bone-marrow transplantation after two cycles of chemotherapy. At that time, they had an excess of blast cells and monocytes in their bone marrow, but these cells were regarded as reactive. In contrast, a low level of suPAR did not predict a rapid response to chemotherapy, as after several cycles of chemotherapy six patients had normal levels of suPAR in their plasma and residual disease in the bone marrow.

**Increased levels of uPA antigen in plasma samples of leukemia patients**

In addition to plasma suPAR levels, plasma uPA levels were also significantly increased in acute leukemia patients (0.62±0.10 ng/ml in ALL, 0.55±0.07 ng/ml in AML vs. 0.32±0.02 ng/ml in healthy individuals). Most patients with high plasma uPA levels also had high plasma suPAR levels. uPA activity was generally quite low in plasma samples from healthy individuals and from leukemia patients, but two patients with hybrid leukemia and one AML patient showed markedly higher plasma uPA activity (Fig. 12). Both these hybrid leukemia patients suffered symptomatic retinal bleeding with loss of visual field at the beginning of therapy. An AML patient with high uPA activity died on the third day after diagnosis, and bleeding diathesis could not be evaluated properly.
Tumor-cell production of uPAR and uPAR fragments in acute leukemia patients during chemotherapy (IV)

Although suPAR levels have been shown to be elevated in a variety of solid tumors, no longitudinal studies have addressed the behavior of suPAR during chemotherapy treatment. In addition, the source of excess suPAR in cancer patients has not been discovered. As we have observed that in leukemia patients, and especially in AML patients, suPAR levels were elevated at the time of diagnosis, we continued our project in acute leukemia and studied uPAR in plasma, urine, and in tumor cells in acute leukemia patients during chemotherapy and also in healthy individuals.

Follow-up samples (blood and urine) were collected from patients with acute leukemia (n=35) at 5, 14, 28, and 56 days after start of chemotherapy (total samples=151). Of 35 patients, 25 had AML, 8 had ALL, and 2 were diagnosed with hybrid leukemia. Samples from 40 healthy individuals served as controls. From the EDTA blood samples, plasma and mononuclear cells were separated and lysates made. In addition, 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. uPAR and uPAR fragments were analyzed with ELISA and immunoprecipitation followed by immunoblotting.

Correlations between suPAR levels and tumor-cell count in the circulation

Plasma suPAR levels were elevated in acute leukemia patients at diagnosis (2.42±0.41 ng/ml compared to 0.82±0.04 ng/ml in healthy controls). When suPAR levels were compared to the tumor-cell count, a significant correlation appeared (Rho=0.59, p=0.002). Especially in AML patients, the correlation was stronger (Rho=0.69), since tumor-cell lysates of AML patients
Results

contained uPAR, but ALL patients' tumor cells contained only a little or no uPAR. Furthermore, plasma suPAR levels correlated highly with the content of uPAR in tumor cell lysates (Rho=0.86, p=0.0004). These data strongly suggest that the excess plasma suPAR is produced by tumor cells. To confirm that high plasma suPAR levels are not only due to the high WBC count in acute leukemia patients, we studied chronic-phase CML patients who had extremely high WBC counts (mean 138x10^9 cells/L, range 82-225x10^9 cells/L) and found that their suPAR levels were still low (mean 1.07 ng/ml, range 0.65-1.49 ng/ml) with no correlation between high WBC count and plasma suPAR level. We concluded therefore that the abundant release of uPAR from the cell surface to the plasma is a special characteristic of malignant tumor cells.

Rapid decrease in suPAR levels during chemotherapy

In AML patients, suPAR levels decreased to the normal plasma suPAR level within 2 weeks of chemotherapy, at the same time as tumor cells disappeared from the circulation. That this decrease in plasma suPAR levels correlated highly with the decrease in tumor cells in the circulation in 2 weeks strengthens the notion that the excess plasma suPAR is derived from tumor cells. In addition, in AML patients plasma suPAR levels at diagnosis correlated significantly with the decrease in suPAR levels after 2 (r=0.94) and 4 weeks (r=0.95) of treatment (Fig. 13). Patients with normal plasma suPAR levels and no tumor cells in circulation at diagnosis showed no marked changes in suPAR levels during chemotherapy (see also Table 3).

Fig. 13. suPAR at diagnosis vs. decrease in suPAR level during chemotherapy. Plasma suPAR levels were measured in AML patients’ plasma samples at diagnosis and during chemotherapy. The graph shows the correlation between suPAR values at diagnosis and a decrease in suPAR values in 2 or 4 weeks. Correlation coefficients, analyzed by Pearson correlation test, were r=0.92 and r=0.95, respectively, p<0.001.
Results

Urinary suPAR levels closely followed plasma suPAR levels at diagnosis and during chemotherapy. The only exception was seen in urinary samples taken 5 days after the start of chemotherapy. Those samples showed a peak in urinary suPAR, a type of peak not observed in blood samples. Because the increase in urinary suPAR levels 5 days after start of chemotherapy correlated with the decrease in tumor-cell count at the same time, the urinary suPAR peak may be a reflection of massive tumor cell death in the body and of accumulation of suPAR in the urine (see Table 3).

Expression patterns of uPAR fragments in leukemia patients and in healthy controls

Since proteolytic cleavage of uPAR induces a potent chemotactic response in vitro, it was of interest to study whether these fragments exist in vivo in blood and urine samples of leukemia patients. In leukemia cells of AML patients, both full-length receptor and a fragment corresponding to domains D2+D3 existed. In contrast, mononuclear cells of healthy controls contained only the full-length receptor, and cell lysates of ALL patients usually had no uPAR. The same phenomenon was observed in peripheral blood plasma samples and in plasma samples of bone marrow aspirates. In AML patients, fragmented uPAR was more visible in plasma samples of bone marrow aspirates than in samples of peripheral blood. This may have been due to the higher local concentration of tumor cells in the bone marrow. In bone marrow aspirates of healthy controls or of patients with chronic leukemia, only full-length uPAR appeared. When we analyzed plasma samples of bone marrow aspirates with zymography, we noticed that the samples with higher amounts of fragmented receptor had more active uPA than did samples containing only full-length receptor (Fig. 14).

Fig. 14. Zymogram of plasma samples from bone-marrow aspirates

<table>
<thead>
<tr>
<th>uPA</th>
<th>tPA</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
</table>

The first two lanes show lysis bands from uPA and tPA standards; lanes A, samples from healthy controls; B, samples from AML patients with cleaved uPAR in bone-marrow aspirates; C, AML patients with only full-length receptor in the bone marrow; and lane D, CML patient with no cleaved receptor in the marrow.
Table 3. Summary of AML patient data

| Code | FAB type | Treated with chemotherapy | Age | Circ. blast cells at dg (10^9/L) | uPAR (ng/mg) in cell lysates | suPAR in plasma (ng/ml) at day 0 | suPAR in plasma (ng/ml) at day 5 | suPAR in plasma (ng/ml) at day 14 | suPAR in plasma (ng/ml) at day 28 | suPAR in plasma (ng/ml) at day 56 | suPAR in urine (ng/mg) at day 0 | suPAR in urine (ng/mg) at day 5 | suPAR in urine (ng/mg) at day 14 | suPAR in urine (ng/mg) at day 28 | suPAR in urine (ng/mg) at day 56 |
|------|----------|---------------------------|-----|---------------------------------|----------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| A1   | M4       | Yes                       | 65  | 39                              | 1.20                       | 10.5                         | 7.1                           | 1.5                           | 1.2                           | †                             | 23.1                         | 29.6                          | 16.3                          | 8.1                           | †                             |
| A2   | M2       | Yes                       | 67  | 14                              | -                          | 1.0                          | 1.1                           | 0.8                           | 0.7                           | 0.6                           | 2.9                          | 4.4                           | 1.4                           | 2.3                           | 2.2                           |
| A3   | M2       | Yes                       | 66  | 61                              | 0.45                       | 2.6                          | 2.3                           | 1.2                           | 0.9                           | 0.8                           | 5.4                          | 10.7                          | 2.6                           | 1.6                           | 1.7                           |
| A4   | M4       | Yes                       | 36  | 17                              | 0.30                       | 3.5                          | 3.5                           | 4.4                           | 3.6                           | 1.2                           | 9.7                          | 10.0                          | 16.0                          | 9.1                           | 4.7                           |
| A5   | NC       | No                        | 74  | 26                              | 0.40                       | 3.2                          | 4.9                           | †                             | -                             | -                             | 19.8                         | 20.3                          | †                             | -                             | -                             |
| A6   | M0       | Yes                       | 65  | 0                               | 0.20                       | 1.0                          | 0.9                           | †                             | -                             | -                             | 1.8                          | 2.1                           | †                             | -                             | -                             |
| A7   | M2       | Yes                       | 66  | 0.4                             | 0.20                       | R                            | R                             | R                             | -                             | -                             | 1.8                          | 5.2                           | 13.6                          | 4.9                           | -                             |
| A8   | NC       | Yes                       | 42  | 5                               | ND                         | 0.8                          | 0.8                           | 0.8                           | 0.9                           | -                             | 0.8                          | 0.8                           | 3.1                           | 6.7                           | -                             |
| A9   | M2       | Yes                       | 51  | 84                              | 0.30                       | 1.7                          | 1.2                           | 0.7                           | 0.6                           | 1.0                           | 7.7                          | -                             | 2.2                           | 1.3                           | 2.8                           |
| A10  | M1       | No                        | 63  | 58                              | 0.20                       | 1.6                          | †                             | -                             | -                             | -                             | 7.6                          | †                             | -                             | -                             | -                             |
| A11  | M2       | Yes                       | 51  | 16                              | 0.25                       | 1.7                          | 1.7                           | 1.2                           | 1.1                           | 1.1                           | 4.0                          | 13.6                          | 1.7                           | 1.2                           | 9.8                           |
| A12  | M7       | Yes                       | 61  | 1                               | 0.15                       | R                            | R                             | R                             | R                             | -                             | 1.9                          | 2.0                           | 4.5                           | 3.8                           | 4.3                           |
| A13  | M2       | Yes                       | 66  | 9                               | -                          | 1.9                          | 1.2                           | †                             | -                             | -                             | 18.7                         | †                             | -                             | -                             | -                             |
| A14  | M4       | Yes                       | 46  | 82                              | 0.25                       | 2.3                          | 1.3                           | 0.9                           | 1.0                           | 0.4                           | 2.5                          | 10.6                          | 12.3                          | 3.3                           | -                             |
| A15  | M2       | Yes                       | 23  | 34                              | 0.08                       | 1.5                          | 1.6                           | 0.7                           | 1.0                           | 0.5                           | 2.5                          | 8.8                           | 3.2                           | 2.4                           | 0.9                           |
| A16  | M0       | Yes                       | 39  | 0                               | 0.20                       | 1.3                          | 1.2                           | 2.2                           | 1.2                           | 1.3                           | 1.6                          | 1.2                           | 4.4                           | 8.9                           | 6.1                           |
| A17  | M2       | Yes                       | 66  | 62                              | 0.30                       | 3.1                          | 3.3                           | 1.4                           | 0.9                           | 0.7                           | 6.8                          | 13.8                          | 8.5                           | 2.0                           | 2.1                           |
| A18  | M1       | Yes                       | 34  | 10                              | 0.20                       | 0.9                          | 0.8                           | 0.5                           | 1.9                           | 0.9                           | 2.9                          | 2.6                           | 3.8                           | 6.6                           | 1.9                           |
| A19  | M2       | Yes                       | 51  | 4                               | ND                         | 0.7                          | 0.5                           | 0.9                           | 0.9                           | 0.6                           | 2.4                          | 1.1                           | 1.8                           | 1.6                           | 1.3                           |
| A20  | M2       | Yes                       | 78  | 118                             | 0.35                       | 2.4                          | 1.4                           | 0.8                           | 0.6                           | 0.8                           | 3.2                          | 3.5                           | 2.3                           | 2.0                           | 2.3                           |
| A21  | M2       | Yes                       | 71  | 74                              | 0.50                       | 4.5                          | 2.5                           | 1.0                           | 1.6                           | †                             | 3.3                          | 3.1                           | 2.0                           | 4.1                           | †                             |
| A22  | M4       | Yes                       | 61  | 49                              | 0.90                       | 3.9                          | 2.1                           | 1.3                           | 0.7                           | 0.5                           | 4.4                          | 2.7                           | 1.6                           | 1.8                           | 1.8                           |
| A23  | M5       | Yes                       | 68  | 67                              | 1.45                       | 7.9                          | 3.5                           | 1.2                           | †                             | -                             | 6.7                          | 7.8                           | 4.6                           | †                             | -                             |
| A24  | M2       | Yes                       | 53  | 1                               | 0.20                       | 0.8                          | 0.5                           | 0.6                           | 1.4                           | -                             | 4.2                          | 2.5                           | 0.9                           | 4.4                           | -                             |
| A25  | M2       | Yes                       | 48  | 3                               | -                          | 0.4                          | 0.3                           | 0.4                           | 0.6                           | -                             | 0.5                          | 0.6                           | 0.8                           | 1.7                           | -                             |
| Mean |          |                           | 56  | 33                              | 0.37                       | 2.6                          | 1.9                           | 1.2                           | 1.2                           | 0.8                           | 4.7                          | 6.7                           | 4.8                           | 3.9                           | 3.2                           |

uPAR levels were measured with ELISA, with their values at days 0, 5, 14, 28, and 56 after start of chemotherapy. Urinary suPAR values were normalized with the amount of creatinine in urine samples. In monocytic leukemias, abnormal monocytes were included in blast cell counts. ND: not detectable, NC: not classified, R: reactive (see Methods, page 41), dg: diagnosis, circ.: circulating, †: died.
Healthy individuals had both full-length uPAR and domains D2+D3 in their urine, but D1 was not usually observed. In contrast, AML patients with a high tumor-cell count had, at diagnosis, large amounts of D1 in their urine. In the follow-up samples, the amount of D1 decreased, and the uPAR fragment pattern started to resemble the pattern seen in healthy controls. In ALL patients, the fragment pattern at diagnosis and during chemotherapy did not undergo any considerable change and was similar to that of healthy individuals. This all gave further evidence that in AML patients, plasma and urinary suPAR are released from tumor cells. Furthermore, because uPAR fragments were observed in vivo in patient samples, and as the fragment pattern in leukemia differed considerably from that seen in healthy controls, it is possible that these fragments may play a role in the pathophysiology of acute leukemia.

**Release of soluble uPAR (suPAR) by endothelial cells and peripheral blood mononuclear cells: effect of cell interactions on suPAR release (V)**

Although our studies in leukemia showed that the excess plasma suPAR must be produced by tumor cells, the cause for the low level of plasma suPAR in healthy individuals was still unclear. Therefore, our aim was to study the cellular source of suPAR in plasma samples of healthy individuals and factors influencing its release.

A variety of cell types in our body such as endothelial cells, fibroblasts, keratinocytes, smooth muscle cells, and monocytes express uPAR. Because blood is in close contact with endothelial cells and with various blood cells, we first chose to study the release of uPAR by these cells. We cultured endothelial cells and peripheral blood mononuclear cells under physiological conditions and measured suPAR in the growth medium, as well as uPAR in the cells with immunoprecipitation, immunoblotting, immunofluorescence staining, and ELISA.

**Production of suPAR by mononuclear blood cells and endothelial cells**

We incubated citrated or heparinized blood from healthy individuals on a rotator at 4°C or at 37°C to find whether blood cells can release suPAR into the plasma. No increase in plasma suPAR was observed in blood incubated at 4°C during 3 days of incubation. In contrast, in blood incubated at 37°C, we observed a significant increase in plasma suPAR level after only one day (0.71±0.12 ng/ml at the beginning vs. 1.32±0.21 ng/ml after one day at 37°C). Plasma levels increased further and were 1.93±0.19 ng/ml after 3 days of incubation (Fig. 15).

Because these experiments showed that blood cells can release suPAR, we decided to analyze more closely the release of suPAR by mononuclear leukocytes under in vitro conditions. When purified blood mononuclear cells were cultured under physiological conditions without stimulation, they secreted a small amount of suPAR into their culture media (0.13 ng/10^6 cells in
Results

24 hours); endothelial cells were able to release suPAR under culture conditions (0.48 ng/10^6 cells in 24 hours). In contrast, similarly cultured or PMA-stimulated thrombocytes were unable to release any suPAR into the culture medium, nor was any suPAR found in thrombocyte lysates.

Enhanced suPAR release by endothelial cells in co-culture with mononuclear cells or with thrombocytes: cell-cell contact required

Because in the normal circulation, blood cells and endothelial cells are simultaneously present, we examined the release of suPAR when these cells are cultured together. Unexpectedly, we noticed that enhanced amounts of suPAR appeared in the culture media when either blood mononuclear cells or thrombocytes were cultured together with endothelial cells. To study whether cell-cell contact was required for enhanced suPAR release, we then co-cultured cells in Transwell chambers. These experiments showed that soluble molecules in shared growth medium were not sufficient to enhance suPAR release. In the co-culture media without cell-cell contact, suPAR levels did not differ from the combined values of separately growing cells. However, when cells were allowed to adhere to each other, enhanced release of suPAR appeared again.

In thrombocyte-endothelial cell co-cultures, it was most likely that endothelial cells were responsible for the enhanced suPAR release, because thrombocytes failed to produce any suPAR. However, in mononuclear-endothelial cell co-cultures this was unclear, because both cell types were able to release suPAR. We therefore studied uPAR in cell lysates and on cell surfaces. In the endothelial cell lysates, after co-culturing the cells with mononuclear cells we found higher amounts of uPAR than in the situation of endothelial cells cultured alone.

Fig. 15. Production of suPAR by blood cells.
Citrated or heparinized blood was obtained from healthy individuals, and plasma was separated after 0 to 3 days of incubation on a rotator at 4°C or at 37°C. suPAR levels measured with uPAR ELISA and the mean±SE values are shown.

Enhanced suPAR release by endothelial cells in co-culture with mononuclear cells or with thrombocytes: cell-cell contact required

Because in the normal circulation, blood cells and endothelial cells are simultaneously present, we examined the release of suPAR when these cells are cultured together. Unexpectedly, we noticed that enhanced amounts of suPAR appeared in the culture media when either blood mononuclear cells or thrombocytes were cultured together with endothelial cells. To study whether cell-cell contact was required for enhanced suPAR release, we then co-cultured cells in Transwell chambers. These experiments showed that soluble molecules in shared growth medium were not sufficient to enhance suPAR release. In the co-culture media without cell-cell contact, suPAR levels did not differ from the combined values of separately growing cells. However, when cells were allowed to adhere to each other, enhanced release of suPAR appeared again.

In thrombocyte-endothelial cell co-cultures, it was most likely that endothelial cells were responsible for the enhanced suPAR release, because thrombocytes failed to produce any suPAR. However, in mononuclear-endothelial cell co-cultures this was unclear, because both cell types were able to release suPAR. We therefore studied uPAR in cell lysates and on cell surfaces. In the endothelial cell lysates, after co-culturing the cells with mononuclear cells we found higher amounts of uPAR than in the situation of endothelial cells cultured alone.
Furthermore, with immunofluorescence staining, we observed that the intensity of uPAR staining was markedly elevated in endothelial cells co-cultured with mononuclear cells, which suggested that endothelial cells are responsible for enhanced suPAR release.

We next studied the presence of uPAR fragments from the culture media and cell lysates by immunoprecipitation and immunoblotting. In the endothelial cell lysates, both full-length uPAR and the D2D3 fragment existed, and noticeably, cells grown in co-cultures showed more fragmented uPAR. Furthermore, in the co-culture growth media samples, we found all forms of uPAR: full-length receptor and fragments D2D3 and D1. Fragment D1 has thus far been detected only in the culture media of PMA-stimulated U937 cells and in the urine samples. Although these results warrant consideration of the role of suPAR release in cell adhesion, further studies are still needed to resolve the mechanical factors behind this phenomenon.
DISCUSSION

Over the last 20 years acute leukemias have been classified according to the FAB classification, which is based on morphological and cytochemical features. However, in recent years, knowledge in cytogenetics has markedly increased, and also much more is known of its significance for the outcome of the disease. Furthermore, other important features have been recognized regarding the clinical behavior of the leukemia. Based on all this, a new WHO classification of neoplastic diseases of the hematopoietic and lymphoid tissues was recently launched (Harris et al., 1999). It tries to integrate genetic and clinical features with the morphology, cytochemistry, and immunophenotype of the neoplastic cells, and in that manner, to define disease entities which have clinical relevance. In the future, leukemias may be even more strictly divided into subclasses according to the biological features of their malignant cells. This could assist in planning individual modes of therapy for individual patients. It is therefore of great importance to search for biological factors which influence disease progression. In this work, we have tried to evaluate various components related to the pathophysiology, clinical characteristics, and severity of the disease.

Impact of plasminogen activation in leukemia

Production of plasminogen activators by adherent tumor cells is closely related to the migration and invasive properties of malignant cells. The importance of proteolysis is less clear in hematopoietic malignancies than in solid tumors. Earlier studies have shown pronounced differences in uPA-catalyzed plasminogen activation between leukemia cells and adherent cells derived from tumors of non-hematopoietic origin (Stephens et al., 1988; Tapiovaara et al., 1991; Stephens et al., 1992). PAs were highly expressed by cultured leukemia cells, and in contrast to adherent tumor cells, leukemia cells had an enhanced capacity to activate pro-uPA, and mainly the active form of uPA was released into the culture medium, indicating the proteolytic activity of leukemia cells. In addition to these in vitro studies, only a few earlier studies have examined the plasminogen activation system in ex vivo samples from leukemia patients (Wilson et al., 1983; Tapiovaara et al., 1993; Plesner et al., 1994b). Furthermore, new components of the plasminogen activation system, such as suPAR, have been discovered, and their presence and possible roles in leukemia had not been analyzed. Therefore, we chose to study these aspects of the plasminogen activation system in leukemia both in in vitro models and in ex vivo samples from leukemia patients.
Discussion

Plasminogen activation system and hemostasis

Leukemia patients have an increased bleeding tendency. Much of this bleeding is due to the thrombocytopenia caused by disruption of normal precursor cell differentiation in their bone marrow. However, this bleeding tendency is not always attributable to thrombocytopenia. In normal hemostasis, a fibrin clot is formed at the site of a tissue injury. When the clot is no longer necessary, activation of plasminogen occurs, and plasmin starts to degrade the clot. In hemostatic disorders, plasminogen can be unnecessarily activated, and plasmin as a wide-spectrum protease can degrade almost any plasma protein. In addition, if the plasmin inhibitors are not working properly, uncontrolled fibrinolysis will occur with a fatal outcome. This kind of situation has been described in APL patients with DIC syndrome. Plasma samples of these patients have been shown to contain reduced levels of $\alpha_2$-antiplasmin and PAI-1 and increased levels of plasmin-$\alpha_2$-antiplasmin complexes (Sakata et al., 1991; Fukao et al., 1992; Dombret et al., 1993; Dombret et al., 1995). Therefore, it has been suggested that the actual problem in DIC is excessive fibrinolysis rather than excessive coagulation. In our in vitro model for acute promyelocytic leukemia, we found that on the APL cell surface plasminogen can be activated and plasmin can be formed. These results suggest that because plasmin has a potent protease activity, it can interfere with the normal fibrinolytic balance and be a part of a cascade leading to the excessive bleeding problems encountered in APL patients. Furthermore, we noticed that plasmin formation is transiently induced by RA treatment, but that the overall effect of RA treatment on plasmin formation is suppressive. This fits in well with the clinical observation that in APL patients, RA treatment diminishes bleeding tendency. Our results on plasmin formation in APL were obtained by use of the promyelocytic NB4 cell line, and we have not studied the effect of RA on plasmin formation in APL cells ex vivo from patients. However, we have been able to examine promyelocytic cells from some APL patients with FACS analysis, showing that cells ex vivo from APL patients have both uPAR and uPA on the cell surface in increased amounts. Recently, Menell et al. (1999) have also shown that the expression of annexin II, a proposed receptor for tPA, is higher on the cell surface in APL patients than in other acute leukemia patients and that after RA treatment, its expression decreases.

Our patient material included three patients with markedly high uPA activity in their plasma. All these three also had high uPA antigen levels. Notably, although receiving prophylactic platelet transfusions, during the first weeks of treatment two of these three patients with high uPA activity exhibited symptomatic retinal bleeding with loss of visual field. The third patient with high plasma uPA activity died on the third day after the start of therapy, and bleeding diathesis could not be evaluated properly. Although this bleeding diathesis episode with high plasma uPA activity may have been coincidental, it is noteworthy that in the other 53 patients no clinical bleeding was observed, and although some of these patients had increased plasma uPA protein levels, they still had normal uPA activity levels, indicating that their production of uPA
was controlled by complex-formation with inhibitors. However, more patient data are needed to clarify the real connection between bleeding diathesis and uPA activity, and the possible value of uPA in therapeutic approaches.

**Cell surface uPA and uPAR: diagnostic tools in leukemia?**

Our results demonstrated that in acute leukemia patients, cell-surface uPAR expression is restricted to blast cells expressing myeloid markers (in AML patients and patients with a hybrid phenotype). Earlier studies examining uPAR on the leukemia cell surface have given rather contradictory results (Plesner et al., 1994a; Jardi et al., 1996; Scherrer et al., 1999). Some studies report that only blast cells from AML FAB groups M4 and M5 have uPAR on the cell surface, and blast cells from more poorly differentiated subclasses (M0-M2) have no intracellular or cell surface uPAR. In our material, which included patients from FAB classes M0, M1, M2, M3, M4, and M5, we found that 28 of 29 AML patients had uPAR on the cell surface. In addition, two of three patients with a hybrid phenotype and one ALL patient with aberrant myeloid marker on the blast cell surface were uPAR-positive. The discrepancy of these results for uPAR positivity may be due to the different antibodies and techniques used, and to the source of the blast cells (peripheral blood vs. bone marrow). At the same time our studies were published, Lanza et al. (1998) reported results on uPAR in acute leukemia cells, which are in good accordance with our results. They also found that uPAR is expressed in blast cells of M0 to M5 subclasses of AML and also in blasts of patients with biphenotypic leukemias. Furthermore, they described a combination of different markers (uPAR positivity, abnormalities in chromosome 11, and M5 FAB class), which predict a subset of AML characterized by a more aggressive clinical course. The estimation of blast cell lineage is not always self-evident, and additional phenotypic markers would be very useful. uPAR (CD87) could serve as such an additional marker, because its expression seems to be restricted only to blast cells expressing myeloid markers.

**suPAR as a marker for prognosis in leukemia**

In clinical work there are a few parameters, chromosomal abnormalities, for instance, which can serve as prognostic markers in leukemia. However, the prediction of relapse is often difficult, and early intervention is delayed. Therefore, we wanted to study both the cell-surface and the plasma compartments of blood samples from patients with acute leukemia in an attempt to discover whether components of the plasminogen activation system correlate with severity of the disease. In our patient material, we noticed that in AML patients, high suPAR levels at diagnosis correlated with poor response to chemotherapy and with relapse within a short time (5 months). Although these results suggest that suPAR level can be related to the aggressiveness of the disease, it needs to be remembered that our series was quite small. In a large colon
Discussion

carcinoma series (591 patients), Stephens et al. (1999) showed an increasing risk of mortality with increasing plasma suPAR level and suPAR level to be an independent prognostic marker. It would therefore be of interest to study with a larger size patient population, whether suPAR level is an independent prognostic marker, and whether it correlates with the long-term survival of leukemia patients.

In our follow-up study, we aimed to define whether suPAR can be used as a follow-up marker during therapy, and if it can predict relapse. This seemed promising at first, because we found that suPAR levels correlate with the number of circulating blast cells, and because suPAR levels decreased rapidly during treatment. Furthermore, there were patients who had only a transient decrease in suPAR levels during treatment, whose levels increased again in relapse, or if no remission was achieved. However, it seemed that the levels do not increase before blast cells appear in the circulation, i.e., they fail to show the presence of blast cells in the bone marrow. In addition, there were also patients who had a normal amount of suPAR at diagnosis. In short, a multicenter study of suPAR in leukemia would be interesting and necessary to clarify whether suPAR measurement can be used in the clinical setting as an additional prognostic tool or in combination with other markers such as cytogenetical abnormalities.

Invasion of leukemia cells

In adults, hematopoiesis occurs mainly in the bone marrow. Precursor hematopoietic cells divide and further differentiate there, and when they are well differentiated and mature enough, they are released into the circulation. From the circulation, the fate of various blood cells is often predestined, but sudden changes in the body and various stimuli can alter the circulation of leukocytes. In leukemia, the normal differentiation of blood cells is disturbed, and immature cells burst from the bone marrow into the circulation in an uncontrolled manner. Leukemic cells also have the capability to invade various tissues outside the hematopoietic and lymphatic system. Various cell-surface markers, including ICAMs, have been thought to play a role in the homing of leukocytes and leukemia cells. They also facilitate the adhesion between leukocytes and endothelial cells (Carlos and Harlan, 1994; Gahmberg, 1997; Gahmberg et al., 1997). In addition to ICAMs, also many other molecules, including integrins and uPAR, are involved in the tissue-invasion by normal leukocytes and adherent tumor cells (Chapman, 1997; Danø et al., 1999; Preissner et al., 2000). In leukemia, because the invasion mechanism of blast cells and factors influencing it have not been studied in detail, we examined leukemia cell invasion in three-dimensional cultures mimicking the in vivo situation and evaluated factors which could play a possible role in the disease progression.
RA-induced invasion of promyelocytic leukemia cells

The effect of RA on APL cell extravasation is very pronounced. Without RA treatment, neither promyelocytic NB4 cells nor cells ex vivo from an APL patient were able to traverse the endothelial cell layer in the organotypic culture. However, when differentiation was induced by RA, both of these cell types started to invade rapidly. Which molecules are responsible for this invasion? Our results with FACS analysis and Northern blotting showed that RA induces the expression of uPAR and PAI-1, both involved in vitronectin-dependent adhesion. Furthermore, without RA treatment, promyelocytic cells had only ICAM-3 among the various ICAMs on their cell surfaces, but RA treatment also induced expression of ICAM-1 and of the integrins CD11c and CD18. Interestingly, earlier results have demonstrated that uPAR is associated with MAC-1 (CD11b/CD18) and also with other integrins. Therefore, the RA-induced extravasation of promyelocytic cells can be the result of the combined effect of different types of adhesive molecules. Our blocking experiments with antibodies to various adhesion molecules demonstrated that ICAM-1 antibodies can inhibit the RA-induced extravasation. In addition, our preliminary experiments with uPAR antibodies show that polyclonal antibodies against uPAR do inhibit the extravasation, but monoclonal uPAR antibodies (R3 and R4) fail to inhibit it. It is possible that the effect of polyclonal antibodies is nonspecific (perhaps due to sterical hindrance), but it is also possible that the effect is specific and that the monoclonal antibodies used are unable to block those important sites required for their association with other adhesion molecules (e.g., with vitronectin and integrins). Although RA has a therapeutic effect on APL by inducing cell differentiation, it may also have harmful side-effects. In the RA syndrome, patients have fever and cell-infiltration which may, in part, be caused by the RA-induced changes in the adhesion molecule profile on the cell surface (Frankel et al., 1992). If these symptoms are caused by the induced expression of ICAMs or of uPAR, it would be difficult to develop agents specific against leukemia cells, because these molecules are expressed on the surfaces of normal leukocytes as well, and because they also have important functions in normal cell adhesion.

Extravasation of blast cells vs. normal mononuclear cells

It seems that same adhesion molecules take part in the extravasation process of normal leukocytes and leukemia cells, because in both cases ICAM-1 antibodies were able to inhibit the invasion of cells through endothelial cell layers. However, blast cells from different subclasses of AML usually have different capabilities of invasion. In monocytic types of AML (M4/M5), more often skin infiltrations of leukemia cells occur. Earlier studies have shown that ICAM-1 expression does not correlate with any specific AML subtype (Maio et al., 1990; Bruserud and Ulvestad, 1999). Furthermore, our studies demonstrated no correlation between sICAM-1 level in plasma and AML FAB class. In adherent tumor cells, uPAR expression has been associated with a more invasive phenotype. Intriguingly, our results in AML showed that blast cells from
M4/M5 subclasses have the highest amounts of uPAR on their surfaces. In addition, Scherrer et al. (1999) have reported that at the mRNA level, uPAR expression is found only in M4/M5 subtypes. It is therefore possible that uPAR is related to M4/M5 blast cells’ invasive capacity.

The biological function of soluble ICAMs in adhesion is unclear. On the one hand, in vitro sICAM-1 can bind LFA-1 when bound to the substratum (Rothlein et al., 1991; Hedman et al., 1992), but on the other hand, sICAM-1 had no effect in our adhesion/extravasation experiments. Furthermore, because we noticed no correlation between plasma sICAM-1 levels and the invasive behavior of leukemia, it seems probable that soluble ICAMs in the circulation may have no function in leukemia cell adhesion. However, shedding of ICAM-1 from the cell surface may have some function in disease progression, since it has been shown that lower ICAM-1 expression on the leukemia cell surface is associated with the resistance of leukemia cells to the lytic activity of lymphokine-activated killer (LAK) cells (Raspadori et al., 1993). This suggests that leukemia cells may avoid being killed by the immune system by regulating their own ICAM-1 expression. It would thus be important to study whether differences exist in the adhesion molecule profile between leukemia cells in the bone marrow and in the peripheral blood. It is possible that leukemia cells need several adhesion molecules when they escape from the bone marrow into the circulation, and then later on, they regulate their adhesion molecule profile by shedding these receptors from their cell surfaces. This may result in a situation in which leukemia cells are no longer under the control of the normal immune system, and they are able to divide and invade in an uncontrolled manner.

**uPAR - a multifunctional protein. Biological function in vivo in cancer patients?**

In order to be able to grow and metastasize, malignant tumors need help from non-malignant cells, e.g., from endothelial cells to form blood vessels in order to guarantee a sufficient nutrition supply. It has been suggested that tumor cells recruit other cells to produce molecules that could help the tumor cells invade. In adherent tumors, uPAR and other components of the PA system are not always expressed by tumor cells, but by the surrounding stromal cells. However, in many in vitro experiments, uPAR has been localized to the leading edge of migrating tumor cells, suggesting that uPAR function is also important for tumor cells. In blood samples of patients with various malignant adherent tumors (ovarian, bladder, colon, breast, lung, prostate, and head and neck carcinomas) plasma/serum suPAR levels have been elevated (Pappot et al., 1997; Stephens et al., 1997; Sier et al., 1998; Wahlberg et al., 1998; Miyake et al., 1999; Schmidt and Hoppe, 1999; Sier et al., 1999; Stephens et al., 1999). It has not been known whether these elevated levels are produced by tumor cells or by stromal cells. Our results in
leukemia provided quite strong evidence that malignant tumor cells produce the excess plasma and urine suPAR in leukemia patients. Furthermore, we have noticed in vitro that promyelocytic NB4 leukemia cells can produce suPAR into their culture medium. However, the question still remains open whether this soluble molecule has any biological function or is just a release mechanism of cells for getting rid of extra uPAR on their surfaces.

The role of suPAR/uPAR in cell migration, adhesion, and chemotaxis

uPAR cleavage from the cell surface (either cleavage of the full-length receptor or of only the ligand-binding domain) may function as a regulation mechanism for cells to control their adherence to other cells or to the extracellular matrix. For example, in extravasation, cells need to attach themselves to endothelial cells, and then after traversing the endothelial cell barrier, they need to detach to be able to move on. Our in vitro experiments showed that endothelial cells shed more uPAR when they grow in physical contact with leukocytes. It is possible that uPAR shedding from cell surfaces may dissociate cells from integrins and decrease adherence, and in that manner, promote cell movement. This phenomenon can occur very rapidly, and adherence and detachment can follow each other in a cascade-like process. However, it is likely that many other adhesion molecules participate in this process as well, and that they also have overlapping roles. This may be the reason why uPAR knockouts did not show major differences in tumor cell invasion, although marked effects were seen in inflammatory cell recruitment in the same knockouts.

In vitro experiments have shown that the soluble urokinase receptor needs to be cleaved, i.e., the connecting peptide between domain 1 and 2 needs to exposed, before it is able to induce a chemotactic effect in THP-1 and smooth-muscle cells (Resnati et al., 1996; Fazioli et al., 1997; Degryse et al., 1999). In contrast, full-length uPAR is required for efficient vitronectin binding (Wei et al., 1994; Høyer-Hansen et al., 1997a; Sidenius and Blasi, 2000), and furthermore, it has been demonstrated that full-length uPAR is associated with β1-integrins, whereas the cleaved form does not (Montuori et al., 1999). It therefore seems that the different forms of uPAR may be required and be important for different functions. In this study, we found uPAR fragments in tumor cells, and in plasma and urine samples of leukemia patients, whereas samples from healthy individuals usually contained full-length uPAR. This difference is not likely to be accidental. On the contrary, this suggests that these fragments may have some unknown function in cancer pathophysiology. In the future, it would be of interest to isolate these fragments from various samples from cancer patients and to analyze their amino acid sequences, to see where the cleavage occurs in vivo, i.e., to discover whether or not it takes place at the site of uPA cleavage. Furthermore, the isolated fragments could be tested in vitro, to see whether they induce such processes as cytoskeletal reorganization, adherence of cells, or chemotaxis.
Discussion

Such studies would shed more light on the possible role of uPAR and uPAR fragments in the pathophysiology of cancer in general, and on their significance in acute leukemia, as well.
PERSPECTIVES

Because cancer cells resemble the body’s non-malignant cells in many ways, they are not effectively rejected by the immune system. However, in addition to their normal functions, cancer cells also possess advantages which assist them to overgrow normal cells. The ideal way to treat the cancer would be to kill the cancer cells but not to affect normal cells. For example in leukemia, this is very difficult to accomplish by means of conventional chemotherapy, since both normal blood cells and leukemia cells are dividing cells. In addition, both these cell types have similar phenotypic markers on their surfaces. Therefore, it is important to characterize the special features of leukemia cells and to find any possible differences between them and normal blood cells which could be useful in the design of future therapy.

During several decades, strong evidence has accumulated that in tumor invasion and metastasis, the plasminogen activation system plays an important role. The new non-proteolytic roles of the plasminogen activation system have reinforced interest in the plasminogen activation field and also explained some old phenomena not understandable by the mechanisms of the proteolytic functions of the plasminogen activation system. In this study, we have tried to examine the role and the possible importance of the plasminogen activation system and of some adhesion molecules in leukemia. Our results show that the components of the plasminogen activation system are expressed in increased amounts in leukemia cells in vitro and in vivo and that various drugs used in the treatment of leukemia regulate the expression level and the activity of these components. Although our results also suggest that some of these components, such as uPAR, can be used as diagnostic or prognostic markers in leukemia, still further studies are needed to clarify their clinical impact. However, as general knowledge of the functions of uPAR increases, and when our understanding of the biological role of soluble uPAR becomes clearer, the results presented here may also contribute to the understanding of the pathophysiology of leukemia and to better approaches in the treatment of leukemia patients.
ACKNOWLEDGEMENTS

This study has been carried out at the Department of Virology, Haartman Institute, University of Helsinki. I want to thank the Heads of the Department, Professor Antti Vaheri and Professor Carl-Henrik von Bonsdorff, for creating such a research-friendly atmosphere in the department and for providing excellent working facilities. Especially, I want to warmly thank Antti Vaheri for supervising this work. It has been a privilege to work in his group, and his profound and wide knowledge in science has been valuable in my work. Although he has offered me great freedom in conducting my research, when needed he has always been ready to help.

I am deeply grateful to Docent Riitta Alitalo for all her interest and help in my work. Although she has not been an official supervisor, I feel that she has helped and encouraged me so many times during these years and taught me the basics of hematology.

I wish to express my gratitude to my collaborators and friends in the Department of Virology. When I came to the department, Dr. Hannele Tapiovaara was finishing her PhD thesis in plasminogen activation in leukemia. Although we did not have a chance to work together for a long time, I wish to warmly thank her for introducing me to this field and helping and encouraging me in those first important steps in science. Dr. Vappu Sirén has been senior in our group and has always kindly taken care of the whole PA group. I warmly thank her for many joyful discussions and for her ability to see the bright sides of all things. I want especially to thank Heli Myöhänen for teaching me many techniques in the lab and for many nice more-or-less-scientific discussions. Her wide knowledge in the plasminogen activation field has helped me to solve many problems. It has been a pleasure to work with Irina Suomalainen, and I warmly thank her for all her help during these studies. She has not only run after my samples, but she has always been very optimistic and encouraged me in every possible way. My sincere thanks go to all other former or present members of our extended PA group: Drs. Miina Weckroth, Leena-Maija Aaltonen, Ren Wei Chen, Tambet Teesalu, Josef Bizik, Francis Akenami, and Leena Riittinen. You have made our lab a joyful place to work, and I want to express my sincere thanks to you all for your friendship and kind support. In addition, I want to warmly thank Docent Marjaleena Koski-Niemi, Dr. Eeva Auvinen, and Docent Eeva-Marjatta Salonen for their interest in my work.

I have been privileged to collaborate with many other scientists outside our department. I want to warmly thank Docent Erkki Elonen for his help with the clinical issues. Dr. Ross Stephens, Professor Francesco Blasi, Professor Carl G. Gahmberg, and Docent Olli Carpén are gratefully acknowledged for their interest and help in these studies. Drs. Nicolai Sidenius and Cornelis Sier are warmly thanked for many helpful discussions and for their patience in answering my endless e-mails.

My medical student background did not help me much in the first steps of the lab work, and I am grateful to all of you, who have advised and helped me many times during these years.
Especially I want to acknowledge Satu Cankar, Pirjo Sarjakivi, Anja Virtanen, Liisa Ruuskanen, Leena Kostamovaara, Virpi Tiilikainen, Monica Schoultz, and Pirjo Tuomi.

Professors Jorma Keski-Oja and Tapani Ruutu are gratefully acknowledged for reviewing and improving this thesis. In these last steps of the thesis work, when you are already tired and blind towards your work, it has been a joy to get some fresh ideas and suggestions how to improve.

I wish to thank Dr. Carol Norris and also Carol Pelli for reviewing the language of our manuscripts, and Carol Norris also for editing the language of this thesis. The valuable help of Dr. Hanna Oksanen in statistical matters is gratefully acknowledged.

Although this work has buried me many times in the laboratory, I want to thank all my friends with whom I have spent many delightful moments far away from work. Especially, I want to thank my dear friend Eeva Martelin with whom I have shared many scientific and non-scientific sorrows and joys during these years.

The support of my parents has been irreplaceable during these years and for my whole lifetime. I sincerely thank them for their everlasting love and encouragement. I also thank my other relatives, my parents-in-law, and all the rest of my husband’s family for their support and happy moments together.

Finally, I want to express my deepest gratitude to my husband Topi for putting up with me and loving me all these years.

This work was financially supported by The Foundation for the Finnish Cancer Institute, the Finnish Cancer Societies, the Academy of Finland, the Finnish Society of Hematology, the Finnish Association of Hematology, The Helsinki University Hospital Research Funds, the Finnish Medical Foundation, the Jenny and Antti Wihuri Foundation, and Biomedicum Helsinki Foundation.

Helsinki, December 2000

(Signature)
REFERENCES


Arnaout MA, Lanier LL, and Faller DV. Relative contribution of the leukocyte molecules Mo1, LFA-1, and p150,95 (LeuM5) in adhesion of granulocytes and monocytes to vascular endothelium is tissue- and stimulus-specific. Journal of Cellular Physiology 137: 305-309, 1988.


References


Blasi F. Proteolysis, cell adhesion, chemotaxis, and invasiveness are regulated by the u-PA-u-PAR-PAI-1 system. Thromb Haemost 82: 298-304, 1999a.


References


References


Meerschaert J, and Furie MB. The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. Journal of Immunology 154: 4099-4012, 1995.


Plesner T, Ploug M, Ellis V, Rönne E, Hoyer-Hansen G, Wittrup M, Pedersen TL, Tschering T, Dano K, and Hansen NE. The receptor for urokinase-type plasminogen activator and urokinase is translocated from two
References


Plesner T, Behrendt N, and Ploug M. Structure, function and expression on blood and bone marrow cells of the urokinase-type plasminogen activator receptor, uPAR. Stem Cells 15: 398-408, 1997.


References


Sidenius N, and Blasi F. Domain 1 of the urokinase receptor (uPAR) is required for uPAR-mediated cell binding to vitronectin. FEBS Lett 470: 40-46, 2000.


