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LEUKEMIC DISSEMINATION IN ACUTE MYELOGENOUS LEUKEMIA: A PUTATIVE ROLE FOR TYPE IV COLLAGENASES

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **LEUKEMIC DISSEMINATION IN ACUTE MYELOGENOUS LEUKEMIA: A PUTATIVE ROLE FOR TYPE IV COLLAGENASES** submitted by **HAROON RASHID HASHMI** in partial fulfillment of the requirements for the degree of **Master of Science** in **Experimental Medicine**.

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ABSTRACT

In normal steady-state hemopoiesis the passage of blood cells from the bone marrow to the peripheral blood is very selective and only mature and differentiated hemopoietic cells egress from the bone marrow. In acute myelogenous leukemia (AML), however, there is a loss of this selectivity and a large number of immature cells in blast stage are able to cross the bone marrow barriers and appear in circulation.

The present study was undertaken to look at the possible mechanism of dissemination of leukemic cells from the bone marrow and focused on the production of type IV collagenases/gelatinases and their role in the *in vitro* invasiveness of leukemic cells. The experimental approach for this study was based on knowledge obtained from studies of solid tumor cells, most of which are not only invasive *in vitro* but also secrete type IV collagenases/gelatinases.

Unlike the normal hemopoietic progenitors ($CD34^+$ cells) from hematologically normal bone marrow, the leukemic cells studied for this thesis were both invasive in the *in vitro* invasion assay and secreted type IV collagenases/gelatinases. These results therefore suggested that in AML the immature cells in blast stage, unlike their counterparts in the normal bone marrow, secrete type IV collagenases which may enable them to invade through the extra-cellular matrix (ECM) and basement membrane barriers.

This study describes for the first time a possible mechanism underlying the dissemination of leukemic cells from bone marrow to peripheral blood in AML and postulates the role of type IV collagenases/gelatinases in the *in vitro* invasiveness of leukemic cells.

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LIST OF ABBREVIATIONS

AMF	Autocrine motility factor
AML	Acute myelogenous leukemia
AP-1	Activator protein-1
b-FGF	Basic-fibroblast growth factor
BFU-E	Blast-forming unit-erythrocyte
BM	Bone marrow
BMT	Bone marrow transplantation
cDNA	Complimentary DNA
CFU-GEMM	Colony-forming unit-granulocyte/erythrocyte/macrophage /megakaryocyte
CFU-GM	CFU-granulocyte/macrophage
CFU-Meg	CFU-megakaryocyte
CML	Chronic myelogenous leukemia
CSF	Colony-stimulating factor
DMEM	Dulbecco's modified essential medium
ECL	Enhanced chemoluminescence
ECM	Extra-cellular matrix
EDTA	Ethylenediamine-tetraacetic acid
EGF	Epidermal growth factor
EHS	Engleberth Holm-Swarm
FAB	French-American-British
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluoracine isothiocynate
FMLP	*N*-formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte-CSF
GAGs	Glycosaminoglycans

GM-CSF	Granulocyte/macrophage-CSF
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
LTMC	Long term marrow culture
M-CSF	Macrophage-CSF
MACS	Magnetic cell sorting system
MMPs	Matrix metalloproteinases
MT-MMP	Membrane type- matrix metalloproteinase
PA	Plasminogen activator
PA-I	PA-inhibitor
PB	Peripheral blood
PBS	Phosphate buffer saline
PBSCT	Peripheral blood stem cell transplantation
PDGF	Platelet derived growth factor
PHA	Phytohemagglutinin
PMNL	Polymorphonuclear leukocytes
PMSF	Phenylmethylsulfonyl fluoride
rhTIMP	Recombinant human- tissue inhibitor of metalloproteinase
SF	Steel factor
t-PA	Tissue type-PA
TGF-b	Transforming growth factor-b
TIMPs	Tissue inhibitors of metalloproteinases
TM	Transmembrane
TNF	Tumor necrosis factor
TPA	12-O-tetra-decanoylphorbol-13-acetate
u-PA	Urokinase type-PA

" READ! IN THE NAME OF THY LORD AND CHERISHER WHO CREATED.

CREATED MAN, OUT OF (MERE) CLOT OF CONGEALED BLOOD."

The Holy Qur'an, chapter 96, verses 1 and 2

1.0 INTRODUCTION

Acute myelogenous leukemia (AML) is a hematological malignancy characterized by a genetically determined disturbance of the proliferation and differentiation of immature progenitor cells. AML patients have a poor prognosis which can, however, be improved with treatment by chemotherapy and/or bone marrow transplantation. We now have a better knowledge of the biology of AML regarding its origin, the various chromosomal abnormalities associated with it and its different subtypes but, to date, we do not understand fully the mechanisms underlying the egress or dissemination of leukemic blasts from the marrow to the peripheral blood. Under normal conditions the passage of blood cells is very selective and only fully differentiated and mature blood cells egress from the marrow. How immature cells navigate their way into the circulation in AML has hitherto been unanswered. Some reports have implicated changes in endothelial cell lining and expression of adhesion molecules in the egress of leukemic blast cells (1), but we are still far from having a complete and detailed picture of this mechanism and understanding its cause.

1.1 RATIONALE FOR THIS STUDY.

The unique structural components of the basement membranes are the three-dimensionally assembled molecules of laminin, type IV collagen, heparan sulfate proteoglycans, and entactin/nidogen (2). The extra-cellular matrix (ECM) or basement membrane barriers are penetrable by normal mature hemopoietic cells such as granulocytes, monocytes and activated lymphocytes and by metastatic tumor cells. It is likely that these cells accomplish

this by their ability to attach themselves to these membranes and to produce enzymes which break down these barriers. Some early *in vitro* experimental systems used natural tissue barriers such as chick embryo chorioallantoic membrane (3), chick embryo heart (4) and human amniotic membrane (5) to quantitate the invasive potential of tumor cells. More recently, Albini et al. (6) introduced an assay based on the use of Matrigel. Matrigel is a reconstituted basement membrane consisting of collagen type IV, laminin and heparan sulfate proteoglycans extracted from the murine Englebreth Holm-Swarm (EHS) tumor. It produces a structure representative of most biological basement membranes including those found in blood and lymphatic vessels (7). In most studies that have involved the use of Matrigel it has been shown that tumor cells capable of penetrating Matrigel *in vitro* are also invasive and highly metastatic *in vivo* (8-10). Hence the Matrigel-based assay provides a good system for the selection of invasive and noninvasive tumor cells *in vitro*. However, to date few studies have been done to evaluate the invasive potential of malignant hemopoietic cells. Reports indicate that cells from an undifferentiated murine myeloid leukemic cell line (M1) (11) and human promonocytic U-937 cell line (12) exhibit very low or negligible levels of invasion through the Matrigel layer.

One step in the process of invasion is the secretion of proteinases by tumor cells. These proteinases disrupt the membrane barrier by cleaving the components of the basement membrane thereby allowing the migration of tumor cells (13,14). The production by invasive and metastatic tumor cells of a number of such matrix degrading enzymes has been shown to correlate with their invasive potential, while inhibition of these enzymes *in vitro* results in inhibition of tumor invasiveness (15).

Since type IV collagen is the major component of basement membrane, type IV collagenases (92- and 72- kDa collagenases/gelatinases), which belong to the family of metalloproteinases and specifically degrade type IV collagen, gelatin and fibronectin, have been the focus of study among the matrix degrading enzymes. Type IV collagenases have been implicated in malignant conversion, tumor invasion and metastasis, and a positive correlation between expression of these gelatinases and capacity to cross the Matrigel layer has been reported for several metastatic cell lines (12,16,17). Although matrix proteolysis occurs with normal cell functions under physiologic conditions, for example, tissue remodeling and trophoblast implantation, tumor cells combine their mobility with proteolysis at stages of cell growth and at sites where proteolysis by normal cells is not common. To date, not much information has been gathered on the secretion of these type IV collagenases/gelatinases by undifferentiated leukemic cells and their role, if any, in invasion.

1.2 HYPOTHESIS AND EXPERIMENTAL APPROACH.

Based on our knowledge of metastatic processes, the hypothesis for the present study was that, in AML, the blast cells, unlike their normal immature counterparts in the marrow, acquire the capability of producing matrix degrading enzymes, particularly type IV collagenases/gelatinases, which enable them to cross the bone marrow barriers and enter the peripheral blood by invading the ECM and basement membrane. This hypothesis is schematically shown in Figure 1A.

To test this hypothesis the major goals of this study were:

I) *To evaluate whether leukemic cells have in vitro invasive potential.* Based on the knowledge that the Matrigel-based invasion assay has been used successfully for the selection of invasive and noninvasive tumor cells, the first aim of this thesis was to develop and standardize a Matrigel-based assay in order to evaluate the invasive potential of leukemic cells. This assay served as the model system of *in vitro* cell migration for the rest of the study.

II) *To examine whether the leukemic cells secrete type IV collagenases/gelatinases.* This would be studied by zymography, a technique that identifies enzyme activity by substrate degradation. To detect type IV collagenases/gelatinases, unheated samples in non-reducing conditions would be applied to polyacrylamide gels copolymerized with gelatin. After electrophoresis the gels would be incubated at 37°C for 24 to 48 hrs followed by staining and destaining procedure. The enzyme activities would be visualized as clear bands against a blue background of Commassie blue.

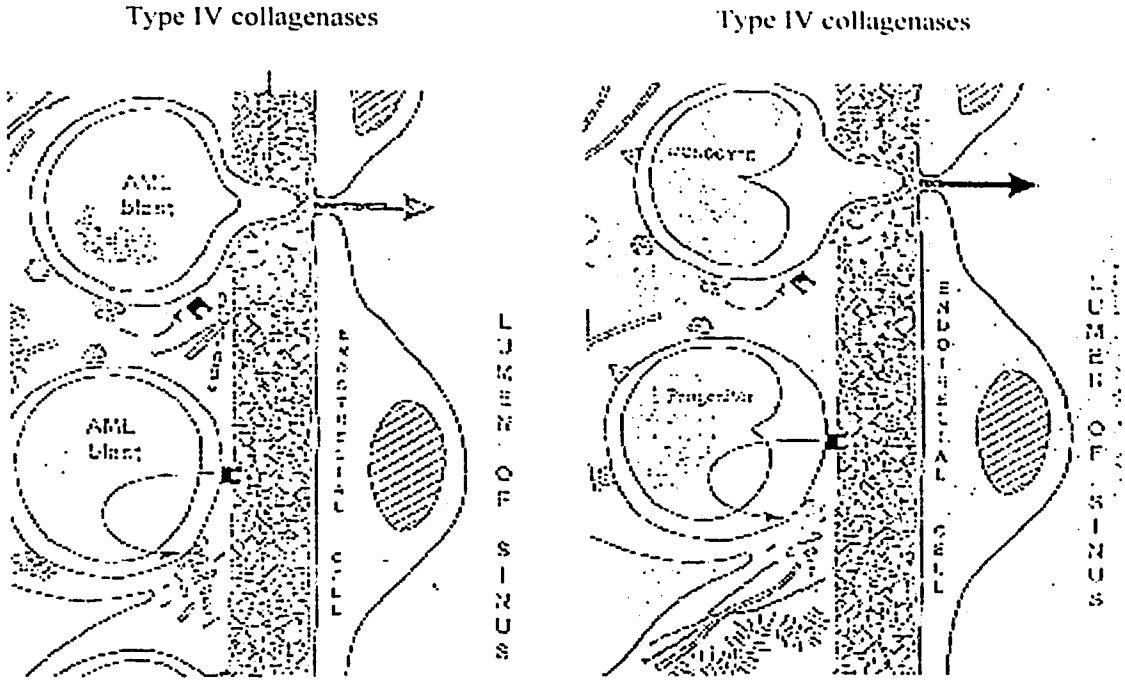
III) *To determine whether type IV collagenases/gelatinases are involved in the in vitro invasiveness of leukemic cells.* If the leukemic cells were found to be invasive *in vitro* and also secreted type IV collagenases/gelatinases, the next step would be to examine whether these gelatinases have a direct role in the *in vitro* invasiveness of leukemic cells. This could be tested by demonstrating inhibition of invasiveness in the *in vitro* invasion assay using inhibitors of these enzymes including natural inhibitors of metalloproteinases and antibodies against type IV collagenases/gelatinases.

IV) *To test whether normal hematopoietic progenitor cells are invasive* in vitro *and whether they secrete type IV collagenases.* $CD34^+$ cells would be isolated from normal

bone marrow to test whether there were any differences in invasive potential and type IV collagenase production by leukemic cells and normal progenitor cells.

The hypothesis could be assumed to be correct if leukemic cells, but not undifferentiated and immature, cells from the normal marrow, were found to secrete type IV collagenases/gelatinases and were invasive in the Matrigel-based *in vitro* invasion assay, particularly if their invasiveness could be modulated by inhibitors of type IV collagenases.

Development of an animal model to test this hypothesis would be desirable to generate information which better simulates *in vivo* conditions but, until such studies are carried out, results obtained from this study could provide us with some information about the behaviour and invasive properties of AML cells and advance our understanding about the biology of leukemia.



A. AML bone marrow

B. Normal bone marrow

Figure 1a. *Schematic presentation of hypothesis.*

A. In AML, undifferentiated and immature leukemic blasts in the bone marrow are able to secrete type IV collagenases which enable them to cross through the basement membrane and enter the peripheral blood.

B. In normal bone marrow, however, type IV collagenases are secreted only by fully differentiated and mature blood cells during their egress from the bone marrow.

⊔, ⬡, △ Representing cell adhesion molecules.

2.0 LITERATURE REVIEW

2.1 NORMAL HEMOPOIESIS

2.1.1 Development of hemopoietic cells and hemopoietic microenvironment

The most primitive stem cells in the bone marrow, known as "totipotent" or "pluripotent" stem cells, have the capability to give rise to all the lymphoid (T lymphocytes, B lymphocytes, natural killer cells) and myeloid (erythrocytes, granulocytes, monocytes, platelets) cell lineages involved in hemopoiesis (18). Primitive stem cells exhibit a high potential for self-renewal, an ability that ensures their presence in sufficient numbers to maintain hemopoiesis throughout adult life. These cells are extremely rare and represent approximately 1 per 10^4 bone marrow cells (18). The presence of the CD34 antigen is a good marker to identify primitive stem cells capable of long-term reconstitution, as well as progenitor cells committed to the myeloid, lymphoid and erythroid lineages (78). The question may be asked: how a pluripotent stem cell "decides" whether to produce another stem cell of the same type when it divides or a more mature daughter cell? It has been shown that the stem cell either self-renews on division or differentiates into a committed progenitor by a random or "stochastic" rather than a deterministic process (21,22).

To produce mature cells, (i.e., erythrocytes, granulocytes, monocytes, lymphocytes and platelets) a pluripotent, self-renewable stem cell differentiates into a colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) stem cell that loses its ability to self-renew (34). On further division the CFU-GEMM differentiates into a progenitor cell that is committed to a particular myeloid lineage colony-forming unit-

granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) or colony-forming unit-megakaryocyte (CFU-Meg) progenitor cell. After four to five subsequent divisions by these committed progenitors, mature blood cells are produced (23,24).

The hemopoietic stem cells and progenitor cells, however, do not develop and function independently; their development and differentiation, processes known as myelopoiesis, erythropoiesis and lymphopoiesis, take place within a *hemopoietic microenvironment* in the bone marrow (19). The hemopoietic microenvironment mainly consists of *stromal cells* (fibroblasts, macrophages, endothelial cells and adipocytes), *accessory cells* (T-lymphocytes and monocytes) and their products, *extracellular matrix* (ECM) *proteins* and *cytokines* (20). These cells and their products influence the development of hemopoietic cells. Interaction between the stem and progenitor cells and the elements of the microenvironment, including both stromal and accessory cells, is critical for the maintenance of the stem cells and the regulation of hemopoiesis (22,25). For example, SL/SL or steel mutant mice, which die from anemia, have a defect in the stromal elements that fail to maintain and regulate hemopoiesis even though the hemopoietic stem cells are competent (25).

Accessory and stromal cells enrich the marrow microenvironment with the mediators known as colony stimulating factors (CSF), hemopoietic growth factors or cytokines, which play a key role in the regulation of hemopoiesis (18). Growth factors, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), Interleukin-1 (IL-1), IL-6, IL-11, basic fibroblast growth factor (b-FGF) and steel factor (SF) have been identified in supernatants of stromal cultures (24). In addition to these

secreted growth factors, extracellular matrix-bound growth factors are also responsible for hemopoiesis, e.g., heparan-sulfate proteoglycans, present in the ECM of adherent stromal layers, bind and concentrate GM-CSF, IL-3 or b-FGF and present them in their biologically active form to hemopoietic progenitors (24). The growth factors may, however, have different regulatory effects on hemopoiesis, e.g., CSFs, such as G-CSF, GM-CSF and macrophage-CSF (M-CSF), act mainly as positive regulators (26,27). Others, such as tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β) act mainly as inhibitors (28). Some cytokines, such as IL-1 and IL-6, are characterized as having little or no intrinsic capacity to stimulate or inhibit hemopoietic colony-formation by themselves but as enhancing the effect of other cytokines (29,30). The physiology of hemopoietic cytokines is very complex; most of them are produced by different cell types, and all have more than one function, act on different target cells and may influence the production and function of other cytokines (27,31). Recent research suggests that growth factors and progenitors pack together in the fronds of the medullary cavity with surrounding endothelial cells, fibroblasts and stromal cells (22). Binding of hemopoietic cells to specific loci within the marrow stroma may therefore expose them to either growth promoting or growth inhibitory molecules or both, resulting in ordered and regulated hemopoiesis (21,22).

A complex and rich network of hemopoietic ECM consists of: a) *Fibronectin*, which is a large 450 kDa fibril-forming glycoprotein composed of two similar subunits (24). The fibronectin molecule has several domains responsible for interaction with ECM components and hemopoietic cells (24). The RGD motif forms the sequence of minimal

recognition site in the "cell-binding domain" of this molecule and most cells bind to fibronectin by interacting with this peptide via the $\alpha 5\beta 1$ integrin receptor (24). Since hemopoietic progenitors express cell surface receptors capable of interacting with the attachment sites of fibronectin, their interaction with fibronectin in bone marrow may be important in hemopoiesis. b) *Hemonectin*, which is a 60 kDa ECM protein, occurs only in the bone marrow microenvironment (24). Although its molecular structure and the cell surface receptors that interact with the molecule have not been identified, it is believed that hemonectin preferentially binds myeloid progenitors and plays an important role in maturation and the release of granulocytic elements from the bone marrow microenvironment (24). c) *Thrombospondin*, a 450 kDa glycoprotein, is abundantly present in the bone marrow microenvironment and has several domains which bind to ECM components or cell surface receptors (24). Thrombospondin contains an RGDA site which serves as a ligand for $\beta 3$ integrins. Additionally, CD36 antigen present on monocytes, platelets, hemopoietic progenitors and endothelial cells binds to thrombospondin (24). This molecule also serves as an adhesive ligand for committed progenitors including CFU-GEMM, CFU-GM and BFU-E and this attachment probably results in a signal transduction that modulates the response of progenitors to cytokines (24). d) *Collagens* and *laminin* are also present in great abundance in the bone marrow microenvironment. Laminin consists of three very long polypeptide chains arranged in a shape of a cross with a number of functional domains that can bind collagen type IV, proteoglycans and cell surface receptors (24). The interstitial collagens, types I-III, are assembled in collagen fibers and constitute the structural backbone of the extracellular

space (24). Type IV collagen molecules differ in many respects from interstitial collagens. The triple-stranded helical structure of type IV collagen is disrupted in a number of regions. Also, instead of forming fibrils, these molecules associate by their COOH-termini to form head-to-head dimers that assemble in a multi-layered network which is stabilized by disulfide and covalent cross links and thus forms a sheet-like mesh-work that constitutes a major part of the basal membrane (14). The crossing of these basal membranes is an important step in the egress of blood cells from the bone marrow. For example, mature granulocytes express integrins $\alpha 2\beta 1$ and $\alpha 6\beta 1$, usually not found on normal human immature progenitors or stem cells, which may allow them to bind to and migrate through these basal membranes (22). Due to their unique structure type IV collagens are not degraded by interstitial collagenases but require distinct and specific enzymes, type IV collagenases, which make a single cleavage in the molecule; this causes the triple helix to denature and renders the individual subunits susceptible to further degradation (14,33). Collagens type I to VI are strongly expressed in the bone marrow but only type VI collagen has been shown to support the binding (via heparansulfate proteoglycans rather than $\beta 1$ integrin) of hemopoietic cell lines such as HL-60 (myelomonocytic), U-937 (promonocytic), K-562 (erythroleukemic) and A431 (human epidermoid) (32). The same report, however, suggested that due to the ubiquitous distribution of collagen I and collagen VI in the body, it is unlikely that the specificity of binding of hemopoietic cells to the bone marrow stroma is mediated by type VI collagen alone. A specific role for laminin and collagens in the process of localization, proliferation or differentiation of hemopoietic progenitors in the bone marrow microenvironment has,

however, not been defined. e) *Glycosaminoglycans* (GAGs) contains four major groups, (i) hyaluronic acid (ii) chondroitin and dermatan sulphate (iii) heparan and heparin sulphate and (iv) keratan sulphate (24). Of these, the first three are found in the bone marrow microenvironment (24). Both hyaluronic acid and heparan sulphate have been implicated in the adhesion of hemopoietic progenitors to the bone marrow microenvironment and heparan sulphate is also thought to be responsible for concentration of hemopoietic cytokines such as IL3 and b-FGF (24). This compartmentalization of cytokines could be a way by which the bone marrow microenvironment regulates induction or inhibition of proliferation and differentiation of hemopoietic progenitors. The exact role of chondroitin and dermatan sulphate in hemopoiesis has not been determined.

2.1.2 Egress of blood cells from the bone marrow

Hemopoiesis occurs in the intersinusoidal spaces with the maturing blood cells entering the systemic circulation by passing through the endothelial cells and then the basement membrane present on their abluminal side. The endothelium, along with the reticular covering, forms the blood-bone marrow barrier. However, how mature blood cells navigate their way into the circulation is not fully understood.

The sinusoidal endothelium is continuous and contains no pre-formed apertures. A transient transcellular aperture, however, forms when the migrating cell comes in contact with the endothelial cell (35,36). In mice, there is an increase in number of reticulocytes and leukocytes in transit following erythropoietin administration but a sharp decline in the adventitial cell cover (37). This change in adventitial cover may be purely passive due to

the pressure of a large number of cells attempting to gain access to the endothelial cells (38) or it may be active due to contraction of microfilaments in the cell's cytoplasmic projections (39). Since cell egress may involve other important factors, the mechanism of release may be different for different cell types (40).

2.2 ACUTE MYELOGENOUS LEUKEMIA

2.2.1 Biology of acute myelogenous leukemia

Acute myelogenous leukemia (AML) is an uncontrolled proliferation of a malignant clone of myeloid cells, resulting in marrow replacement by the leukemic clone (41). Based on its morphology and cytochemistry, a population of poorly differentiated neoplastic or leukemic cells is categorized into subtypes according to the French-American-British (FAB) classification (42). According to the FAB classification, there are six subtypes of AML: acute myelocytic leukemia without (M1) and with (M2) maturation, acute promyelocytic leukemia (M3), acute myelomonocytic leukemia (M4), acute monocytic leukemia (M5) and acute erythroleukemia (M6).

Chromosomal abnormalities, consisting of translocations, inversions, insertions and deletions, are normally associated with leukemias (42). Such chromosomal translocations lead to malignancies by generating chimeric proteins that may affect the normal maturation and differentiation. For example, in the majority of AML subtypes M1 and M2 the (8;21) translocation is found (42). The chromosomal breakpoints involved in this translocation have been cloned and shown to involve the *AML1* gene on chromosome 21 and *ETO* gene on chromosome 8. The chimeric AML/ETO product may induce transformation through

its altered interaction with genes normally regulated by *AML1* (42a). Similarly, the translocation t(15;17), which involves the promyelocytic leukemia gene (*PML*) on chromosome 15 and the retinoic acid receptor-α gene (*RAR α*) on chromosome 17, is specifically seen in AML-M3 (42b). Although the exact mechanism by which the chimeric product of PML/RARα mediates leukemic transformation is not known, the chimeric product has been demonstrated to inhibit myeloid cell differentiation (42b). Similarly, 11q23 translocations are typically seen in AML-M4 and M5 (42). In Philadelphia chromosome-positive acute leukemia, the reciprocal (9;22)(q34;q11) chromosomal translocation results in transposition of the cellular *abl* gene from its location on chromosome 9 to chromosome 22 where it is recombined with the *bcr* gene resulting in the expression of a chimeric BCR/ABL protein product (p210 or p185) with acquired tyrosine kinase activity (42,43). Additionally, point mutations alter the structure and function of oncogenes related to hemopoiesis and lead to AML. For example, the *ras* family of oncogenes (*N-*, *K-*, and *H-ras*) is thought to play a role in growth factor signal transduction. Mutations in RAS have been described in approximately 25% to 30% of AML cases (42,43).

2.2.2 Clinical presentation and treatment

The clinical symptoms of all subtypes are similar and are mainly the result of suppression of normal hemopoiesis (44). The white cell count is generally elevated and patients complain of fatigue and weakness. Due to the involvement of myeloid cells, there is usually a decrease in number of mature neutrophils that results in various bacterial, viral

and fungal infections (44). Some clinical features, however, are specific to one subtype, e.g., hemorrhage due to disseminated intravascular coagulation in acute promyelocytic leukemia (AML-M3), and gum and skin infiltration in monocytic leukemia (AML-M5) (43). AML originates in the bone marrow but the leukemic cells escape the marrow, circulate in the blood and infiltrate the tissues and organs causing their dysfunction (41,44). Hepatomegaly, spleenomegaly and lymph node enlargement are often seen in cases of AML. Leukostasis occurs when the circulating blast count increases above 100,000 mm^3. It causes serious organ damage and requires immediate medical attention as it can be fatal if it occurs in the lungs or brain (44).

Treatment of AML commonly involves administration of chemotherapy to kill the leukemic cell pool and prevent regrowth of these cells. The first phase of this treatment is known as *induction therapy* and consists of an intensive course of chemotherapy with the purpose of achieving remission. In general, a patient is in remission when blast forms in the marrow are 5% or fewer, clinical and microscopic signs of leukemia disappear and normal cells start to repopulate the marrow (44). The second therapy phase is known as *post-remission therapy* and is done to prevent the regrowth of leukemic cells that can lead to relapse in patients in complete remission. Along with chemotherapy, treatment of AML may also include *bone marrow transplantation* (BMT) or *peripheral blood stem cell transplantation* (PBSCT) after myeloablative therapy (45). BMT may be either an autologous transplant, consisting of the patient's own marrow harvested during remission, or an allogenic transplant. In both cases, transplantation done during the first complete remission results in better long-term disease-free survival (42). For PBSCT, mobilization

techniques are applied to increase the blood stem cell count during the harvest and reinfusion of these cells usually elicits a rapid and complete hemopoietic recovery (45).

2.2.3 Dissemination of leukemic cells.

In AML large numbers of immature blasts appear in the circulation. The egress of leukemic blasts from the bone marrow is, to some extent, similar to the egress of normal mature blood cells (35). Although in the leukemic marrow the sinusoidal wall does undergo some changes, the entry of leukemic blasts into the sinusoidal marrow occurs in the presence of an intact endothelial lining (1). In murine Shay myelogenous leukemia, one report shows complete degeneration of the sinusoidal wall whereas another shows that the sinusoidal wall remains continuous even in advanced stages of the disease (35). The role of the endothelial lining in leukemia remains controversial; however, most reports indicate the loss of adventitial cells with disease progression. For example, both AML and CML bone marrows show up to a 40% reduction in adventitial cell cover rate compared with normal marrows (1). Additionally, surface molecules present on normal and malignant hemopoietic cells may play an important role in marrow cell egress. For example, granulocytes in bone marrow transiently express cell surface located glycoproteins (fucosyl glycopeptides) which disappear after their release in the peripheral blood.

These observations suggest that immature leukemic blasts may acquire some properties allowing them to leave the bone marrow that are absent in their normal counterparts. One such property may be the production of matrix and basement membrane degrading enzymes.

2.3 INVASION AND METASTASIS

Invasion and metastasis are processes whereby a tumor cell escapes from a primary tumor site, penetrates basement membrane barriers and colonizes a new site. Invasion and metastasis are complex, active processes and not an accidental consequence of tumor growth (15,46).

2.3.1 Three-step theory of invasion and metastasis.

Three major steps are involved in the process of invasion of solid tumor cells. These steps include: i) adhesion of the tumor cell to the basement membrane or extracellular matrix; ii) activation and secretion of proteinases that cleave components of the extra cellular matrix and disrupt the basement membrane; iii) cell migration by pseudopodial protrusions (13,15).

i) **Adhesion**. Glycoproteins of ECM such as fibronectin, collagen, laminin and a variety of cell adhesion molecules mediate adhesion of tumor cells to the basement membrane (15,46). These adhesion molecules are grouped in three major families: a) *Integrins* are transmembrane glycoprotein heterodimers with α and β subunits (46,47). Their binding to ECM proteins often occurs through recognition of a specific tripeptide sequence, the Arg-Gly-Asp or RGD motif (13). Inhibition of *in vitro* invasiveness by RGD peptides indicates the importance of integrins in metastasis (47). Utilization of these adhesion receptors may, however, differ from tumor type to tumor type. For example, it has been shown that $\alpha_v\beta_3$ leads to a more metastatic phenotype (46) and production of $\alpha_v\beta_3$ integrin (vitronectin receptor) is elevated in malignant melanoma cells (15). Conversely, the human osteogenic

morphogenesis. The difference is that tumor cells, to become invasive, combine their mobility with proteolysis at stages of cell growth and at sites where proteolysis by normal cells is not common. This proteolysis locally alters and disrupts the organization and integrity of the basement membrane. This creates a transient zone of membrane loss around the invading tumor cell thereby allowing the tumor cell to invade through the basement membrane (13). A detailed discussion of these proteases appears in section 2.4 on matrix degrading enzymes.

iii) **Migration**. To complete the invasive process the tumor cell has to exhibit motility and migrate through the hole in the basement membrane induced by the proteolytic enzymes. Tumor cell lines that are highly invasive and metastatic show a higher degree of motility than their counterparts showing low metastatic potential (15). Pseudopodia initiate migration in a particular direction and the entire cell moves in that direction by the assembly and disassembly of cross-linked actin filaments and other elements of the cytoskeleton (13). Factors that induce migration include the extracellular matrix components (laminin, fibronectin, collagen, and their proteolytic digestion products) and growth factors (hepatocyte growth factor, insulin growth factor family, transforming growth factor beta family) (33,48). These factors, however, may not be sufficient for intrinsic locomotion of tumor cells even if their production is due to the interaction between tumor cell and host tissue. This is overcome by the autocrine motility factor (AMF) which belongs to the family of tumor cell driven motility factors (33,48,51). The presence of such autocrine factors results in a malignant tumor having greater invasive and metastatic properties. For example, human melanoma A2058 cells and human breast

carcinoma cells in culture produce AMF that stimulates their motility (52). AMF mediates its effect through cell surface receptors that localize the leading and trailing edges of motile cells (48).

2.3.2 Genetic aspects of invasion and metastasis.

The search for genes that induce invasion and metastasis continues, but evidence linking oncogenes to unrestrained tumor growth and human malignancies is already quite convincing. Oncogenes like *ras*, *raf*, *mos*, *fes*, *fms*, *src*, *myc* and *fos* induce or increase metastatic potential when transfected in appropriate recipient cells (13,15,48). For example, transfection of mutated ras oncogene sequences in NIH 3T3 (mouse embryo-derived fibroblast) cells makes these cells highly invasive and metastatic (53). The process of invasion and metastasis, however, involves interaction between multiple gene products and other proteins in the signal transduction pathway (48).

Unlike oncogenes that augment metastatic potential, *nm23* (non metastatic 23), is a well documented suppressor gene for metastasis (13,15,46,54). For example, highly metastatic murine melanoma cell lines show significantly low expression of *nm23* (55). Conversely, transfection of *nm23* in metastatic melanoma cells leads to a significant reduction in metastatic potential (56). Similarly, normal breast tissues and non invasive breast lesions have high levels of nm23 protein whereas low levels of nm23 protein were found to be associated with metastasis in primary breast cancers (54). The nm23 protein may, therefore, play an important part in the treatment and diagnosis of cancer.

2.4 MATRIX DEGRADING ENZYMES

Invasion and metastasis of tumor cells involves the process of degradation of extracellular matrix components, and proteolytic enzymes are likely accomplices in this process. The four major classes of proteinases, i.e., metalloproteinases, serine proteinases, cysteine proteinases and aspartic proteinases, degrade matrix molecules *in vitro* (57). Their role *in vivo*, however, depends on the type of cells and tissue environment.

2.4.1 Metalloproteinases.

i) *Subgroups and substrate specificity*. The MMPs that degrade ECM are classified into subgroups based on their substrate preference (the interstitial collagenases, type IV collagenases and stromelysins) although all have overlapping substrate specificity (59,123). **a)** Interstitial collagenases include fibroblast-type MMP-1 and neutrophil collagenase or MMP-8 (59,61). The ECM substrates for MMP-1 include fibrillar collagens I, II, III, V, VII and X whereas MMP-8 can degrade collagens I, II, III and gelatin (61). **b)** Type IV collagenases include a 72 kDa form, gelatinase A or MMP-2, and a 92 kDa form, gelatinase B or MMP-9 (59,61,123). Both forms of type IV collagenases degrade gelatin, collagens type IV, V, VII, X and XI, fibronectin and elastin (59,61). **c)** The stromelysins are composed of stromelysins 1, 2 and 3 as well as matrilysin and metalloelastase (58,123). ECM substrates for stromelysin-1 or MMP-3 and stromelysin-2 or MMP-10 include proteoglycans, fibronectin, laminin, collagens III, IV, V, IX and gelatins (58), but the substrate for stromelysin-3 or MMP-11 is unknown. Matrilysin or

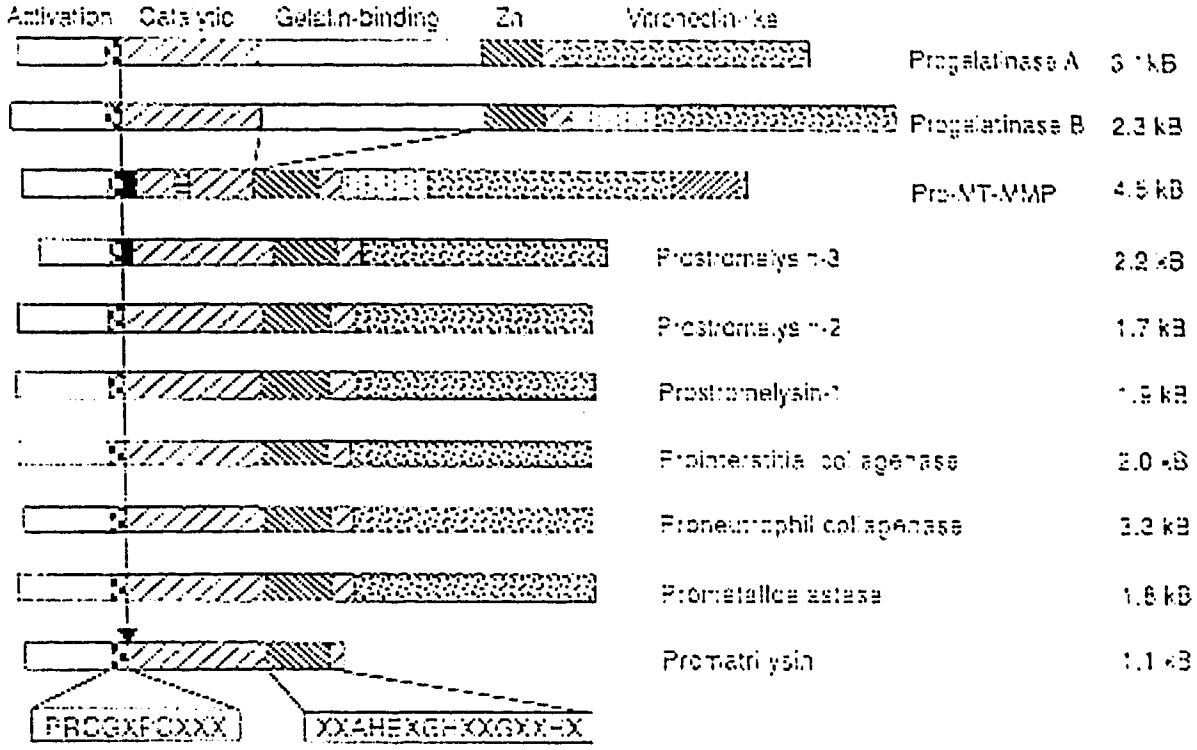
MMP-7 degrades proteoglycans, fibronectin, gelatin, elastin and type IV collagen whereas metalloelastase or MMP-12 has activity against elastin and fibronectin (58). The ECM substrates mentioned above for MMPs, however, are representative but not necessarily comprehensive. In addition to these MMPs, a complementary DNA (cDNA) which encodes a unique protein of 66 kDa and can be closely aligned with known MMP family members has recently been found and cloned by Sato, while screening cDNAs with homology to conserved regions in MMP (66). However, this protein contains a potential transmembrane domain at the C-terminus which does not exist in other MMPs and it is therefore known as MT-MMP (membrane-type metalloproteinase). Recently, HL-60 leukemic cells were shown to secrete into the culture media a previously unknown metalloproteinase of 45 kDa which cleaves the substrate at a different site and has a new NH_2-terminal amino acid sequence (compared with other metalloproteinases). Moreover the specific physiological inhibitors of MMPs, recombinant human tissue inhibitors of metalloproteinases (rhTIMP 1 and 2), barely inhibit activity of this enzyme (60).

ii) *Structure*. The primary amino acid structure of the MMP family consists of modular domains, including **a)** the NH_2-terminal domain, a transient hydrophobic signal peptide that the enzyme loses on secretion; **b)** the propeptide domain, that contains the highly conserved sequence PRCGXPDXX (X:any amino acid, P:proline, R:arginine, C:cysteine, G:glycine and D;aspartic acid) and maintains MMPs in the latent state until their activation; **c)** the catalytic domain contains conserved His residues, XXAHEXGHXXGXXHX (X:any amino acid, A:alanine, H:histidine, E:glutamic acid and G:glycine), which binds to the Zn^{++} atom; **d)** a proline-rich hinge region and; **e)** a

hemopexin or vitronectin-like C-terminal domain that contains a TIMP-binding site and is present in all MMPs except matrilysin (59,123). Type IV collagenases have an additional fibronectin-like gelatin-binding domain, immediately upstream of the Zn^{++} binding domain, which confers gelatin binding capability (123). Similarly, MT-MMP and gelatinase B both have an insert that is similar to the type V collagen α-chain (123). In addition, MT-MMP also contains a unique insertion inside the catalytic domain and a potential transmembrane domain (123). The domain structure of the MMP family is schematically shown on the following page.

iii) *Gene expression.* The MMP genes are clustered on the long arm of human chromosome 11 and include genes for MMP-1 and Stromelysins-1 and 2 whereas gene for gelatinases A and B and stromelysin-3 are located on chromosomes 16 and 22 respectively (59). Growth factors and cytokines play an important role in MMP gene expression (61,62); for example, IL-1, TNF-α, PDGF (platelet derived growth factor), EGF (epidermal growth factor) and b-FGF increase the expression of most MMP genes. A specific DNA sequence, the activator protein-1 (AP-1) binding site, is present in the promotor of gelatinase B, interstitial collagenase, stromelysin-1 and 2 and matrilysin. This sequence forms complexes with transcription factors of C-*fos* and C-*jun* (63). Induction of C-*fos* protein results in the induction of collagenase and/or stromelysin gene expression (63).

iv) *Activation of metalloproteinases.* Metalloproteinases are activated following disruption of the interacting Zn^{++} atom at the catalytic site and a cysteine residue in the propeptide domain, which leads to autoproteolysis with cleavage of the propeptide domain, and



This picture of the domain structure of the matrix metalloproteinase family is taken from a review article published by J.M. Ray and W.G. Stetler-Stevenson published in European Respiratory Journal, 1994 7:2062

conversion of the proenzyme form into an active metalloproteinase (64). *In vivo*, this activation requires a proteolytic cascade which involves several enzymes including plasmin. Plasmin partially activates both procollagenase and prostromelysin and active stromelysin further activates collagenase molecules increasing their activity by five to eight-fold (14,64).

Plasmin activation, however, is not the only mechanism of MMP activation *in vivo*. For example, cell surface-mediated activation of progelatinase A occurs and this membrane-mediated activation involves binding of the proenzyme through its C-terminal domain to the cell surface of Con-A-stimulated human fibroblasts (65). Similarly, the transmembrane (TM) domain at the C-terminus of MT-MMP has been shown to be responsible for 72-kDa progelatinase A activation since *in-vitro* truncation of this TM-domain abolishes this function in MT-MMP (67). In addition, a recent report shows autolytic activation of recombinant human 72-kDa type IV collagenase expressed in a baculovirus/insect cell system, where the proenzyme is processed through cleavages at specific sites in a stepwise mechanism and is finally converted to an active form (68). Hence it is clear that a variety of processes convert latent or prometalloproteinases into active enzymes.

v) *Secretion of type IV collagenases/gelatinases by normal blood cells.* In addition to azurophil or primary granules (containing hydrolases such as elastase, cathepsins, aryl sulphatase) and specific or secondary granules (containing receptors and functional proteins in their membrane such as CR3, and FMLP-receptor and proteins such as lactoferrin, β_2 microglobulin and collagenase), neutrophils contain granules with high specific activity of gelatinase (69,70). Although gelatinase and lactoferrin colocalize in a

substantial number of peroxidase-negative granules, these gelatinase containing peroxidase negative granules are now referred to as "gelatinase granules" due to the presence of a high concentration of gelatinase (70). These gelatinase granules were separated from the lactoferrin-containing specific granules by subcellular fractionation and it was shown that, unlike lactoferrin which was synthesized almost exclusively in the group containing myelocytes and metamyelocytes, they were synthesized and appeared in a group containing band cells and segmented cells (69,70a,70). Therefore the appearance of gelatinase granules can mark a step in neutrophil differentiation. The enzymes from gelatinase granules act specifically on collagen and on its denatured form gelatin (71). Apart from 92 and 72 kDa gelatinases (72), a 98 kDa collagenase has been isolated from leukocytes capable of degrading type IV collagen (73). Attempts made to isolate type IV collagenases/gelatinases from human monocytes were not successful until recently when it was shown that monocytes express mainly the 85 kDa and 65 kDa species of gelatinases and these occur as a truncated form of type IV collagenases previously isolated from tumor cells, with a core enzyme identical to that isolated from neutrophils (74). Additionally, it was shown that when monocytes differentiate to macrophages either *in vitro* or *in vivo* they acquire the capacity to synthesize and secrete large amounts of MMPs including matrilysin, interstitial collagenases and 92 kDa gelatinase (75). Similarly, MMP-9 was detected in conditioned media of TPA stimulated primary T-lymphocytes (76). In an another report both type IV collagenases were detected in conditioned media of activated (PHA or IL-2) T-lymphocytes and it was shown that the inhibition of these

enzymes by GM6001, a specific inhibitor of metalloproteinases, resulted in a reduced migraton of T-lymphocytes in the Matrigel-based assay (77).

Cells in the earliest stages of hemopoietic differentiation can be identified by the presence of the CD34 antigen, a stage specific antigen present on a small population of bone marrow and peripheral blood cells (78). The $CD34^+$ population contains progenitor cells committed to the myeloid, lymphoid and erythroid lineages as well as the primitive stem cells capable of long-term reconstitution (78). To date there is no information available on the production of gelatinases by these cells.

vi) *Secretion of type IV collagenases/gelatinases by leukemic cells.* To my knowledge there are only few reports available on the secretion of type IV collagenases by leukemic cells, e.g., MMP-9 has been isolated from the conditioned media of TPA treated monocytic U-937 leukemic cells and this secretion was further enhanced by treating these cells with IL-1 or TNF-α (12). Similarly, MMP-9 activity was found in culture fluids of the promyelocytic cell line HL-60 treated with TPA or TNF-α (79). The human granulocytic sarcoma cell line HSM-1, however, has been shown to secrete 92 kDa gelatinase without any stimulation (80). However, the secretion of MMP-2 by leukemic cells has not yet been reported. In addition, to date there is no information about the secretion of type IV collagenases by primary leukemic cells from patients diagnosed with AML.

vii) *Inhibitors of metalloproteinases.* A family of tissue inhibitors of metalloproteinases (TIMPs) including TIMP-1, TIMP-2 and TIMP-3 inhibit all active forms of matrix metalloproteinases (58,81,82). For example, TIMP-1, a 28.5 kDa glycoprotein, is present

in most body fluids (81). Additionally, most connective tissue cells and macrophages also secrete TIMP-1 (41). TIMP-2, a 23 kDa unglycosylated protein, is a less abundant but nonetheless ubiquitous form of TIMP. Although TIMP-1 and TIMP-2 have only 40% primary sequence homology, the 12 cysteine residues that form disulfide bonds are completely conserved (58). Both TIMP-1 and TIMP-2 bind non-covalently to active MMPs in a 1:1 molar ratio and specifically inhibit their activity (14). Besides their ability to bind at active sites, TIMP-1 and TIMP-2 also form complexes with specific metalloproteinases. TIMP-1 forms a complex with the 92-kDa type IV collagenase (83) and TIMP-2 with the 72-kDa type IV collagenase (84). TIMP-3 or Chimp-3 exists only in chickens and is exclusively present in the 'insoluble' extracellular matrix (82). Regulation of TIMP-1 and TIMP-2 expression is independent of one another; e.g., phorbol esters upregulate expression of TIMP-1 but not TIMP-2 (85). Some reports show opposing patterns of MMP and TIMP regulation; e.g., in many cell types including fibroblasts and epithelial cells, TGF-β upregulates TIMP expression and represses stromelysin and collagenase expression (86).

TIMPs are important in the control of numerous physiological and pathological processes including tumor cell invasion and metastasis. In this context, their major role is to regulate degradation of the basement membrane and extracellular matrix by MMPs. A variety of studies reveal an inverse correlation between the expression of TIMPs and the efficiency with which the tumor cells invade and metastasize (87,88). For example, in comparison to non-invasive and non-tumorigenic controls, Swiss 3T3 cells become invasive in human amnion invasion assay and metastatic in athymic mice by inducing reduction in TIMP-1

levels (89). Similarly, TIMP-1 significantly inhibits metastatic lung colonization of B16-F10 (murine melanoma) cells (90). These inhibitory properties give TIMPs considerable clinical relevance in diseases where MMPs play a major role.

In addition to TIMPs, antibodies against MMPs selectively block their activity and abolish the invasive action of tumor cells. For example, antisera against the 72 kDa type IV collagenase have been shown to inhibit *in vitro* endothelial cell invasion of human amnion membrane induced by basic fibroblast growth factor (b-FGF) (54,91).

Synthetic inhibitors of MMPs have a similar inhibitory effect on tumor invasion; e.g., 1,10-phenanthroline has been shown to completely block B16 tumor cell invasion into the amnion (92). Inhibition by 1,10-phenanthroline is thought to proceed via a mixed, 1,10-phenanthroline-enzyme-zinc complex rather than by removal of the active site metal ion (93).

2.4.2 Serine proteinases.

Serine proteinases fall into two major groups of enzymes. The first group includes digestive (chymotrypsin and trypsin), lysosomal (elastase and cathepsin G located in azurophil granules of mature circulating PMNs), coagulation (thrombin and factors VII, IX, X, XI, XII) and fibrinolytic (tissue type plasminogen activator, urokinase type plasminogen activator and plasmin) enzymes (58). The second group of serine proteinases are homologous to the bacterial proteinases subtilisins, and include furin and prohormone-converting enzymes, PC-1 to -6. Serine proteinases play various roles in normal physiological processes such as fibrinolysis, inflammation, wound healing and prohormone

processing. A recent report indicates that cell surface antigen 175, the expression of which increases as bone marrow cells differentiate during hemopoiesis, contains serine protease activity, suggesting a role for serine proteinases in hematopoietic cell differentiation (94). Tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA) are important in tumor growth and tumor cell invasion (95). t-PA is a 70-kDa enzyme that associates primarily with endothelial cells in tissues, although other cells such as fibroblasts and tumor cells also express it. By contrast, u-PA is a 54-kDa enzyme that most cell types express (57). Both plasminogen activators convert the zymogen plasminogen to plasmin which has a broad trypsin-like substrate specificity and can degrade ECM components such as laminin and fibronectin and the protein core of proteoglycans (14,96).

The plasminogen activator (PA) producing cells also produce PA inhibitors. Three inhibitors in the serine proteinase inhibitor superfamily have high affinity for PA *in vivo*. These inhibitors include PAI-1 (type 1 PA inhibitor), PAI-2 (type 2 PA inhibitor) and PN-1 (protease nexin 1) [14]. Interaction of PA inhibitors with u-PA blocks the pro-uPA-uPA-plasmin loop and inactivates the enzyme molecule (14). Studies measuring PA in tumor tissues and various model invasion systems show the important role plasminogen activators play in the process of cellular invasion (96). For example, PA activity is two-fold higher in aggressive PC-3 cells than in inactive DU-145 cells and injection of the former cells into nude mice leads to expression of u-PA activity that is greater in metastatic tumors than in primary tumors (97). Tumor cells, however, produce mostly u-PA except for melanoma and neuroblastoma cells that produce t-PA (96). Experimental

use of PA inhibitors also supports the involvement of plasminogen activators in cellular invasion; e.g., invasive PC-3 cells show lack of PAI-1 expression and the addition of the latter results in inhibition of the *in vitro* invasiveness of these cells (97). Similarly, synthetic inhibitors of serine proteinases such as leupeptin and benzamidine have also been shown to inhibit the ability of tumor cells to penetrate the basement membrane Matrigel (98,99). Most serine proteinase inhibitors interact with both the catalytic site and the substrate binding site of a typical serine proteinase and the serine and histidine residues in the catalytic site are by far the most frequent targets for inhibitors (93).

2.4.3. Cysteine Proteinases.

The lysosomal proteinases, cathepsin B, H, L and S, and the cytosolic calpains I and II are the two major groups of cysteine proteinases (58). Their catalytic activity depends on the reactive thiol group of a Cys residue at the catalytic site (14). In general, cysteine proteinases are intracellular enzymes that digest material that is phagocytosed (14,57). These enzymes, however, do have extracellular activity particularly where the pH is acidic and unstable at neutral or alkaline pH (14). In some pathological conditions cells secrete these enzymes. For example, some malignant tissues secrete cysteine proteinases that facilitate cell detachment from primary tumors and invasion by degrading ECM (14). Correlation between membrane-associated cathepsin L and high metastatic potential exists in melanoma cells (57). Cathepsins are also present in conditioned-medium of some tumor cells and breast carcinoma cultures (14). A recent report indicates the existence of a

correlation between cysteine proteinase activity and stages of transformation of rat fibroblast cells (100).

The calpain group consists of those cysteine proteinases that require calcium for their activity (58). They mainly contain calpain I that requires micromolar levels of calcium for its activity and calpain II that requires millimolar levels of calcium. Calpains play multiple roles; e.g., regulation of cytoskeleton structure, modulation of signal transduction, downregulation of receptors, modulation of ion transport and specific degradation of Z lines of muscle fibers (58).

Cystatins, a superfamily of cysteine inhibitors, regulate the intracellular and extracellular activities of cysteine proteinases (57,14). There are three members of this superfamily: 1) Type I cystatins, including cystatins A and B; 2) type 2 cystatins, including S and C cystatins; and 3) type 3 cystatins, including L-, H-, and T-kininogens. In extracellular spaces α_2-macroglobulin also acts as a cysteine proteinase inhibitor.

2.4.4 Aspartic Proteinases.

Aspartic proteinases belong to one of the following groups: cathepsins, pepsins and chymosins, renins, fungal aspartic proteinases and retroviral aspartic proteinases (101). The major aspartic proteinases in humans are cathepsin D and E, renin, pepsin and gastricin. The active sites of aspartic proteinases contain a water molecule that forms a hydrogen bond to two aspartic groups (58). Although the major function of cathepsin D is to break down the phagocytosed material (57), reports indicate its involvement in cancer. For example, a correlation exists between expression of cathepsin D and various stages of

oncogenic transformation of rat fibroblast cells (100). Another report shows increased secretion of pro-cathepsin D in human mammary cancer cells (102).

3.0 MATERIALS AND METHODS

3.1 Cells and cell separation techniques

3.1.1- *Human leukemic cell lines* KG-1 (myeloblastic), K-562 (myelogenous), HEL (erythroid), HL-60 (promyelocytic) and U-937 (promonocytic) were obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), except for HL-60 cells, which were grown in 20% FCS. KG-1 cells were grown in Iscove's modified Dulbecco's medium (IMDM) with 20% FCS. The human fibrosarcoma HT-1080 cell line was obtained from ATCC and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FCS. All cell lines were incubated at 37°C in 5% CO_2. The cells were harvested at the exponential growth phase, washed with IMDM and resuspended in medium supplemented with 10 to 20% FCS or 0.1% bovine serum albumin (BSA) and used for experiments.

3.1.2- *Normal peripheral blood (PB) polymorphonuclear leukocytes (PMNLs)* collected from healthy adult volunteers were isolated by Ficoll-Hypaque centrifugation at 300 g for 30 min. Contaminating red cells were removed with the lysing solution (0.15 M NH_4Cl, 0.01 M $NaHCO_3$, 0.12 mM Na_2EDTA) and PMNLs (purity > 90%) were resuspended in IMDM supplemented with 0.1% BSA or 10% FCS and used in the invasion assay.

3.1.3 - *Normal bone marrow (BM) cells* were collected from hematologically normal cardiac surgery patients, with their consent, during open heart surgery performed at University of Alberta Hospitals. Light-density cells were separated using 60% Percoll gradient centrifugation at 300 g for 30 min. After washing 3x with IMDM, cells were resuspended in IMDM supplemented with 0.1% BSA and used in experiments.

3.1.4- *Peripheral blood and/or bone marrow samples from AML patients* were collected during diagnostic procedures carried out at the Cross Cancer Institute or University of Alberta Hospitals. Light-density BM and PB cells were separated using 60% Percoll gradient and Ficoll centrifugation respectively, at 300 g for 30 min. Cells, after washing 3x with IMDM, were resuspended in IMDM supplemented with 0.1% BSA and used in experiments fresh or were frozen in the media containing 80% IMDM, 10% dimethyl sulfoxide (Fischer Scientific, Canada) and 10% FCS. Differentials on separated BM and PB were done using Wright's stain and clinical information including FAB classification was obtained from the Medical Records of University of Alberta Hospital.

3.1.5- *$CD34^+$ cells.* $CD34^+$ cells were isolated from hematologically normal bone marrow (obtained as described above) by the magnetic cell sorting (MACS) system and CD34 isolation reagents kit (CytoBio Technics, Oakville, ON)(schematically shown in appendix 1). Briefly, light density cells were separated on 60% Percoll as described above. These cells, however, were resuspended in 300 μL of 1X PBS-0.5% BSA-5mM EDTA per 10^8 cells. Approximately 25 x 10^4 cells were taken at this stage for FACS analysis and were

labelled as preMACS. To the remaining cells, for magnetic labeling, 100 μL of blocking reagent-A1 (human IgG) per 10^8 cells was added and gently mixed. Next, 100 μL of antibody reagent-A2 (modified CD34 antibody, QBEND/10 mouse IgG1) per 10^8 cells was added and mixed. This was followed by a 15 min incubation at 4^0C. After incubation, cells were washed once in 10 mL of PBS-0.5% BSA-5mM EDTA buffer per 10^8 cells. After spinning at 300 g for 10 min., the supernatant was removed and the cell pellet was resuspended in 400 μL of PBS-0.05% BSA-5mM EDTA per 10^8 cells. Next, 100μL of reagent-B (colloidal superparamagnetic microbeads recognizing antibodies in reagent A2) per 10^8 was added and after gentle mixing was incubated for 15 minutes at 4^0C. After incubation cells were washed once with PBS-0.05%BSA-5mM EDTA buffer, spun at 300 g for 10 min and the pellet was resuspended in 500 μL PBS-0.05%BSA-5mM EDTA buffer. To separate $CD34^+$ cells, first a separation column was placed in the MINI MACS separator and washed with 500 μL of 1X PBS after which the cell suspension was allowed to pass through the column. The column was washed 4x with 500 μL of 1X PBS buffer. The cells collected until this stage were $CD34^-$. To elute $CD34^+$ cells retained on the column, the column was removed from the magnetic separator, filled with 1 mL of 1X PBS and, using the provided plunger, the cells were eluted into a sterile tube. $CD34^+$ cells were used in the invasion assay, zymography and, along with $CD34^-$ cells and preMAC cells, were subjected to FACS analysis. To label the cells for FACS analysis, 5×10^5 cells were added to a U bottom 96 well plate. To block non-specific sites, 10 μL of normal mouse serum was added to these cells and incubation was carried out for 60 min at 4^0C. To label the cells, 40 μL of CD34 anti-HPCA-2 (FITC linked) antibody, which recognizes

a different epitope on the CD34 antigen than the one used in separation, was added and the cells were incubated for 60 minutes at 4^0C. A mouse IgG1 negative control FITC conjugate (Accurate Chemicals and Scientific Corporation, Westbury, NY) was used as a control. After incubation, 100 µL of immunofluorescence (IF) buffer (PBS + 1% BSA + 0.2% sodium azide) was added and cells were spun at 2000 rpm for 7 min at 4^0C. Supernatant was removed and the cells were washed twice more with 200 µL IF buffer at the same speed, time and temperature. After the final wash, cells were resuspended in 100 µL 1% formalin and placed in FACS tubes. The volume was brought up to 0.4 mL by adding 0.3 mL of 1% formalin. Flow cytometric measurement of samples were performed using a Facscan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). This instrument is equipped with an air coded argon laser (488 nm excitation). A 530 nm filter with a band width of 30 nm was used to collect the green fluorescence signal. Data were analyzed using Lysis II software (Becton Dickinson). The MACS system for isolating $CD34^+$ cells yielded a 90% pure population of $CD34^+$ cells.

3.2 Invasion assay

3.2.1- *Matrigel-based assay.* The procedure of Albini et al. (6) was modified as follows. Thirteen mm polyvinylpyrrolidone-free polycarbonate filters of 8-µm pore size (Costar/ Nucleopore, Toronto, ON) were coated with Matrigel preparation (Collaborative Biomedical Products, Bedford, MA) from batch 1 (lot # 903942), batch 2 (lot # 905043) or batch 3 (lot # 910583). The Matrigel, diluted to the desired concentration with ice-cold distilled water, was applied to the filters which, after drying for approximately 45 min at

room temperature, were kept overnight at 4^0 C. On the day of the assay the Matrigel (coated on the filters) was reconstituted with serum-free IMDM supplemented with 0.1% BSA for at least 30 min at 37^0 C. Uniformity of the coating was checked by Coomassie blue staining and low power microscopic observation. The lower compartment of the blind well chambers were filled with IMDM. Cells were resuspended in 0.2 mL IMDM and placed in the upper compartment and the chambers were incubated at 37^0 C in 5% CO_2. To standardize this *in-vitro* invasion assay, the following parameters were varied as described in the results of the assay standardization (4.1): the amount of Matrigel coated on the filters, supplementation of the media in the lower compartment and in which cells were resuspended and the length of time for which the chambers were incubated. However, after standardization of the assay, the following standards were used for the rest of the study: 25 μg of Matrigel was used to coat each filter; IMDM supplemented with 0.1% BSA was used to fill the lower compartment of the chambers and to resuspend cells; the concentration of cells used in the upper compartment was 1.5×10^6/mL; and the chambers were always incubated at 37°C in 5% CO_2 for 2 hours. After incubation, the contents of the lower compartments were collected, the invasive cells were counted in a hemocytometer and the rate of invasion was expressed as a percentage of the ratio of invaded cells to the total number of cells placed in the chamber. For adherent HT-1080 cells, filters were stained with Wright's stain and the lower surface was inspected microscopically for the presence of attached cells. As a control, the passive migration of cells to the lower compartment was assessed in experiments in which filters which had not been coated with Matrigel were used.

To assess the effect of different concentrations of chemoattractant on invasion, Costar transwell units (Costar, Cambridge, MA) were used instead of Boyden chambers, as described by Repesh (103). Polycarbonate filters (6.5-mm in diamete and 8 μm pore size) were coated with 15 to 30 μg Matrigel (batch 1), dried and kept overnight as for filters for blind well chambers, and reconstituted with serum-free IMDM for 30 min at 37° C. The lower compartment of each transwell assembly was filled with 0.6 mL IMDM supplemented with 10^{-8} to 10^{-5} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The Costar plates were incubated for 2 or 4 hours at 37°C, 5% CO_2 , and the remaining procedure was carried out identically to that described above for the blind well chambers.

3.2.2- *Inhibition studies of* in vitro *invasiveness.* Inhibition of *in-vitro* invasiveness was carried out using inhibitors of metallo- and serine proteinases. The following *inhibitors of metalloproteinases* were used: 1,10-phenanthroline (Sigma) at 0.05 to 1.0 mM final concentration, recombinant human-TIMP-2 (rh-TIMP-2) at 10 or 50 μg/mL final concentration, and monoclonal antibody against 72 kDa gelatinase, MMP-2 (Oncogene Science, Uniondale, NY) at 10 μg/mL final concentration. *Inhibitors of serine proteinases* used were: benzamidine (Sigma) at 0.05 mM to 1.0 mM final concentration and phenyl-methyl-sulfonyl-fluoride (PMSF) (Sigma) at 0.25 and 0.5 mM final concentrations. KG-1 cells and normal granulocytes suspended in IMDM and 0.1% BSA were first pre-incubated with inhibitors for 30 min after which this cell suspension was placed in the upper compartment of the modified Boyden chambers and incubated for 2 hours at 37° C in 5% CO_2. Following incubation, the filters were removed and cells that had crossed the Matrigel layer were recovered from the lower compartment and counted in a

hemocytometer. The percent inhibition of invasiveness was calculated by comparing the percentage of invasiveness in the presence and absence of inhibitors.

3.3 Cell conditioned media

To study secreted enzymes, normal granulocytes, KG-1 cells and cells from AML patients were cultured at concentrations of 10^6 cells/mL in serum-free IMDM. Cell conditioned media were collected after 2 and/or 24 hours of incubation and were either concentrated approximately 10-20-fold using the Centricon 10 concentrating vessel (Amicon Inc., Beverly, MA) or were left unconcentrated. In addition, cell free supernatant was also collected from the upper compartment of the blind well chamber at the completion of the invasion assay (2 hours) with KG-1 cells , concentrated 10x and used in zymography. Concentrated (10x) 0.1% BSA-IMDM was also prepared. $CD34^+$ cells were placed in serum free IMDM at a concentration of 5×10^5 cells/0.5 mL for 2 hours and $CD34^-$ cells at a concentration of 5×10^4 cells/0.5 mL, 5×10^5 cells/0.5 mL and 1×10^6 cells/0.5 mL for 2 hours after which supernatants were collected. Cell conditioned media from AML patients nos. 2 and 3 were also collected after incubation of 5×10^5 cells/0.5 mL in addition to the conditioned media of these cells collected from 10^6 cells/mL as described above.

3.4 Zymography

3.4.1- *Gelatin-copolymerized SDS-PAGE.* Gelatin degrading activities of unconcentrated and concentrated cell-conditioned serum-free media were identified using the protocol of

Heussen and Dowdle (104) adapted in Dr. Janowska's laboratory for use in the dual vertical slab system. Fifteen μL of unheated samples (conditioned media of KG-1, granulocytes, PB or BM cells from AML patients and CD34 cells) were mixed with 5 μL of 4X sample buffer (0.16M Tris-HCl, 50% glycerol, 8% SDS and 0.08% bromophenol blue) without 2-mercaptoethanol and applied to the 7% polyacrylamide separating gels co-polymerized with 2 mg/mL of gelatin (Sigma). Prestained standard molecular weight marker proteins (Bio-Rad, Mississauga, ON) were used for reference. Electrophoresis was carried out according to Laemmli (105) using a mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories, ON). After electrophoresis the gels were first washed 3x for 10 min each with 2.5% Triton X-100 (Sigma) to remove SDS and then incubated overnight in zymography buffer (0.15 M NaCl, 5 mM $CaCl_2$, 0.05% NaN_3 and 50 mM Tris-HCl, pH 7.5). To observe the gelatinolytic activity of the secreted enzymes, gels were stained with 0.05% Coomassie brilliant blue G-250 (Sigma) in a mixture of methanol: acetic acid: water (2.5: 1: 6.5), and de-stained in 20% isopropanol with 10% acetic acid. The enzyme activity appeared as a clear band against the blue background.

To quantitate the activities of the detected enzymes, the zymograms were all stained overnight with an equal quantity of Coomassie brilliant blue G-250 and de-stained with an equal quantity of 20% isopropanol with 10% acetic acid for an equal length of time. After this they were processed in the ScanJet II cx scanner (Hewlett Packard, Boise, ID). The gelatinolytic activity of the separate bands was analyzed and expressed in numbers using SigmaScan/Image measurement software (Jandel Corporation, San Rafael, CA).

3.4.2- *Inhibition of gelatinolytic activity of 92- and 72 kDa gelatinases in zymograms.* Zymography was carried out exactly as described above except that samples were run in duplicate on the same gel. After electrophoresis the gel was cut in half and each half was incubated separately in zymography buffer with or without (control) 1,10-phenanthroline at either a final concentration of 0.1 mM or 1.0 mM.

3.5 Western blot analysis

Western blot analysis was performed, with modifications as described by Towbin and Gordan (106). Gelatin-column affinity-purified fractions (prepared by Dr. Grzegorz Sawicki in Dr. Janowska's lab) of media conditioned by KG-1 or HT-1080 (10x diluted) were used. HT-1080 conditioned media was used directly in Western blot whereas KG-1 sample was precipitated by using ice-cold trichloroacetic acid (TCA) at a final concentration of 10%. Next, sample was spun down in a microcentrifuge for 5 min after which it was washed 2x in ice cold ether:ethanol (50:50) and resuspended in sample buffer. Samples were then applied to 10% acrylamide gels and electrophoresed using a mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories, ON) in non-reducing conditions according to Laemmli (105). Following electrophoresis, samples were electroblotted onto nitrocellulose membranes (Trans Blot, 0.45 micron, Bio Rad) with freshly prepared semi-dry transfer buffer (25 mM Tris-HCl, 150 mM glycine, 20% methanol, pH 8.3) at 150 m amperes constant current for 20 min using the semi dry transfer system (Tyler, Edmonton, AB). This was followed by one hour of blocking with blocking buffer (5% skim milk powder in TBS{20 mM Tris-HCl, 150 mM NaCl, pH 7.4}

buffer) at room temperature. After blocking, the membranes were probed with rabbit anti-human polyclonal antibodies against 72 kDa (AB-45) and 92 kDa (AB-110) type IV gelatinases. These antibodies were a gift from Dr. WG Stetler-Stevenson (NIH, Bethesda, MD), and have been used to detect the production of type IV collagenases (132, 133). These antibodies were added at a concentration of 5 μg/mL in a total volume of 3 mL of incubation buffer (5% skim milk powder in TBS buffer) for one hour at room temperature. After incubation the membranes were washed three times for 10 min each with gentle rocking using the wash buffer (0.05% Tween-20 in TBS buffer). This was followed by reacting the blots with goat anti-rabbit IgG HRP-conjugated (Bio Rad) secondary antibody at a dilution of 1:3000 for 1 hour at room temperature. After incubation the blots were washed (3x for 10 min each) with wash buffer and the bands were visualized by using the ECL Western blotting system (Amersham International plc, Buckinghamshire,UK).

3.6 Data analysis and statistics

3.6.1- *Invasion assay*. The results of six chambers within an experiment and of at least three experiments, except for CD34 cells with two experiments, were evaluated for mean ± standard deviation (SD).

3.6.2- *Zymography*. Zymography was done ≥ 5x with the cell-conditioned media from KG-1 cells, ≥ 3x from AML cells and ≥ 2x from CD34 cells. Photographs taken for the

most representative gels were then scanned in the ScanJet II cx scanner and printed by laser printer.

3.6.3- *Student's t-test.* The student's *t*-test was used to determine the significance of differences among groups of paired observations or means in *n* number of experiments. Results with $p < 0.05$ were considered statistically significant.

3.6.4- *Coefficient of correlation.* To determine whether there is a predictive relationship between invasiveness and type IV collagenase production, the coefficient of correlation was calculated.

4.0 RESULTS

4.1 Development and standardization of the *in-vitro* invasion assay.

The first aim of the study was to develop and standardize an *in-vitro* invasion assay to investigate the invasive properties of leukemic and normal hematopoietic cells. The *in-vitro* invasion assay system used in the present study is schematically shown in Figure 1. The following observations were made during the standardization of this assay.

4.1.1- *Incubation time of the assay.* Most solid tumor cells require a 5 to 72 hour incubation period for invasion in the Matrigel based assay (107,109). Based on this knowledge, the incubation time required by cultured leukemic cells to cross the Matrigel barrier was tested. Briefly, 30 x 10^4 to 35 x 10^4 cells suspended in IMDM with 0.1% BSA were plated on top of the Matrigel-covered filters (batch 1; 50 µg/filter) in a blind well chamber. After 2, 4, 6 and 8 hours of incubation in 5% CO_2 at 37°C, the invasive cells were counted in the lower compartment. As shown in Figure 2, marked invasion rates were notable for the KG-1 and K-562 cell lines after 2-hour incubation and, although the invasion rate increased with up to 4 hours of incubation, the results were more consistent and had lower standard deviations when the incubation was carried out for 2 hours. After 4 hours no more cells were found to cross the Matrigel layer; in fact, an insignificant decrease in the number of cells recovered from the lower compartment was noted for both cell lines after 6 and 8 hours compared with the 4-hour period. HT-1080 fibrosarcoma cells were not recovered from the lower compartment in any of the incubation times indicated above (Fig. 2). Since HT-1080 are adherent cells, filters were stained and the lower surface was observed under the microscope. Only a few cells could be seen after 2

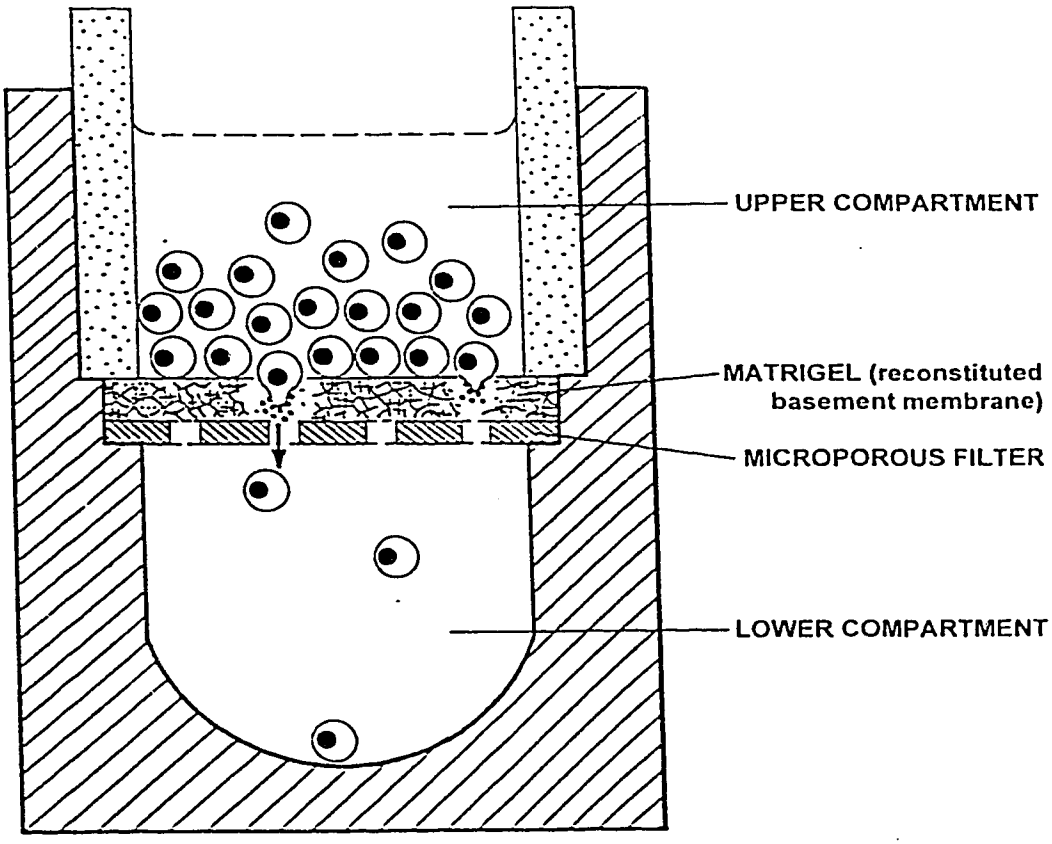


Figure 1. *Schema of Matrigel-based in-vitro invasion assay in a blind well chamber.*

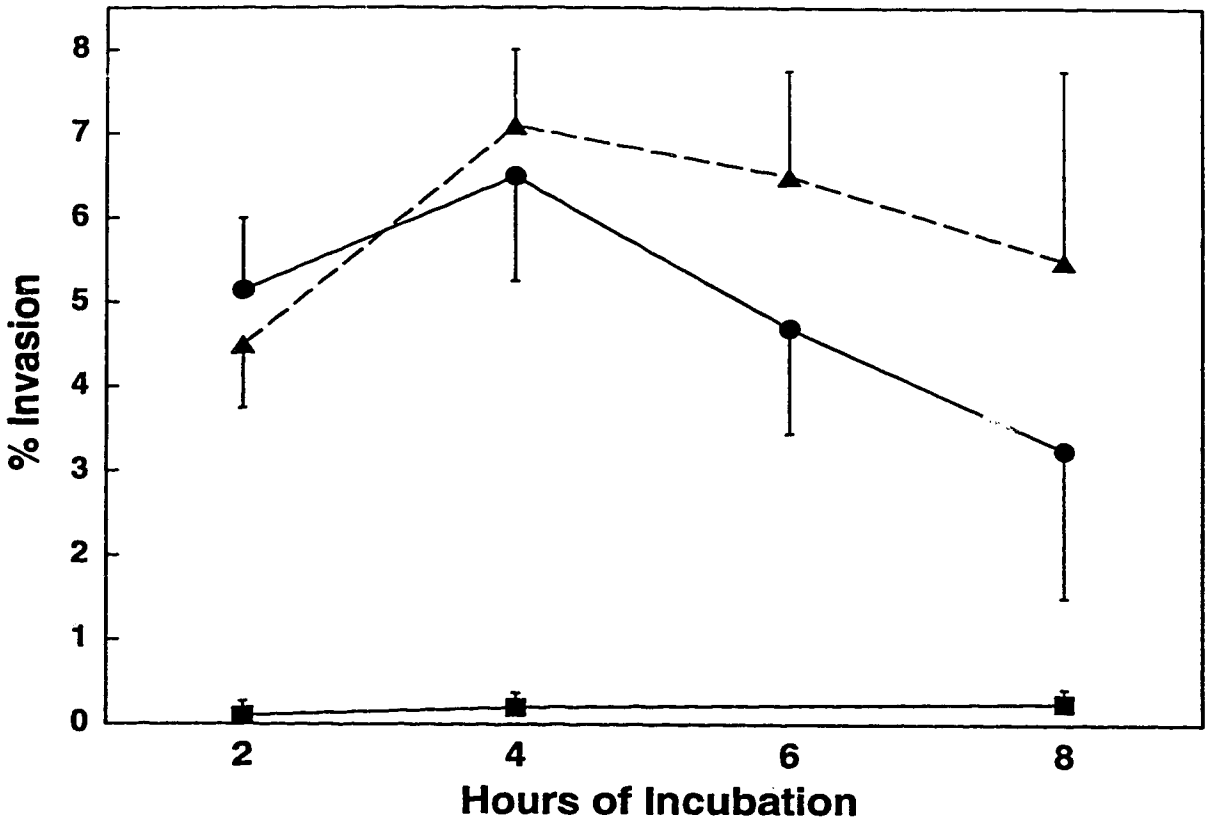


Figure 2. *Invasion of KG-1 (▲), K-562 (●) and HT-1080 (■) through the Matrigel layer during different incubation times.*

Briefly, cells were cultured overnight in serum-free IMDM and washed twice prior to use in the assay. Cells suspended in 0.2 mL 0.1% BSA-IMDM (30×10^4 to 35×10^4 cells) were plated on top of the Matrigel covered filters (batch 1:50 µg/filter) in a blind well chamber. After indicated times of incubation in 5% CO_2 at 37°C, the invasive cells were counted in the lower compartment. Percentage invasion was calculated as the ratio of number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of blind wells. Mean ± SD values of percentage invasiveness shown in the figure were calculated from three experiments (total of 18 chambers).

to 6 hours but numerous cells were present on the lower surface of the filter after 20 to 24 hours of incubation. To establish the optimum time required for incubation, IMDM supplemented with 0.1% BSA was used to fill the lower compartment of the blind well chamber and to resuspend the cells that are placed in the upper compartment. This was done to eliminate the stimulatory effects of chemoattractant or FCS on the invasiveness of cells.

4.1.2- *Effect of chemoattractant on invasion.* Because most *in-vitro* invasion assays are based on the chemotactic response of tumor cells, the effect of chemoattractant on the *in-vitro* invasive potential of leukemic cells and normal granulocytes was tested. Briefly, 30 x 10^4 cells were placed on the polycarbonate filters (8-μm pore size) of Costar transwell units coated with 15 to 30 μg Matrigel (batch 1). FMLP, a potent chemoattractant of leukocytes was placed in the lower compartment of the Costar transwell assembly system, at 10^{-5}, 10^{-7} and 10^{-8} M concentrations and the rates of invasiveness of normal PMNL and cultured leukemic cells were evaluated after 2 hours of incubation in 5% CO_2 at 37°C. As shown in Table 1, in contrast to normal granulocytes which were significantly stimulated to migrate through the Matrigel layer (batch 1; 25 μg/filter) by 10^{-7} and 10^{-8} M concentration of FMLP, no stimulatory effect of the chemoattractant on migration was detected in the case of the leukemic cells tested. Hence, chemoattractant was not used in the lower compartment of the chambers in the remainder of the study.

	Percentage Invasion At Following Concentrations of FMLP			
Cells	0	10^{-5} M	10^{-7} M	10^{-8} M
MNL	8.5 ± 0.5	12.1 ± 1.1	57.2 ± 3.3*	36.9 ± 2.5*
KG-1	14.5 ± 4.0	11.0 ± 5.0	15.1 ± 5.1	14.4 ± 2.9
K-562	5.8 ± 1.3	7.5 ± 0.6	5.9 ± 0.8	7.5 ± 2.5
HL-60	1.3 ± 0.4	2.2 ± 0.4	2.7 ± 0.3	2.0 ± 0.5
HEL	2.4 ± 1.1	1.2 ± 0.5	1.5 ± 0.6	3.3 ± 0.7

: **1.** *Invasiveness of normal granulocytes and leukemic cells in response to attractant (FMLP).*

y, cell lines were cultured overnight in serum-free IMDM and prior to their use in the were washed twice with IMDM whereas PMNL isolated from peripheral blood rials and Methods) were used immediately in the assay. Cells, suspended in 0.2mL BSA-IMDM (30 x 10^4 cells) were placed on the polycarbonate filters (8-μm pore of Costar transwell units coated with 15 to 30 μg Matrigel (batch 1). FMLP, a potent attractant of leukocytes, was mixed in 0.6 mL of IMDM at 10^{-5}, 10^{-7} and 10^{-8} M concentration and placed in the lower compartment. The Costar transwell assembly hen incubated for 2 hrs in 5% CO_2 at 37°C. Following incubation, cells were ered from the lower compartment and counted in a hemocytometer. Percentage on was calculated as the ratio of the number of cells recovered from the lower artment to the total number of cells placed in the upper compartment of the vell. Mean ± SD values of percentage invasiveness shown in the table were ated from four experiments (total of 24 chambers).
ificant difference ($p < 0.05$) in comparison with results of experiments in which no attractant was added to the lower compartment.

4.1.3- *Effect of Matrigel concentration on invasiveness.* The effect of different Matrigel concentrations on invasiveness of KG-1 and K-562 cells was tested. Briefly, 5 x 10^4 to 50 x 10^4 cells suspended in 0.1% BSA were placed on polycarbonate filters (8-μm pore size) coated with 12.5-110 μg/filter of commercial Matrigel preparation from batches 1 and 2 and incubated in 5% CO_2 at 37°C for 2 to 24 hrs. As shown in Table 2, the numbers of KG-1 and K-562 cells recovered from the lower compartments of the blind well chambers decreased with an increase in the amount of reconstituted basement membrane preparation placed on the filters. The effect was independent of both the batches of Matrigel and the incubation time. Significant difference in migration through uncoated and coated filters was noted only if at least 25 μg of Matrigel per 13 mm filter was applied. In fact, when as little as 12.5 μg of Matrigel (batches 1 and 2) were coated on filters, a very small amount of protein was detected on the filters by Coomassie blue staining. As shown in Table 2, the difference in the number of cells passing through the Matrigel coated and un-coated filters suggests that the Matrigel layer was indeed a barrier that had to be actively penetrated by the invasive cells.

4.1.4- *Invasion rate of leukemic cells as a function of initial cell density.* The effect on *in-vitro* invasion of the varying cell concentrations placed in the upper compartment of the blind well chamber was tested. Briefly, 5 x 10^4 to 50 x 10^4 cells from either KG-1 or K-562 or HEL or U-937 cell lines were suspended in IMDM supplemented with 0.1% BSA and plated on top of the polycarbonate filters covered with batch 1 of Matrigel (50 μg/filter). After 4 hrs of incubation in 5% CO_2 at 37°C, the invasive cells were recovered from the lower compartment of the blind well chamber. As shown in Figure 3, the invasive

	% Invasion At These Concentrations Of Matrigel / Filter (μg)				
Cells	0	12.5	25-30	50-70	90-110
KG-1[$] (2 hrs)	19.5 ± 1.2	NT	5.6 ± 1.2*	2.3 ± 1.1*	1.0 ± 0.4*
KG-1[$] (4 hrs)	26.3 ± 2.3	14.0 ± 2.5	NT	9.5 ± 3.8*	3.8 ± 2.0*
K-562[&] (2 hrs)	4.9 ± 1.0	2.9 ± 0.7	2.0 ± 0.4*	0.9 ± 0.5*	NT

Table 2. *Invasiveness of KG-1 and K-562 cells across filters covered with Matrigel of various concentrations.*

Briefly, both cell lines were cultured overnight in serum-free IMDM and washed twice prior to their use in the assay. Cells suspended in 0.2 mL 0.1% BSA-IMDM (5×10^4 to 50×10^4 cells) were placed on polycarbonate filters (8-μm pore size), coated with 12.5-110 μg/filter of commercial Matrigel preparation from batch 1 and batch 2, in the upper compartment of blind well chambers and incubated in 5% CO_2 at 37°C for 2 to 24 hrs. Following incubation, cells were recovered from the lower compartment and counted in a hemocytometer. Percentage invasion was calculated as the ratio of the number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of blind wells. Mean ± SD values of percentage invasiveness shown in the table were calculated from three experiments (total of 18 chambers).

[$] Invasion of KG-1 cells was tested using batch 1 of Matrigel.

[&]Invasion of K-562 cells tested using batch 2 of Matrigel.

* Significance difference ($p < 0.05$) in comparison with uncoated filters (0 Matrigel / filter).

NT- Not Tested

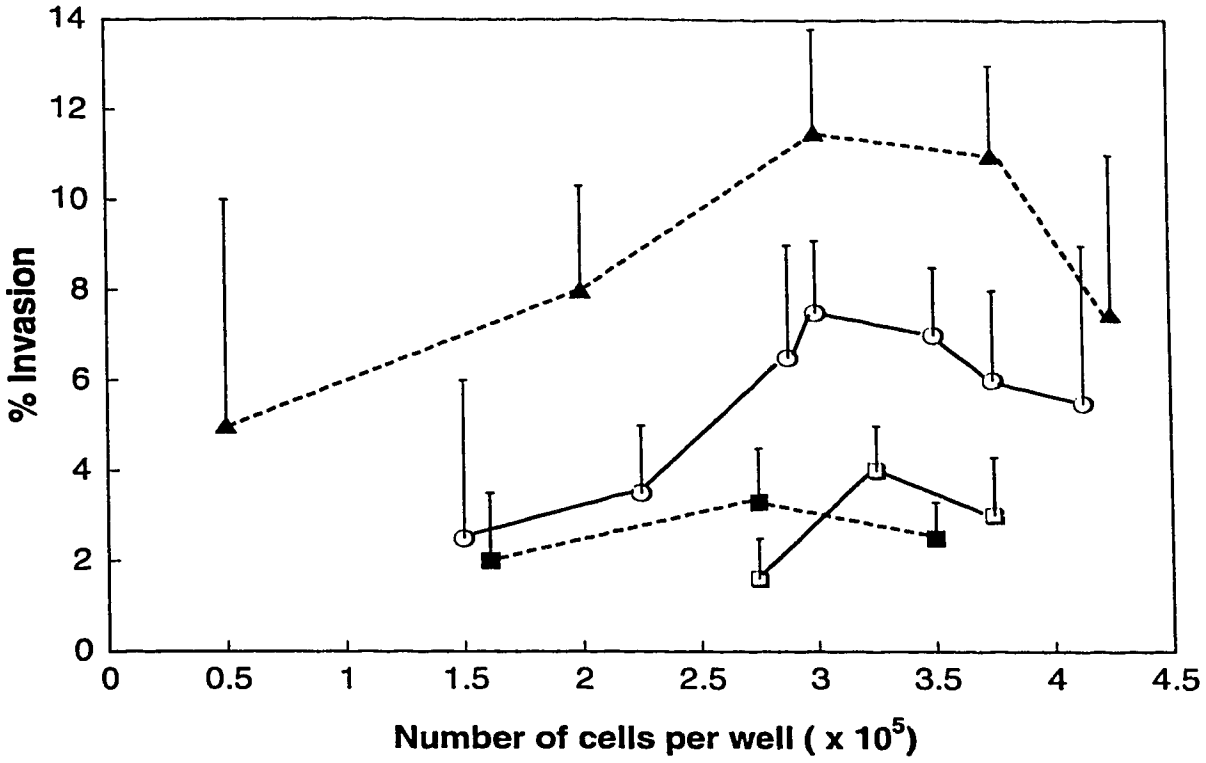


Figure 3. *Invasion of leukemic cells through the Matrigel as a function of initial cell concentration per filter.*

Briefly, cell lines were cultured overnight in serum-free IMDM and prior to their use in the assay were washed twice with IMDM. Cells from KG-1 (▲), K-562 (○) and HEL (□) or U-937 (■) cell lines (5 x 10^4 to 50 x 10^4) were suspended in O.2 mL 0.1% BSA-IMDM and plated on top of the polycarbonate filters covered with batch 1 of Matrigel (50 µg/filter). After 4 hrs of incubation in 5% CO_2 at 37°C, the invasive cells were recovered from the lower compartment of the blind well chamber and counted in a hemocytometer. Percentage invasion was calculated as the ratio of the number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of the blind wells. Mean ± SD values of percentage invasiveness shown in the figure were calculated from three experiments (total of 18 chambers).

activity of cultured leukemic cells was most pronounced and its variability was lowest when the number of cells placed in the upper compartment ranged from 25 x 10^4 to 40 x 10^4. Cell concentrations lower than 15 x 10^4 or greater than 50 x 10^4 per filter (batch1; 50 μg) resulted in highly variable and inconsistent results. Thus, for the rest of the study the cell suspension used in the assay was adjusted to 1.5 x 10^6 /mL and 0.2 mL of this cell suspension was placed in the upper compartment resulting in 30 x 10^4 cells per chamber.

4.1.5- *Comparison of the invasiveness of leukemic and normal hemopoietic cells using different batches of Matrigel.* Briefly, 25 x 10^4 to 35 x 10^4 cells suspended in media supplemented with 0.1% BSA were plated on filters covered with 50 μg of batch 1 or 30 μg of batch 2 of Matrigel. After 4 hrs of incubation, the invasive cells were recovered from the lower compartments of the blind well chambers and counted. As shown in Figure 4, the five leukemic cell lines tested differed in their invasive potential *in vitro*. KG-1 cells were consistently the most potent invaders of the reconstituted basement membrane barrier regardless of the Matrigel batch. With batch 1 (50 μg/filter), the invasive activity of KG-1 cells was comparable with that of normal granulocytes and appeared to be significantly higher than that of the two least invasive cell lines tested, U-937 and HL-60 (Fig. 4A). When batch 2 (30 μg/filter) was used, U-937 and HEL, but not HL-60, cells exhibited significantly lower invasiveness than KG-1 cells, but the difference between HEL and HL-60 was not significant (Fig. 4B). The invasive potential of K-562 cells was consistently less than that of KG-1 cells, but in most cases the difference was

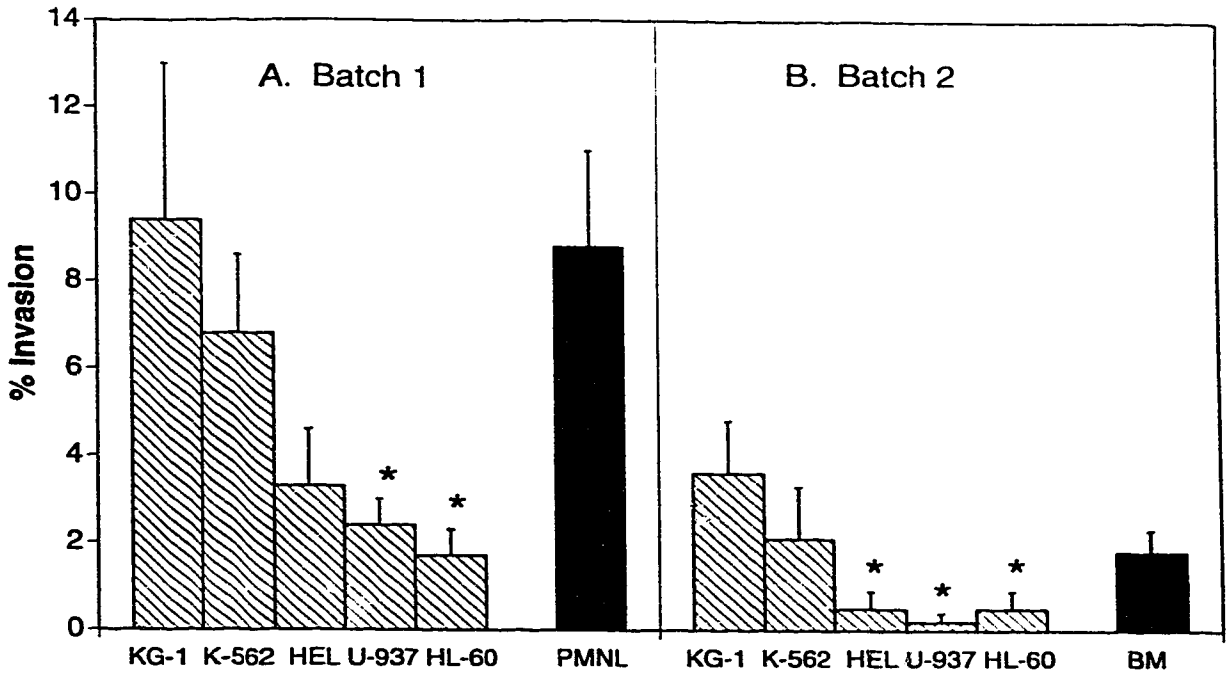


Figure 4. *Invasiveness of cells through filters covered with different batches of Matrigel.*

Briefly, cell lines were cultured overnight in serum-free IMDM and prior to their use in the assay were washed twice with IMDM whereas normal PMNL and BM cells isolated from healthy adult volunteers (Materials and Methods) were used immediately in the assay. Cells suspended in 0.2 mL 0.1% BSA-IMDM (25×10^4 to 35×10^4 cells) were plated on filters covered with 50 µg of batch 1 or 30 µg of batch 2 Matrigel. After 4 hrs of incubation in 5% CO_2 at 37°C, the invasive cells were recovered from the lower compartments of the blind well chambers and counted in a hemocytometer. Percentage invasion was calculated as the ratio of the number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of the transwell.

* Significant difference ($p < 0.05$) in comparison with results obtained for KG-1.

Mean ± SD values of percentage invasiveness of BM cells were calculated from two experiments (total of 12 chambers).

Mean ± SD values of percentage invasiveness of other cell lines were calculated from four experiments (total of 24 chambers) for each batch of Matrigel.

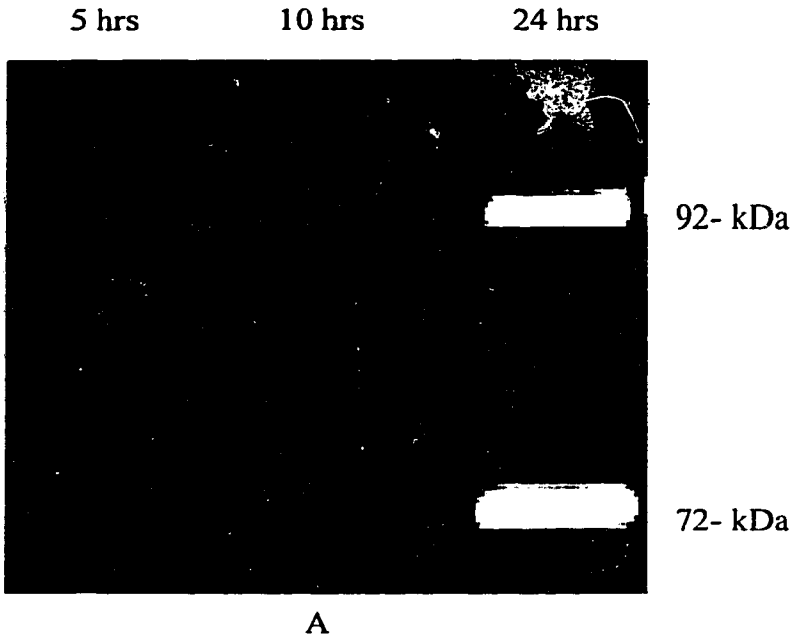
insignificant regardless of Matrigel batch. Although the pattern of differential invasiveness among the leukemic cells tested was similar for both batches of Matrigel, batch 2 created a much more potent barrier for all leukemic cells tested, even though the amount of this batch used per filter was lower than that of the preparation from batch 1 (30 vs. 50 μg/filter) (Fig. 4). Batch 2, however, did not seem to hamper the migration of mature granulocytes significantly. In contrast, migration of the light fraction of normal hemopoietic BM cells through batch 2 was lower than that of KG-1 cells but higher than that of HEL, U-937 and HL-60 cells (Fig. 4B). Although the exact composition of the Matrigel preparations used were not indicated by the producer, it may be speculated that the observed differences in the effectiveness of the barrier formed by the two batches of Matrigel tested resulted from variations in the amounts of collagen type IV, laminin and/or proteoglycans in the natural tumor extracts used to prepare these batches.

4.2 Detection of type IV collagenases/gelatinases by zymography.

Once the *in-vitro* Matrigel assay was established and standardized, the next step was to test whether leukemic cells secreted matrix-degrading enzymes. KG-1 cells were selected for this study. Since type IV collagen is the major component of the basement membranes and of Matrigel, I decided to examine whether type IV collagenases/gelatinases were produced by these cells.

4.2.1- *KG-1 cells secrete 92- and 72 kDa gelatinases.* To test whether KG-1 cells secreted 92- and 72 kDa gelatinases, conditioned media of KG-1 cells were subjected to zymography. Briefly, 10 x 10^6 cells (1 x 10^6/mL) were cultured in serum free IMDM for 5, 10, 15 and 24 hrs. Cell-free supernatants were collected after the indicated time and concentrated 20x. Fifteen μL of concentrated supernatants were mixed with 5 μL of loading buffer and applied to gelatin-copolymerized SDS gels which, after electrophoresis and washing, were incubated in zymography buffer for 24 hours at 37°C. As shown in Figure 5, both 92- and 72 kDa gelatinolytic activities were detected in the cell conditioned media of KG-1 cells. These gelatinases were detectable in the conditioned media incubated for a minimum of 15 hours. They were undetectable up to 10 hours (Fig. 5a) and appeared as very faint bands after 10 hrs of incubation. Hence, the conditioned media were collected after approximately 24 hours of incubation and then tested for the presence of these gelatinases by the appearence of visible bands of greater intensity (Fig. 5a). When the KG-1 cell suspension was collected from the upper compartment of the blind well chamber at the end of the invasion assay (2 hrs.) and cell free supernatant was subjected to zymography, both 92 and 72 kDa gelatinases were detected in the media (Fig. 5b).

4.2.2- *Secretion of 92 and 72 kDa gelatinases by normal granulocytes.* To test whether normal granulocytes secrete 92- and 72 kDa gelatinases, granulocytes were separated as described in Materials and Methods and their conditioned media were tested for the presence of type IV collagenases. Briefly, 10 x 10^6 cells (1 x 10^6/mL) were cultured in serum-free IMDM for 2 hours. Cell-free supernatant was diluted or concentrated as



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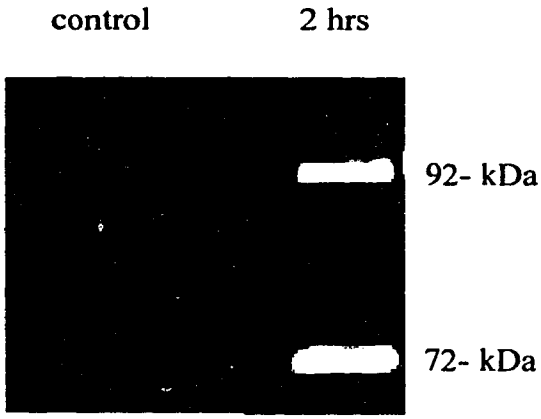


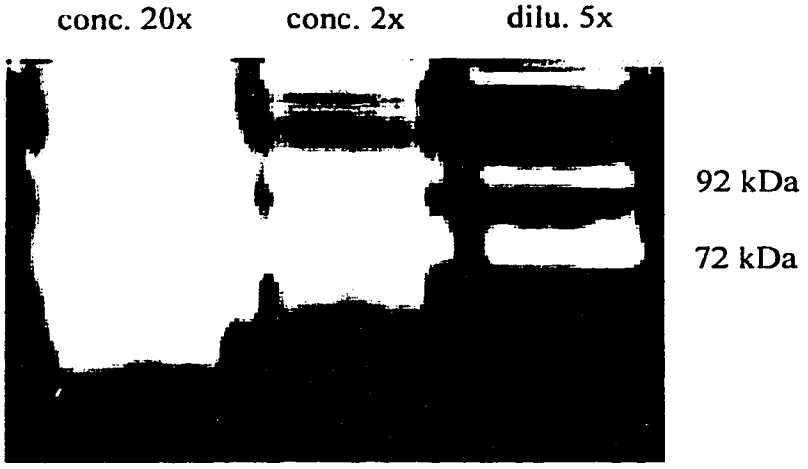
B

Figure 5. *Secreted 92- and 72- kDa gelatinases of KG-1 cells.* **A)** Serum-free conditioned media of KG-1 cells (1 x 10^6/mL) were collected at indicated times, concentrated 20x and applied (15 µL) on the gelatin embedded gel. **B)** Cell suspension was collected from the upper compartment of the blind well after 2 hrs, cell free supernatant was concentrated 10x and 15 µL was applied on gelatin embedded gel. For control, 10x concentrated 0.1% BSA-IMDM was used. In both A and B, after electrophoresis and washing, gels were incubated in zymography buffer at 37°C for approximately 24 hrs.

indicated and applied (15 μL) to 7% gelatin copolymerized gels which, after electrophoresis and washing, were incubated for 24 hours in zymography buffer at 37°C. As shown in Figures 6A and 6B, 20x concentrated and unconcentrated conditioned media of granulocytes contained excessive gelatinolytic activity. However, 5x and 10x dilution of the unconcentrated sample resulted in a better resolution of 92- and 72 kDa gelatinase bands in the zymogram (Fig. 6A and 6B). Secretion of these gelatinases by normal granulocytes was much faster, i.e., it occurred within 2 hours of incubation, and was stronger than for KG-1 cells.

4.2.3- *Inhibition of secreted 92- and 72 kDa enzyme activities by inhibitors.* Detection of gelatinases in the zymograms at 92- and 72 kDa molecular weight, as defined by Bio Rad markers, strongly indicated that these gelatinases were type IV collagenases. A conclusion based on using molecular weight alone for identification, however, is not very convincing. Hence other methods were applied to confirm that the 92- and 72 kDa gelatinases seen in zymograms were type IV collagenases/gelatinases.

If these gelatinases were in fact type IV collagenases of the MMP family, then inhibitors of MMPs should affect the substrate-degrading activity of these enzymes. This logic was tested by first electrophoresing the conditioned media of KG-1 cells, then incubating the gels in zymography buffer containing 1,10-phenanthroline, which is a specific inhibitor of MMPs (Materials and Methods). As shown in Figure 7, 92- and 72- kDa gelatinase activities were completely inhibited, compared with the control (incubated in buffer without 1,10-phenanthroline) by both 1.0 mM and 0.1 mM concentrations of 1,10-phenanthroline. Therefore the appearance of these gelatinases at 92- and 72-kDa



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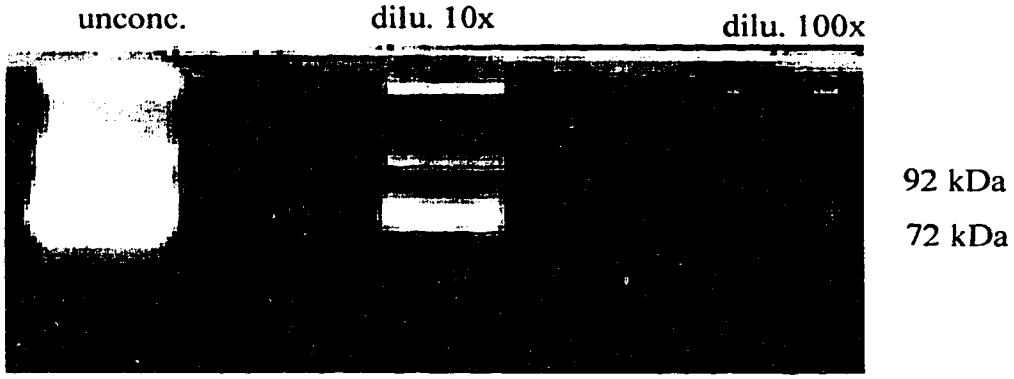


B

Figure 6. *Secreted 92- and 72 kDa gelatinases of normal granulocytes.*

PMNL, after separation from PB (Materials and Methods), were washed 3x with IMDM and cultured (1 x 10^6/mL) in serum-free IMDM. **A** and **B**) Serum-free conditioned media were collected after 2 hrs, concentrated or diluted (with IMDM) as indicated and were applied (15 µL) on gelatin embedded gels. After electrophoresis and washing, the gels were incubated in the zymography buffer at 37°C for approximately 24 hrs.

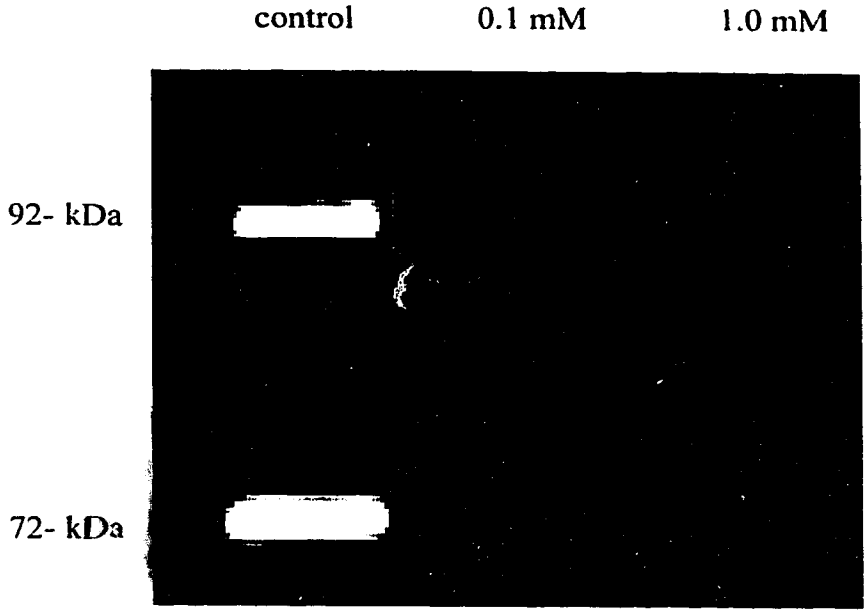


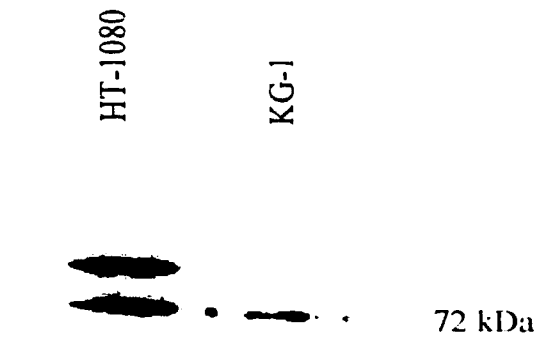
Figure 7. *Effect of 1,10-phenanthroline on secreted gelatinases of KG-1 cells.*

KG-1 cells (10 x 10^6) from continuous culture were washed 3x with IMDM and cultured in serum-free IMDM (1 x 10^6/mL) at 37°C in 5% CO_2. After approximately 24 hrs of incubation, serum-free conditioned media was collected, concentrated approx. 20x and applied in triplicate (15 µL in each lane) on the same gelatin embedded gel. After electrophoresis and washing, the gel was cut in three pieces and each piece was incubated separately in zymography buffer containing 0.1 mM and 1.0 mM of 1,10-phenanthroline or without 1,10-phenanthroline (control) at 37°C for approximately 24 hrs.

molecular weight and the inhibition of their enzyme activities by inhibitors of MMPs strongly indicated that they are 92- and 72- type IV collagenases.

4.3 Confirmation by Western blot analysis of 92- and 72 type IV collagenases.

Polyclonal antibodies against 92- and 72-kDa collagenases (gifts from Dr. W. Stetler-Stevenson) were used in Western blot analysis to confirm that 92- and 72-kDa gelatinases present in the secreted fraction of KG-1 cells were type IV collagenases/gelatinases. For both HT-1080 and KG-1 cells, affinity-purified secreted fractions (on the gelatin column) were used not only to enhance the message but also to eliminate the possibility of cross reaction of antibodies with other enzymes in the secreted fraction. Concentrations of purified proteins used in experiments ranged from 5 to 15 μg/15 μL for KG-1 and 2 to 10 μg/15 μL for HT-1080. The same dilution of secondary antibody, i.e., 1:3000, was used for both 92- and 72-kDa gelatinase blots. Seventy two-kDa gelatinase was detected by loading approximately 5 μg and 10 μg from HT-1080 and KG-1 fractions respectively on the gel and incubating the blot with 12 μg of anti-72 kDa primary antibody (Figure 8a). Similarly, 92-kDa gelatinase was detected by loading 7 μg from HT-1080 and 15 μg from KG-1 fractions respectively and incubating the blot with 15 μg of anti-92 kDa primary antibody (Figure 8b). Hence both 92- and 72-kDa gelatinases in the secreted fraction of KG-1 cells were confirmed as type IV collagenases by using the ECL (enhanced chemoluminescence) method of Western blotting.



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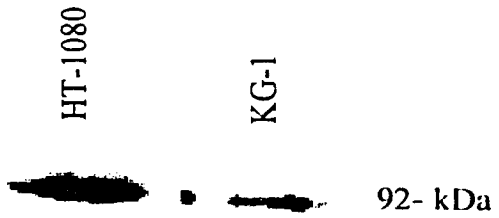


B

Figure 8. *Detection of 92 and 72 kDa gelatinases by Western blotting.*

Gelatin affinity column purified supernatants were used. **A**) 5 μg of HT-1080 and 10 μg of KG-1 gelatin bound protein was reacted with 12 μg of anti-72 kDa antibody. **B**) 7 μg of HT-1080 or 15 μg of KG-1 gelatin bound protein was reacted with 15 μg of anti-92 kDa antibody. Both blots (Fig.8a and 8b) were reacted with 1:3000 dilution of HRP-conjugated secondary antibody. After reacting with ECL solution, X-ray films were exposed to these blots for 30 sec (A) and 1 min (B).

4.4 Role of 92- and 72 kDa type IV collagenases/gelatinases in Matrigel-based assay.

Once it was established that KG-1 leukemic cells secreted gelatin-degrading 92- and 72-kDa enzymes that are type IV collagenases, the next step was to test whether these enzymes were involved in the *in-vitro* invasiveness of these cells. Since type IV collagen is the major component of Matrigel, inhibition of these gelatin-degrading enzymes should have affected the invasive property of KG-1 cells.

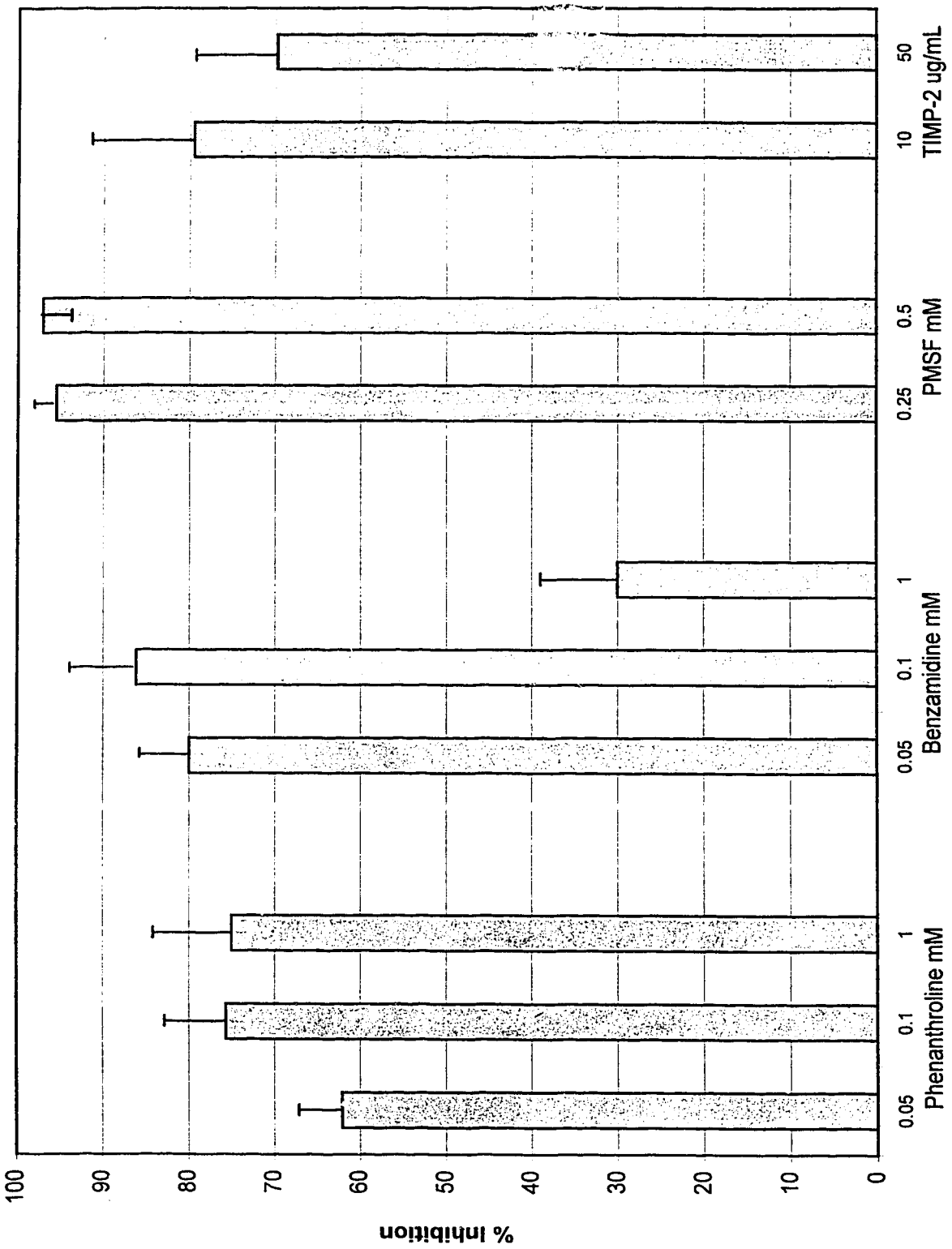
4.4.1- *Inhibition of* in-vitro *migration of normal granulocytes and KG-1 cells with natural and synthetic inhibitors.* The effect of specific inhibitors on the *in-vitro* invasiveness of normal granulocytes and KG-1 cells was tested. For this purpose not only the inhibitors of MMPs but also of serine proteinases were used because, as described in the introduction, serine proteinases may be involved in the degradation of various components of Matrigel and/or activation of MMPs. Briefly, 30 x 10^4 cells in 0.1% BSA-IMDM were mixed with inhibitors of MMPs (0.05 to 1.0 mM of 1,10-phenanthroline, 10 or 50 μg of rhTIMP-2) or serine proteinases (0.05 to 1.0 mM benzamidine, 0.25 to 0.5 mM PMSF) and incubated at 37°C for 30 min. Following incubation, the cell suspension with inhibitor was placed in the upper compartment of a blind well chamber and incubated for 2 hours at 37°C in 5% CO_2. Figure 9 shows the effect of various concentrations of inhibitors of both MMPs (1,10-phenanthroline and rh-TIMP-2) and serine proteinases (benzamidine and PMSF) on the *in-vitro* invasiveness of KG-1 cells. Table 3 shows the maximum percentage of inhibition of *in-vitro* invasiveness that was achieved in normal granulocytes and KG-1 cells using these inhibitors, i.e., 79.5% with 10 μg/mL of rh-TIMP-2, 75% with 1.0 mM 1,10-phenanthroline, 86.1% with 0.1 mM benzamidine and

LEGEND TO FIGURE NO. 9

KG-1 cells were cultured in serum-free IMDM overnight and washed twice prior to their use in the assay. For the inhibitions assay, 30 x 10^4 cells were suspended in 0.2 mL 0.1% BSA-IMDM. After addition of inhibitors of MMPs (1,10-phenanthroline; 0.05 mM, 0.1 mM or 1.0 mM and rhTIMP-2; 10 or 50 μg/mL) or serine proteinases (benzamidine; 0.05 mM, 0.1 mM or 1.0 mM and PMSF; 0.25 mM or 0.5 mM), this cell suspension (KG-1 cells with inhibitors) was incubated for 30 min in 5% CO_2 at 37°C. Following incubation, the cell suspension was placed on Matrigel coated filter (batch 2 or 3; 25 μg) in the upper compartment of a blind well chamber and incubated for 2 hrs in 5% CO_2 at 37°C. Following incubation, the cells were recovered from the lower compartment and counted in hemocytometer. Percent inhibition of invasiveness was calculated by comparing the percentage of invasiveness in the presence and absence of the inhibitors.

Mean ± SD values of percentage inhibition were calculated from 18 chambers for each point indicated in the figure.

Figure 9. Effects of different concentrations of various inhibitors on invasiveness of KG - 1 cells.



	% INHIBITION	
INHIBITORS	**KG-1**	**Granulocytes**
Metalloproteinases		
rh-TIMP-2 (10 µg/mL)	79.5 ± 11.4	NT
o-Phenanthroline (1 mM)	75.0 ± 9.1	37.0 ± 7.2
Serine proteinases		
Benzamidine (0.1 mM)	86.1 ± 7.4	83.0 ± 6.2
PMSF (0.25 mM)	95.5 ± 1.5	NT

Table 3. *Effect of inhibitors on the in vitro invasiveness of KG-1 cells and granulocytes.*

Briefly, KG-1 cells were cultured overnight in serum-free IMDM, washed twice and used in the assay. PMNLs, isolated as described in Materials and Methods, were immediately used in the assay. PMNLs or KG-1 cells (30×10^4) were suspended in 0.2 mL 0.1% BSA-IMDM, mixed with MMP or serine proteinase inhibitors at the indicated final concentrations and incubated at 37°C in 5% CO_2 for 30 min. Following incubation, the suspension of cells and inhibitor was placed on the Matrigel-coated filter (batch 2 or 3; 25 µg) in the upper compartment of the blind well chamber and incubated for 2 hrs. at 37°C in 5% CO_2. After 2 hrs, cell were recovered from the lower compartment and counted in a hemocytometer. Percent inhibition of invasiveness was calculated by comparing the percentage of invasiveness in the presence and absence of the inhibitors.

Mean ± SD values of percentage inhibition shown in the table were calculated from 30 chambers except for the value obtained for rh-TIMP-2 which was calculated from 18 chambers.

NT = Not Tested

95.5% with 0.25 mM PMSF in KG-1 cells. The higher percentage of inhibition achieved by serine proteinase inhibitors than metalloproteinase inhibitors (Table 3) indicated that serine proteinases were also involved in this process of *in-vitro* invasion since their inhibition affected enzyme activities as well. Although the percentage inhibition of invasiveness achieved with 1,10-phenanthroline in normal granulocytes was significantly lower than that achieved in KG-1 cells, no significant difference was seen with benzamidine in the two cell types. To check whether these inhibitors, with the exception of rhTIMP-2, had any toxic effect on the cells, normal granulocytes and KG-1 cells were exposed to similar concentrations of these inhibitors for 2 hours and 30 minutes (the total length of time to which these cells were exposed to inhibitors in the inhibition assay). After incubation, the cell viability was checked by trypan blue staining and both cell types were found to be 99-100% viable.

4.4.2. *Inhibition of* in-vitro *invasiveness of KG-1 cells with monoclonal antibody against 72-kDa gelatinase*. As the inhibitors of MMPs used above, except for rhTIMP-2, are not specific for a specific type of MMP, the effect of monoclonal antibody against 72-kDa gelatinase on the *in-vitro* invasiveness of KG-1 cells was tested in the invasion assay (Materials and Methods). Briefly, 30 x 10^4 cells in 0.1% BSA-IMDM were mixed with moAbs against 72 kDa gelatinase (10 μg/mL final concentration) and incubated for 30 min. Following incubation this suspension was added to the upper compartment of the blind well chamber and incubation was carried out for 2 hours at 37°C in 5% CO_2. As shown in Table 4, up to 51.6% inhibition of *in-vitro* invasiveness was achieved with this

ANTIBODY	% INHIBITION[a]
monoclonal Ab MMP-2 (72- kDa)	51.6 ± 1.5*

Table 4. *Effect of monoclonal antibody against 72- kDa gelatinase on in-vitro invasiveness of KG-1 cells.*

Briefly, KG-1 cells, cultured overnight in serum-free IMDM, were washed twice with IMDM. Cells (30 x 10^4) were then suspended in 0.2 mL 0.1% BSA-IMDM, mixed with moAbs against 72 kDa gelatinase (10 µg/mL final concentration) and incubated for 30 min at 37°C in 5% CO_2. Following incubation this suspension was placed on the Matrigel-coated filter (batch 3; 25 µg) in the upper compartment of the blind well chamber and incubation was carried out for 2 hrs at 37°C in 5% CO_2.

n = 2

[a] Calculated by comparing the percentage of invasiveness in the presence and absence of the antibody.

* Significant difference ($p < 0.05$) when compared to control.

antibody indicating that 72-kDa gelatinase plays a crucial role in the crossing of the Matrigel barrier by KG-1 cells.

4.5 Study of the primary AML cells.

Study of myeloblastic KG-1 cells indicated that they were the most invasive in the *in-vitro* invasion assay and secreted 92- and 72 kDa gelatinases. Hence, the next step was to test whether primary leukemic cells from patients diagnosed with AML possessed similar properties.

4.5.1- *Study of the* in-vitro *invasiveness of leukemic blasts from AML patients.*

Ten patients diagnosed with AML, whose clinical characteristics are presented in Table 5, were included in this study. According to the FAB classification there was one M0, four M1s, two M2s, one M4 and two M5s. Peripheral blood cells of all patients and the bone marrow cells of AML patients nos.4 to 10 were tested for *in-vitro* invasion as described in Materials and Methods. As shown in Table 6, both bone marrow and peripheral blood cells were invasive in the Matrigel assay. With the exception of AML patients nos.7 and 8, bone marrow cells were found to be more invasive than peripheral blood cells (Table 6); however, the percentage of invasiveness within each cell type varied, ranging from 1.8% to 8.6% with bone marrow cells and 1.4% to 11.5% with peripheral blood cells. As shown in Table 5, the bone marrow and peripheral blood samples of these patients (after Ficoll or Percoll separation) contained greater than 90% blast populations except for the peripheral

Patient No.	Sex /Age (yrs.)	FAB class	Peripheral blood			% of Blasts	
			WBC ($x10^9$L)	Hb (g/dL)	PLT ($x10^9$/L)	PB	BM
1	Female / 67	M1	620.0	8.1	90.0	100	NA
2	Female / 59	M2	5.0	6.9	130.0	96	NA
3	Male / 53	M4	65.9	11.0	61.0	100	NA
4	Male / 54	M2	80.0	9.2	<10.0	93	91
5	Female / 47	M1	255.2	11.7	64.0	99	98
6	Female / 51	M5	25.6	10.2	236.0	85	93
7	Male / 50	M0	128	9.4	272	100	100
8	Male / 54	M5	126	6.7	43	95	98
9	Male / 59	M1	292	4.3	32	93	97
10	Male / 63	M1	22	4.6	171	97	96

Table 5. *Clinical data of patients diagnosed with AML.*

	% INVASIVENESS	
Patient No. / FAB	Bone marrow	Peripheral blood
1 / M1	NT	3.3 ± 0.6
2 / M2	NT	11.5 ± 2.0
3 / M4	NT	7.3 ± 1.6
4 / M2	6.7 ± 0.9	1.4 ± 0.3
5 / M1	8.6 ± 0.8	2.6 ± 0.5
6 / M5	3.9 ± 0.6	3.2 ± 0.3
7 / M0	2.0 ± 0.6	4.0 ± 1.3
8 / M5	1.8 ± 0.3	4.1 ± 1.6
9 / M1	5.8 ± 1.3	3.5 ± 1.2
10 / M1	2.5 ± 0.9	1.7 ± 0.3

Table 6. *In vitro invasiveness of blast cells obtained from 10 patients diagnosed with AML.*

Briefly, light density cells, from the bone marrow and peripheral blood of AML patients nos. 1 to 10, were separated using 60% Percoll gradient centrifugation at 300 g for 30 min. After 3 washes with IMDM, 30 x 10^4 cells were suspended in 0.2 mL 0.1% BSA-IMDM and placed on top of Matrigel-coated filter (batch 3; 25 µg) in the upper compartment of the blind well chamber and incubated in 5% CO_2 at 37°C. After 2 hrs cell were recovered from the lower compartment and counted in a hemocytometer. Percentage invasion was calculated as the ratio of number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of blind wells. Mean ± SD values of percentage invasiveness shown in the table were calculated from 18 chambers.
NT = Not Tested

blood of AML patient no.6. No significant difference ($p > 0.05$) was seen between the *in-vitro* invasiveness of peripheral blood cells from M1, M2 and M5 patients (Table 6).

4.5.2- *92- and 72- kDa secreted gelatinases of leukemic cells from AML patients.*

To test whether leukemic cells from AML patients secreted 92- and 72- kDa gelatinases, serum-free conditioned media were collected after 2 and/or 24 hrs of incubation from 10 x 10^6 cells (1 x 10^6/mL), concentrated 20x and subjected to (15 μL) to zymography. Conditioned media from KG-1cells were used as a positive control since at this point it had been shown that the gelatinases (in KG-1 cell-conditioned media) seen in the zymograms were 92- and 72 kDa type IV collagenases/gelatinases. As shown in Figure 10, these gelatinases were detected in the peripheral blood sample of AML patient no.1 and, like the KG-1 gelatinases, were inhibited by 1,10-phenanthroline in the zymogram (Figure 11). Gelatinase of 92-kDa was also detected in peripheral blood cell-conditioned media of all other AML samples (Figures 13 to 17) except AML patient no.5 (Figure 14) and in bone marrow cell-conditioned media of AML patients nos.6 to 10 (Figures 15 to 17). Similarly, 72- kDa gelatinase was also present in both peripheral blood and bone marrow cell-conditioned media of all AML samples (Figures 13 to 17) except in the peripheral blood cell-conditioned media of AML patient no.6 (Figure 15). In Figure 15, bands seen at the 92-kDa position between KG-1 and the bone marrow lane and the bone marrow and peripheral blood lane are probably due to a spill over from adjacent wells since no samples were loaded in these lanes. The activated forms of 92 and 72 kDa gelatinases, i.e., 82 and 62 kDa gelatinases respectively, were detected in conditioned

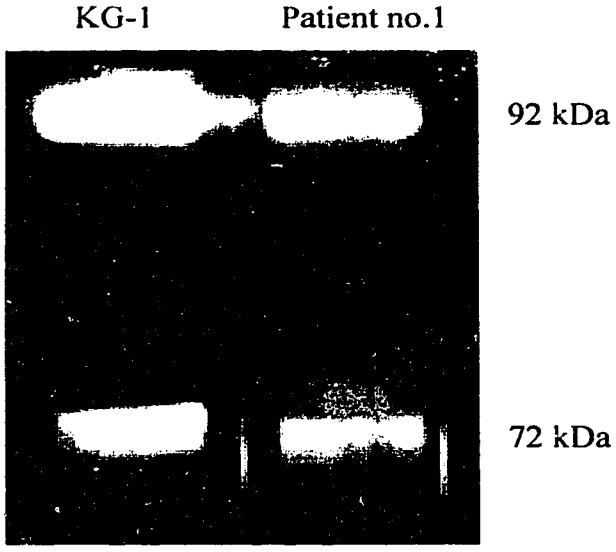


Figure 10. *Secreted gelatinases of KG-1 and peripheral blood cells from AML patient no. 1.*

KG-1 or PB cells (8 x 10^6 to 10 x 10^6 cells) were cultured (1 x 10^6 cells/mL) in serum-free IMDM. After approx. 24 hrs serum-free conditioned media were collected, concentrated 20x and applied (15 µL) to gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for approximately 24 hrs.

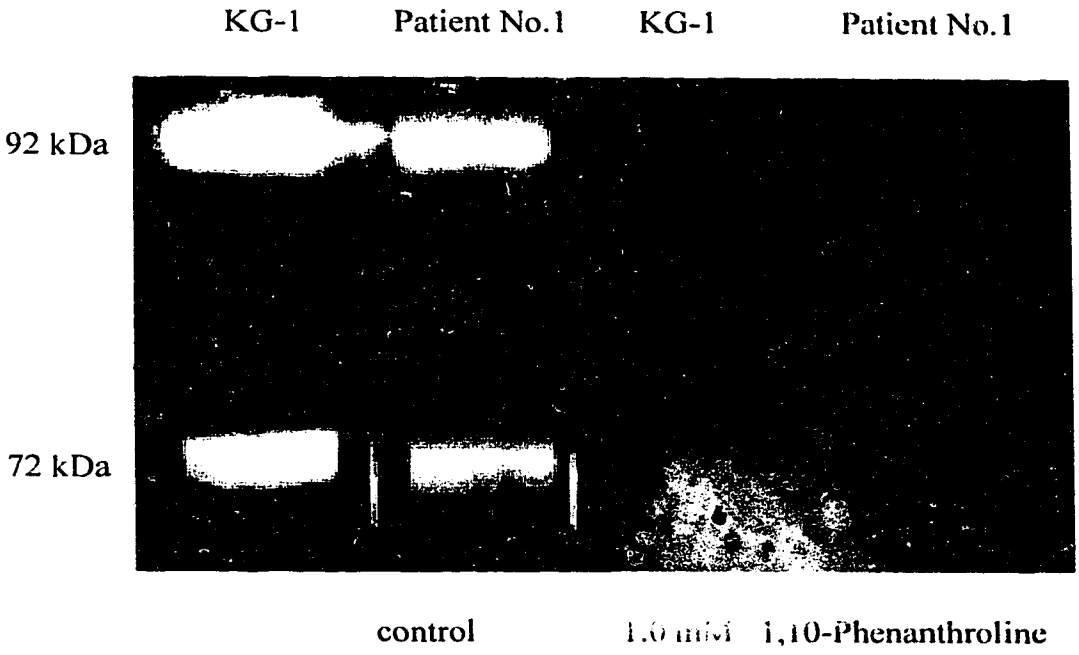


Figure 11. *Effect of 1,10-phenanthroline on secreted gelatinases of KG-1 and peripheral blood cells from AML patient no. 1.*

KG-1 or PB cells (8 x 10^6 to 10 x 10^6) were cultured (1 x 10^6 cells/mL) in serum-free IMDM. After approx. 24 hrs serum-free conditioned media were collected, concentrated 20x and applied (15 µL) in duplicate on the same gelatin embedded gel. After electrophoresis and washing, the gel was cut in two pieces, each of which contained electrophoresed conditioned media of both KG-1 and PB cells of Patient no. 1. These pieces were incubated separately in zymography buffer with or without (control) 1.0 mM 1,10-phenanthroline at 37°C for approximately 24 hrs.

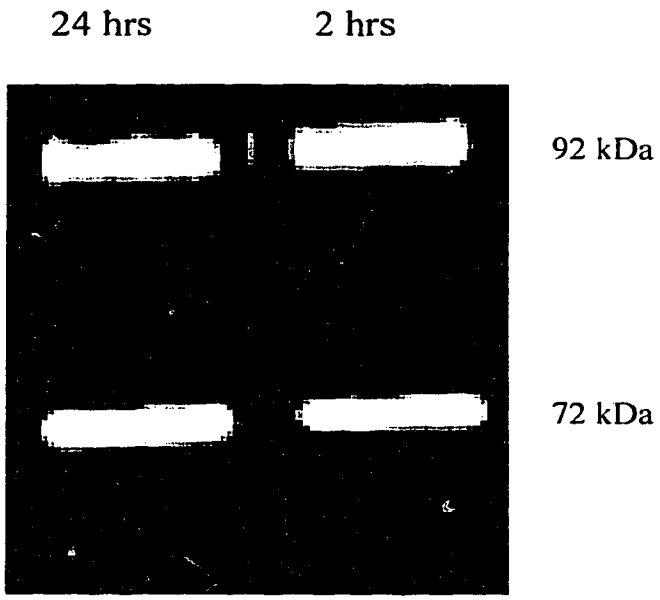


Figure 12. *Secreted gelatinases of peripheral blood cells from AML patient no. 1.*

PB cells from AML patient no. 1 were cultured in two flasks, each containing 10 x 10^6 cells in serum-free IMDM (1 x 10^6 cells/mL). Conditioned media was collected from one flask after 2 hrs and from the other after 24 hrs. After collection, these conditioned media were concentrated 20x and applied (15 μL) to the gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 24 hrs.

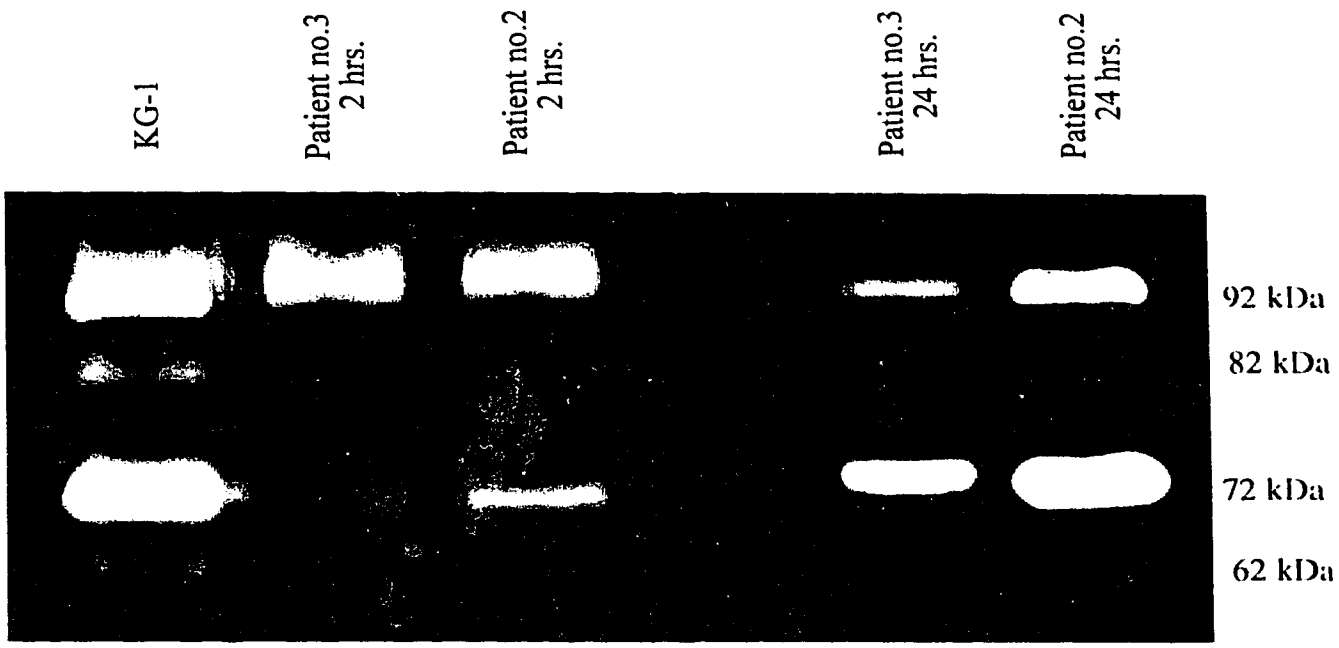


Figure 13. *Secreted gelatinases of KG-1 and peripheral blood cells from AML patients nos. 2 and 3.*

KG-1 or PB cells from patients no. 2 and 3 (10×10^6) were cultured in serum-free IMDM (1×10^6 cells/mL). To collect conditioned media after 2 and 24 hrs, cultures were setup similar to PB cells from Patient no.1 (Fig. 12), except for KG-1 cells which were cultured in one flask and conditioned medium was collected only after 24 hrs. All conditioned media were concentrated 20x and applied (15 µL) on gelatin embedded gel. After electrophoresis and washing, the gels were incubated in zymography buffer at 37°C for 24 hrs.

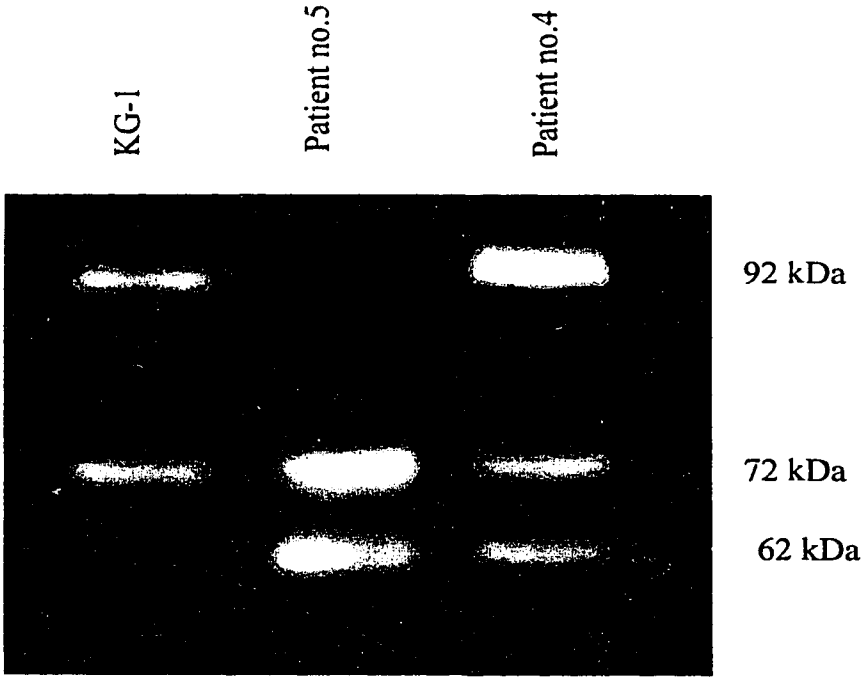


Figure 14. *Secreted gelatinases of KG-1 and peripheral blood cells from AML patients nos. 4 and 5.*

Ten million cells were cultured in serum-free IMDM (1 x 10^6 cells/mL). Conditioned media were collected after 24 hrs, concentrated 20x and applied (15 µL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 24 hrs.

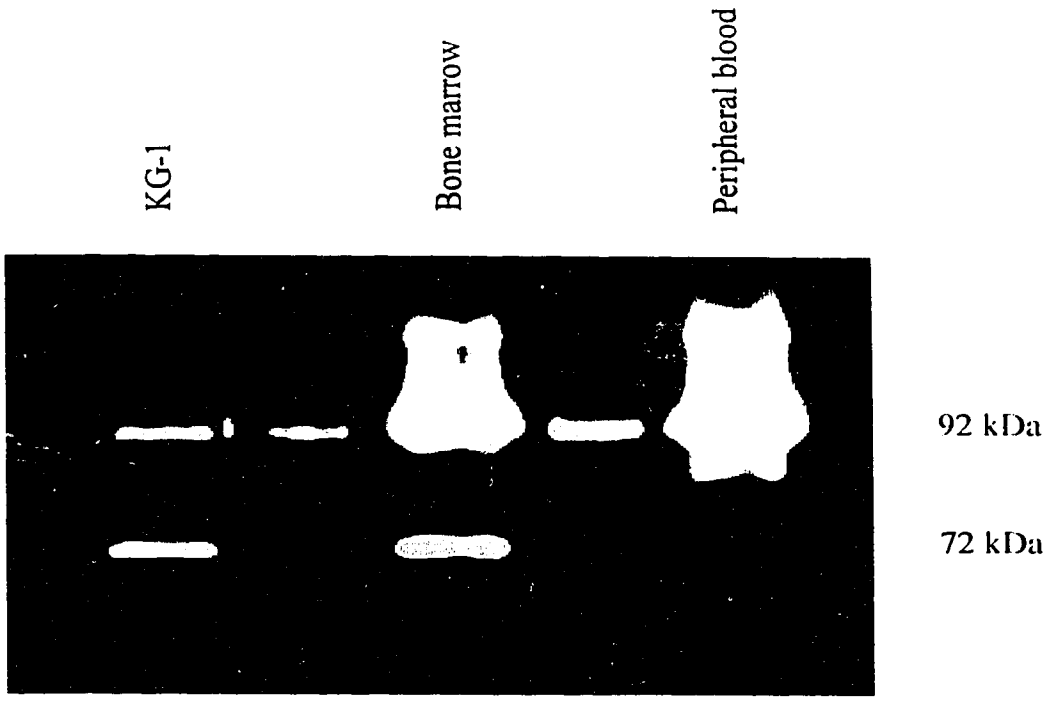


Figure 15. *Secreted gelatinases of KG-1 and PB cells from AML patient no.6.*

Ten million cells were cultured in serum-free IMDM (1 x 10^6 cells/mL). Conditioned media were collected after 24 hrs., concentrated 20x and applied (15 µL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 24 hrs.
(No sample was placed in the middle lanes between KG-1 and bone marrow and, bone marrow and peripheral blood; bands appear probably due to a spill over from the adjacent wells).

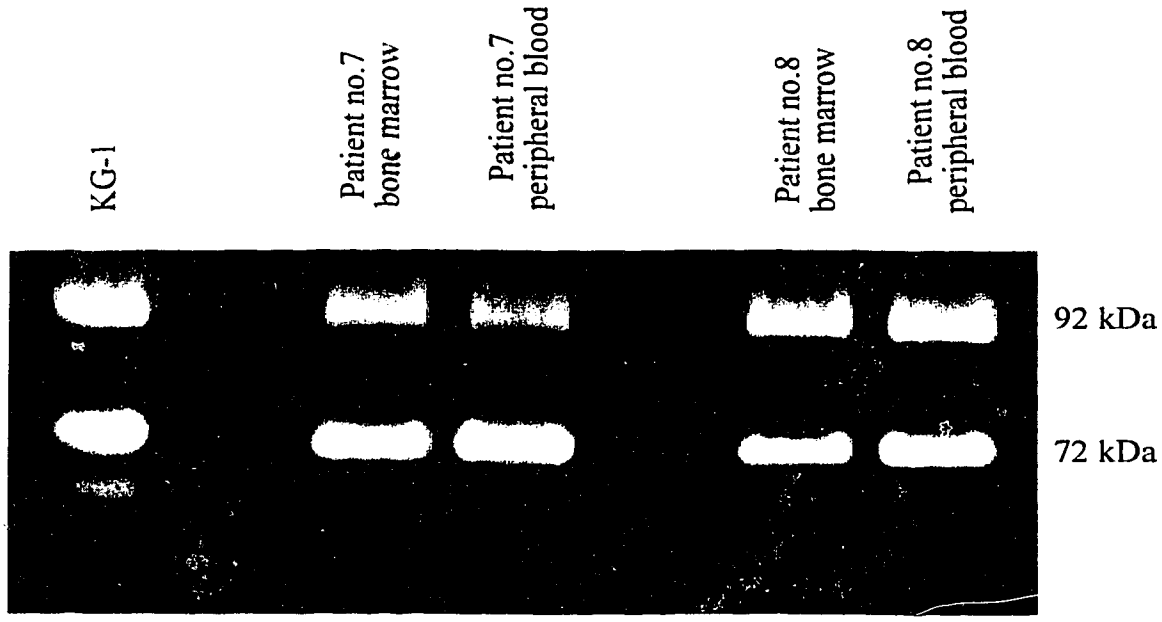


Figure 16. *Secreted gelatinases of KG-1, bone marrow and peripheral blood cells of AML patients nos. 7 and 8.*

Ten million cells were cultured in serum-free IMDM (1 x 10^6 cells/mL). Conditioned media were collected after 24 hrs, concentrated 20x and applied (15 μL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 24 hrs.

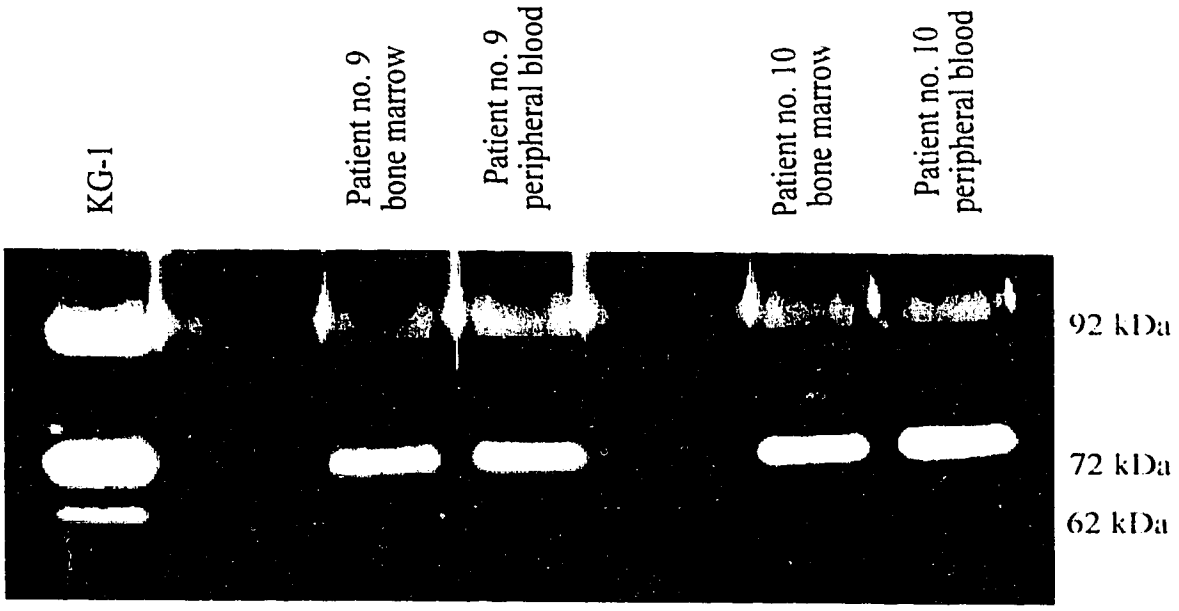


Figure 17. *Secreted gelatinases of KG-1, bone marrow and peripheral blood cells of AML patients nos. 9 and 10.*

Ten million cells were cultured in serum-free IMDM (1 x 10^6 cells/mL). Conditioned media were collected after 24 hrs, concentrated 20x and applied (15 µL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 24 hrs.
(No sample was added to lanes 2 and 5).

media of KG-1 (Fig. 13, 16, 17). The 62 kDa gelatinase was also detected in 24 hrs cell conditioned media of patients nos. 3-5 and 7-9 (Fig. 13, 14, 16, 17). It was also shown that both 92- and 72- kDa gelatinases in peripheral blood samples of AML patients nos.1, 2 and 3 were secreted within 2 hours (Figures 12 and 13). These gelatinases, however, were not only more intensely expressed after 24 hours but also they appeared as sharper bands on zymograms (Figures 12 and 13). Therefore, for the rest of the AML samples conditioned media were collected after approximately 24 hours of incubation.

As shown in Table 7, the gelatinolytic activity of these gelatinases varied greatly in the bone marrow and peripheral blood cell-conditioned media of the AML samples tested (AML patients nos.6 to 10). Although the peripheral blood sample of AML patient no.9 and the bone marrow sample of AML patients nos.9 and 10 showed the secretion of 92-kDa gelatinase in the zymogram (Figure 17), their activity could not be measured by Sigma Scan (Materials and Methods). The zero value of activity observed for the 72- kDa gelatinase of the peripheral blood sample from AML patient no.6 (Table 7), however, was in accord with the zymogram of the same sample because the 72- kDa gelatinase band could not be detected in the peripheral blood cell-conditioned media of AML patient no.6 (Figure 15).

No statistical correlation was found when the intensities of secreted 92- and 72- kDa gelatinases were compared with the *in-vitro* invasiveness of peripheral blood and bone marrow samples from AML patients nos.6 to 10 ($p > 0.05$) (Table 7).

CELLS		GELATINOLYTIC ACTIVITY OF 92 kDa	GELATINOLYTIC ACTIVITY OF 72 kDa	% INVAS. (From Tab. 6)
Patient 6	Bone marrow	42.2	4.1	3.9 ± 0.6
	Peripheral blood	16.8	-	3.2 ± 0.3
Patient 7	Bone marrow	3.4	14.0	2.0 ± 0.6
	Peripheral blood	2.0	14.2	4.0 ± 1.3
Patient 8	Bone marrow	11.2	16.6	1.8 ± 4.1
	Peripheral blood	13.6	16.0	4.1 ± 1.6
Patient 9	Bone marrow	-	7.3	5.8 ± 1.3
	Peripheral blood	-	7.8	3.5 ± 1.2
Patient 10	Bone marrow	-	9.7	2.5 ± 0.9
	Peripheral blood	1.5	13.9	1.7 ± 0.3

Table 7. *Intensities of gelatinolytic activities of 92 and 72 kDa gelatinases as analyzed by Sigma Scan.*

Briefly, the cell conditioned media of light density cells from the peripheral blood and bone marrow of AML patients nos.6 to 10 were subjected to zymography as described in Figs.15-17. However, the gels, after incubation, were all stained overnight with an equal quantity of Coomassie brilliant blue G-250 and de-stained with an equal quantity of 20% isopropanol with 10% acetic acid for an equal length of time. After this they were processed in the ScanJet II cx scanner. The intensity of the gelatinolytic activities (appearing as clear bands against blue background) were analyzed and expressed in numbers using SigmaScan/Image measurement software.

4.6 Study of *in-vitro* migration and gelatinase production by marrow CD34 cells.

After showing that blast cells have the ability to produce 92- and 72- kDa gelatinases which may enable them to cross the basement membrane and enter the peripheral blood, experiments with $CD34^+$ cells, isolated from normal individuals, were carried out to test whether they lack the ability to secrete such gelatinases or not.

4.6.1- *Purity of $CD34^+$ cells isolated by MACS system.* To check the purity of $CD34^+$ cells isolated by the MACS system (Materials and Methods), fluorescence flow cytometric analysis was done on a population of $CD34^+$ cells along with $CD34^-$ and unseparated or pre-MAC cells. Figure 18d shows that the cells in the $CD34^+$ fraction were recognized by anti-CD34 FITC linked antibody. This $CD34^+$ fraction was found to contain 90% of the total population of $CD34^+$ cells compared with 1% and 0% $CD34^+$ cells in the pre-MAC and $CD34^-$ fractions respectively.

4.6.2- In-vitro *migration of $CD34^+$ cells.* The two populations of CD34 cells were tested for migration in the Matrigel-based assay. Briefly, 30 x 10^4 $CD34^+$ or $CD34^-$ cells were resuspended in 0.1% BSA-IMDM and placed in the upper compartment of the blind well chamber. In addition, HEL and $CD34^-$ cells were mixed together resulting in 90% HEL and 10% $CD34^-$ cells or 70% HEL and 30% $CD34^-$ cells in a total of 30 x 10^4 cells and placed in the upper compartment. Incubations were carried out for 2 hours in 5% CO_2 at 37°C. As shown in Table 8, $CD34^-$ cells were found to be 3.5-fold more migratory than $CD34^+$ cells. Since the purity of $CD34^+$ cells was 90%, it was tested whether the migration of these cells was due to contamination by $CD34^-$ cells. A differential count of $CD34^-$ cells done using Wright's stain indicated a mixed population of cells including

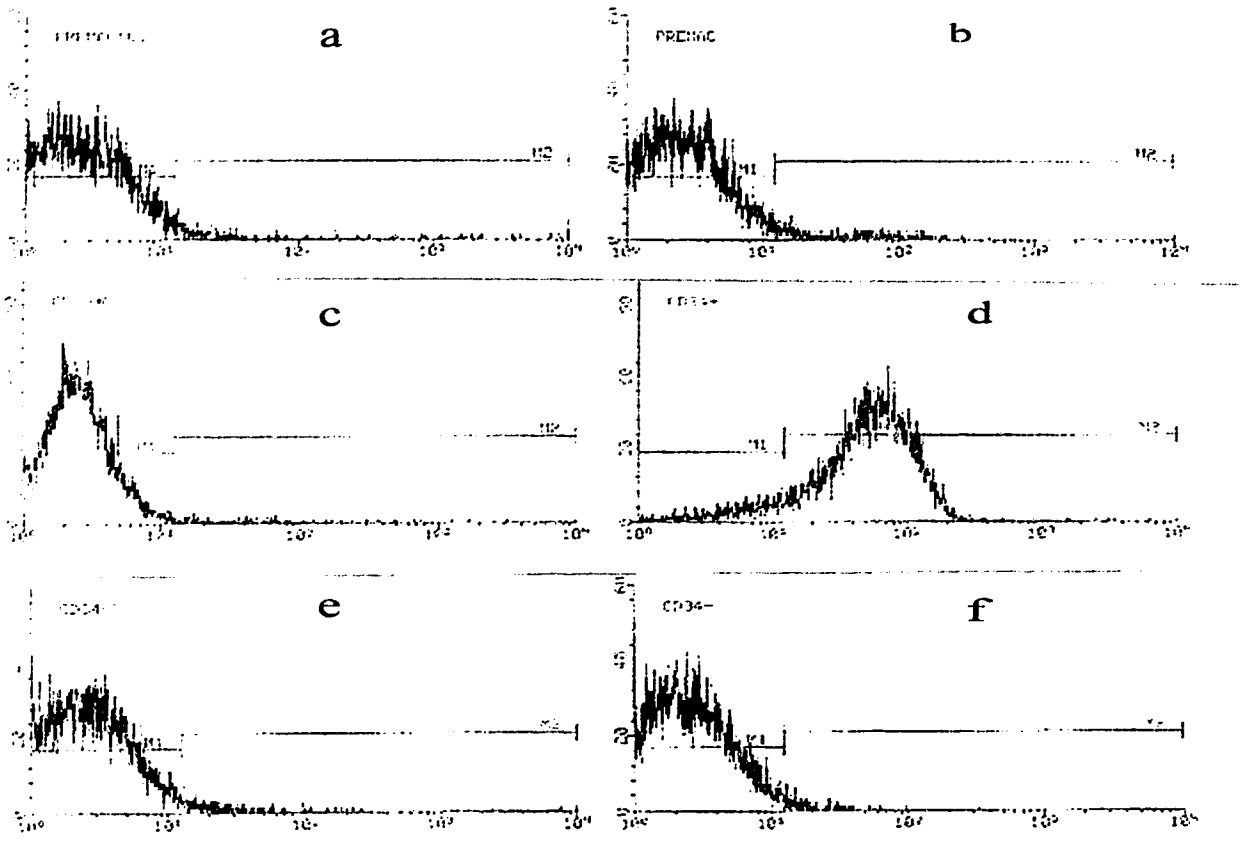


Figure 18. *Florescence flow cytometric analysis of CD34$^+$ cells.*

Briefly, 10 µL of normal mouse serum was added to 5 x 10^5 cells in a U-bottom 96 well plate and incubation was carried out for 60 min at 4°C. Next, 40 µL of CD34 anti-HPCA-2 (FITC linked) antibody was added and incubated for 60 min at 4°C. A mouse IgG1 negative control FITC conjugate was used as control. Cells were then washed twice with immunoflourescence buffer and resuspended in 0.4 mL of 1% formalin and placed in FACS tubes. Flow cytometric measurement of samples were performed using a Facscan flow cytometer.

a = PreMAC control (light density bone marrow cells prior to MACS separation)
b = PreMAC (cells labeled with CD34 anti-HPCA-2 antibody)
c = CD34$^+$ control (cells eluted at the end of the separation procedure; see appendix 1)
d = CD34$^+$ cells (labeled with CD34 anti-HPCA-2 antibody)
e = CD34$^-$ control (cells collected at step 4; see appendix 1)
f = CD34$^-$ cells (labeled with CD34 anti-HPCA-2 antibody)

CELLS	% INVASIVENESS*
$CD34^+$	2.3 ± 0.6
$CD34^-$	8.0 ± 1.8
HEL	0.2 ± 0.2
HEL (70%) + $CD34^-$ (30%)	4.0 ± 0.5
HEL (90%) + $CD34^-$ (10%)	2.2 ± 0.6

Table 8. *In-vitro migration of $CD34^+$ and $CD34^-$ cells.*

Briefly, $CD34^+$ and $CD34^-$ cells separated by MACS system were used. HEL cells, cultured overnight in serum-free IMDM, were washed twice with IMDM before being used in the assay. $CD34^+$, $CD34^-$ and HEL cells (30×10^4) were suspended in 0.1% BSA-IMDM and placed on the Matrigel-coated filters (batch 3; 25 µg) in the upper compartment of the blind well chamber. In addition, HEL and $CD34^-$ cells were mixed together in 0.1% BSA resulting in 90% HEL and 10% $CD34^-$ cells or 70% HEL and 30% $CD34^-$ cells and 30×10^4 cells were placed in the upper compartment (batch and amount of Matrigel on the filter same as above). Incubations were carried out for 2 hrs in 5% CO_2 at 37°C. After 2 hrs, cells were recovered from the lower compartment and counted in a hemocytometer. Percentage invasion was calculated as the ratio of the number of cells recovered from the lower compartment to the total number of cells placed in the upper compartment of blind wells. Mean ± SD values of percentage invasiveness shown in the table were calculated from 12 chambers.

* Significant difference ($p < 0.05$) among all values of percentage invasion except for percent invasiveness of $CD34^+$ cells (2.3 ± 0.6) and HEL (90%) + $CD34^+$ cells (2.2 ± 0.6).

myelocytes (22%), metamyelocytes (2%), myeloblasts (13%), lymphocytes (52%), monoblasts (8%) and erythroblasts (3%). As shown in Table 8, there was no significant difference between the migration of $CD34^+$ cells and non-invasive HEL cells mixed with 10% of $CD34^-$ cells. In fact, the percentage migration of HEL cells increased further when the number of $CD34^-$ cells was increased from 10% to 30% (Table 8). Therefore, it seems that the $CD34^+$ population of cells has either a very low migratory activity through the Matrigel or none at all.

4.6.3- *Production of type IV collagenases/gelatinases by $CD34^+$ cells.* The cell conditioned media of $CD34^+$ cells were tested for the presence of 92- and 72- kDa gelatinases. Briefly, after separation of cells on MACS, 5×10^5 $CD34^-$ cells and 5×10^4 of both $CD34^+$ and $CD34^-$ cells were cultured in 0.5 mL of serum-free IMDM for 2 hours. Following incubation, the cell-free supernatant was used directly in zymography. As shown in Figure 19, these gelatinases were not detected in the conditioned media of $CD34^+$ cells and only 92-kDa gelatinase was found in the conditioned media collected from 5×10^4 and 5×10^5 $CD34^-$ cells (Figure 19). The conditioned media of $CD34^+$ cells did not show the presence of any gelatinases even when the incubation time of the zymograms was extended from 24 hours to 48 hours. However, unlike the conditioned media of $CD34^+$ cells, the conditioned media of peripheral blood samples from AML patients nos.2 and 3 collected under conditions similar to that of $CD34^+$ cells (i.e., similar cell concentrations and times of incubation) showed 92-kDa gelatinase activity on the

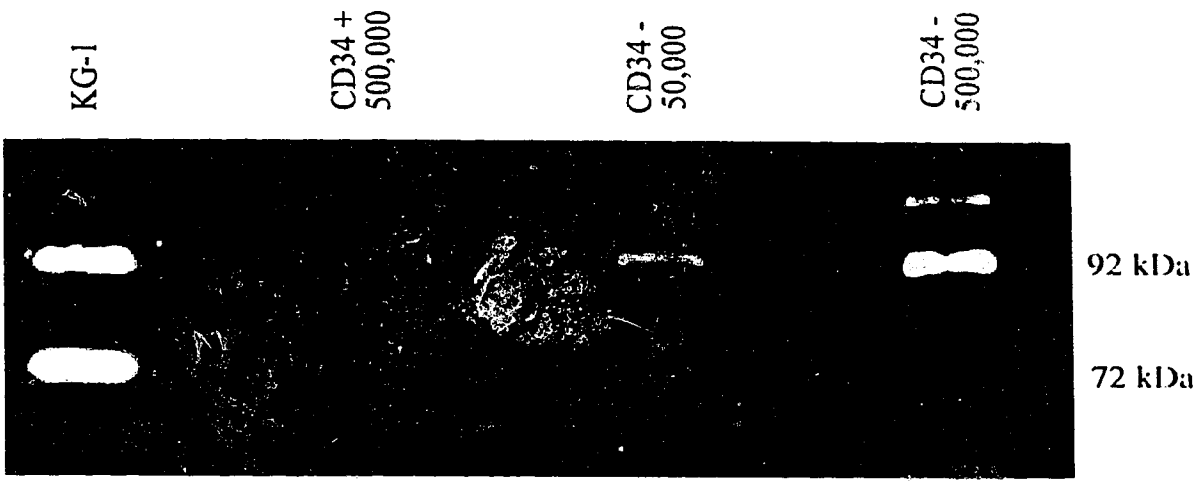


Figure 19. *Secreted gelatinases of KG-1, CD34+ and CD34- cells.*

$CD34^+$ and $CD34^-$ cells, separated by MACS, and KG-1 cells were cultured in serum-free IMDM ($CD34^+$; 5 x 10^5 cells/0.5 mL, $CD34^-$; 5 x 10^4 cells/0.5 mL and 5x10^5 cells/0.5 mL and KG-1;1 x 10^6 cells/mL). Conditioned media were collected after 2 hrs ($CD34^+$ and $CD34^-$) or 24 hrs (KG-1), concentrated 20x (KG-1 only) and applied (15 µL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 48 hrs.

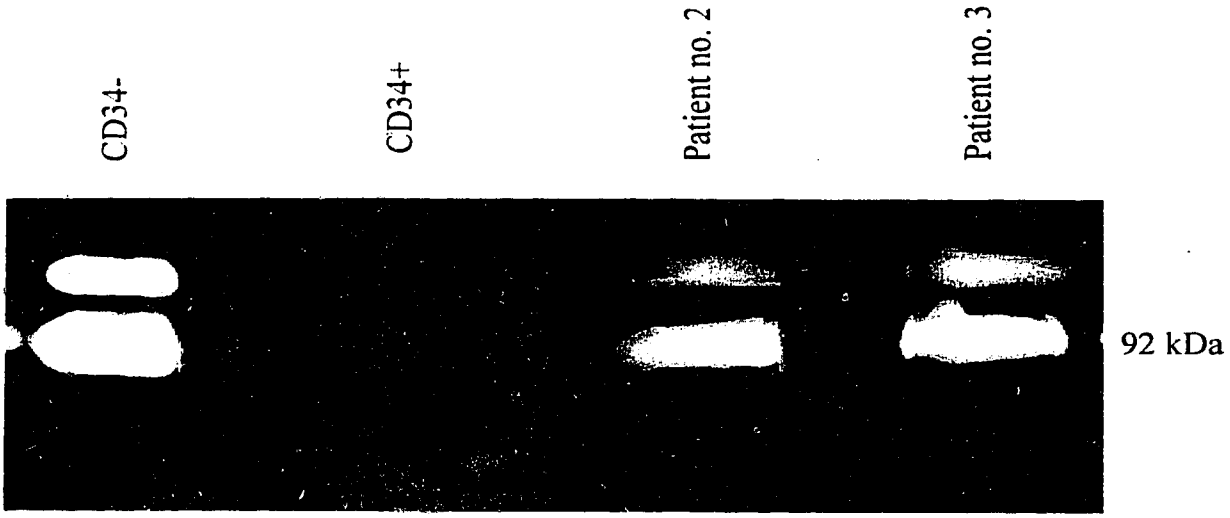


Figure 20. *Secreted gelatinases of $CD34^-$, $CD34^+$ and cells from AML patients nos. 2 and 3.*

Cells were cultured in serum-free IMDM ($CD34^-$; 1 x 10^6 cells/0.5 mL, $CD34^+$; 5 x 10^5 cells/0.5 mL, PB cells from patients nos. 2 and 3; 5 x 10^5 cells/0.5 mL). Conditioned media were collected after 2 hrs and applied (15 μL) on gelatin embedded gel. After electrophoresis and washing, the gel was incubated in zymography buffer at 37°C for 48 hrs.

zymograms (Figure 20). This shows that leukemic blasts, in contrast to their normal counterparts, have the ability to secrete 92-kDa gelatinase.

5.0 SUMMARY AND DISCUSSION

The aim of the first part of the study was to develop an *in-vitro* invasion assay that allows malignant hematopoietic cells to be evaluated for their invasive potential. For this purpose I modified and standardized an experimental system (6) previously described for metastatic tumor cells (using the reconstituted basement membrane Matrigel) to quantitate migration of human leukemic cells. I demonstrated that the Matrigel-based assay is suitable for evaluation of the invasive behavior of human leukemic cells and defined the optimal conditions under which the mechanisms by which normal and malignant hemopoietic cells cross the basement membrane could be studied. These modifications included incubation time of the assay, effect of chemoattractant on invasion, effect of Matrigel concentration on invasiveness, invasion rate as a function of initial cell density, and invasiveness of cells with different batches of Matrigel. The results of these modifications and standardizations are discussed below in detail and have been published (134).

The cultured leukemic cell lines KG-1, K-562, but not the fibrosarcoma cell line HT-1080 were able to cross the Matrigel layer within 2 to 4 hours; prolonging the incubation period above 6 hours did not result in a significant increase in the number of invasive cells (Fig. 2). A decrease in the percentage invasiveness of KG-1 and K-562 cells observed after 4 hours of incubation could be due to the secretion of TIMPs by these cells. Expression of TIMPs by these cell lines has already been detected on mRNA level in preliminary experiments carried out in Dr. Janowska's laboratory. The balance between levels of

MMPs (which could be involved in the process of invasion by KG-1 and K-562 cells) and free TIMPs determines the net MMP activity and hence an alteration to this equilibrium affects the process of cellular invasion. Most transmigration assays with solid tumor cells show that the latter need at least 5 to 72 hours of incubation to cross matrix barriers (107-111). The short incubation time requirement for the assay eliminated possible differences in invasion rates due to cell proliferation during the assay. Cells from the adherent fibrosarcoma line HT-1080, however, in accord with previous findings (107,111), were unable to cross the Matrigel layer in less than 5 to 6 hours and a substantial number of cells were detected on the lower surface of the filter only after 20 hours of incubation. Since the leukemic cells did not adhere to the bottom side of the filters, all the invasive leukemic cells could be recovered from the lower compartment of the blind well chamber and staining the filters followed by tedious counting of the cells on the lower surface was not necessary.

The presence of a chemoattractant, FMLP, in the lower compartment of the blind well chamber had no effect on the *in-vitro* invasive potential of leukemic cells (Table 1). These findings are in accord with other studies which show the ineffectiveness of the chemoattractants prostaglandin E_2, leukotriene B_4 and leukotriene D_4 in inducing migration of unstimulated murine M1 leukemic cells through Matrigel (75). Similarly, in another report, a negligible effect of FMLP on the invasiveness of differentiated U-937 cells is shown (12). Hence these results indicated that leukemic cells differ in their chemotactic responsiveness from solid tumor cells, most of which move through the basement membrane matrices in substantial numbers only toward gradients of potent

chemotactic factors (6,111,112). In this respect, it is interesting that highly metastatic B16F10 melanoma cells readily penetrate the Matrigel barrier regardless of whether chemoattractant is included in the medium in the lower compartment of the Boyden chamber (111). In contrast, neutrophils are attracted to inflammatory sites and/or sites of infection through the production of chemoattractant mediators at these sites. These mediators, such as N-formylated peptides, the fifth component of complement C5a, leukotriene B_4 and IL-8, bind to G-protein-coupled cell surface receptors on leukocytes and as a result of chemoattractant receptor activation, neutrophils are stimulated to move towards the site of inflammation and/or infection (113). In this study, in agreement with previous findings (114), normal polymorphonuclear leukocytes were found to be responsive to the presence of chemoattractant and had higher percentage invasiveness when FMLP was used in the lower compartment of the blind well chamber in the *in-vitro* assay (Table 1). Since most of the cultured leukemic cells tested were able to cross the Matrigel layer in the absence of chemoattractant, it was not used in the assay. The assay thus measured the level of spontaneous rather than induced invasiveness *in vitro*.

The relationship between the amount of Matrigel coated on the filter separating the two compartments and the number of cells recovered from the lower compartment of the Boyden chamber was found to be directly proportional (Table 2), in agreement with earlier studies of tumor cells (111,115,116). Since the number of cells that crossed the filters covered with the lowest concentration of Matrigel (12.5 μg/filter) was only about half the number that passively migrated through the uncoated filters, the reconstituted basement

membrane preparation evidently formed an efficient barrier that had to be actively penetrated by leukemic cells.

During the standardization of the *in-vitro* invasion assay it was found that the number of cells placed in the upper compartment of the blind well chamber greatly affected the rate of invasion of leukemic cells. Most consistent results were obtained when the number of cells placed in the upper compartment ranged from 25 x 10^4 to 40 x 10^4 (Fig. 3). These results are in accord with other reports indicating that high cell density is required for manifestation of the invasive potential of cells (115,116) and that there is a saturation cell density beyond which no further increase in invasiveness can be observed.

This study showed that the level of *in-vitro* invasiveness of human leukemic cells also depended on the batch of Matrigel used in the assay (Fig. 4). However, KG-1 cells, the least differentiated cultured leukemic cells tested, were consistently more invasive than HEL, HL-60 and U-937 cells irrespective of Matrigel batch. The level of invasiveness of all leukemic cells was substantially lower when batch 2 of the Matrigel preparation was used rather than batch 1 (Fig. 4). Since various lots of Matrigel may differ in their protein contents and composition, it is not surprising that the results of the Matrigel-based assay depended on the batch, a finding also reported by others (116,117).

Hence, this *in-vitro* invasion assay provided a useful model for analyzing the interactions of normal and malignant hematopoietic cells with extracellular matrix barriers including adhesion to the components of basement membranes and/or the activity of matrix degrading enzymes produced by migratory cells. Additionally, it allowed isolation and further characterization of the invasive hemopoietic cell populations.

In the next part of my study , the KG-1 cell line was selected as the model for invasive cells in order to study the secretion of type IV collagenases/gelatinases. In addition, primary leukemic cells from patients diagnosed with AML were also tested. To assess whether myeloblastic KG-1 cells and AML blasts from patients secrete 92 and 72 kDa gelatinases, I used an experimental system (already established in Dr. Janowska's laboratory) based on substrate degradation by its specific enzyme.

Both 92 and 72 kDa gelatinases were found in the conditioned media of KG-1 cells (Fig. 5). Once it was established that KG-1 cells produce 92 and 72 kDa gelatinases I confirmed that these gelatinases were type IV collagenases (MMP-2 and MMP-9) by using 1,10-phenanthroline, a specific inhibitor of metalloproteinases, in the zymography assay (Fig. 7) and by Western blotting using rabbit anti-human polyclonal antibodies against 92 and 72 kDa type IV collagenases (Fig. 8). In the Western blots, media from HT-1080 fibrosarcoma cells were used as positive controls as these fibrosarcoma cells have been shown to produce type IV collagenases/gelatinases (118). In addition to 72 kDa gelatinase, an additional protein was detected in the secreted fraction of HT-1080 by the polyclonal anti-MMP-2 in Western blot analysis (Fig. 8A). There is a possibility that this antibody might be detecting both free MMP-2 and TIMP-MMP-2 complex. However, the possibility of unspecific cross-reactivity cannot be ruled out.

Watanabe, using U-937 promonocytic cells, has shown that MMP-9 can be isolated from the conditioned media of these cells. However, this gelatinase was not detectable in untreated conditioned media and was present only after inducing differentiation of U-937 cells to macrophages with TPA and MMP-9 production was further enhanced by treating

these cells with IL-1 or TNF-α (12). Similarly, Nagase treated U-937 with phorbol 12-myristate 13-acetate (PMA), which induces differentiation along the monocytic pathway, to isolate MMP-9 (118). In this study myeloblastic KG-1 cells were found to secrete both 92 kDa and 72 kDa type IV collagenases without any stimulation or induction of differentiation (Fig. 5). However, the possibility of induction of these gelatinases in KG-1 cells by cytokines during incubation period of 10-24 hrs cannot be ruled out. In a recent report Petrides has shown that the basal MMP-9 release in the promyelocytic cell line HL-60 is to a large extent regulated by constitutively secreted TNF-α which acts in an autocrine manner for the maintenance of 92 kDa gelatinase release (79). Although TNF-α was undetectable due to its low levels, this MMP-9 activity in culture fluids of unstimulated HL-60 cells was reduced up to 35% by moABs against TNF-α (79). In the same report, up-regulating the levels of TNF-α, either by activators of TNF-α or by induction of monocytic differentiation in HL-60 with TPA, significantly increased the 92 kDa gelatinase activity. In addition, blast cells from patients with AML produce and secrete TNF-α and its levels in the serum of patients with AML were found to be significantly increased (119,79). Similarly, TGF-β has been shown to up-regulate both 92 kDa and 72 kDa gelatinase activities in cultured fibroblasts (61). Therefore, KG-1 cells could be using a similar autocrine mechanism for the induction of production of type IV collagenases by TNF-α, as HL-60 cells do, or by other cytokines such as IL-1, G-CSF or GM-CSF which are produced by leukemic cells. This hypothesis could be tested by investigating the effect of different inhibitors and antibodies of cytokines on the production of type IV collagenases by KG-1 cells.

In contrast to my findings with KG-1 cells, in U-937 and HL-60 cells the 72 kDa type IV collagenase could not be detected even after induction of differentiation and/or treatment of these cells with factors such as TNF-α and IL-1α (12,79). Therefore I conclude that the most likely reason why KG-1 cells were more invasive in the Matrigel-based assay compared with U-937 and HL-60 cells is their ability to i) secrete both 92 and 72 kDa type IV collagenases/gelatinases; and ii) secrete both type IV collagenases without any paracrine stimulation or induction of differentiation.

In the conditioned media of KG-1 cells type IV collagenases/gelatinases were detectable only after 10-15 hours of incubation and visible bands of greater intensity appeared after approx. 24 hours of incubation (Fig. 5). This observation is in accord with other studies in which 92 kDa gelatinase from U-937 cells was isolated after culturing them for 3 days in media containing agents such as IL-1α and PDGF in the presence and absence of TPA (12). Similarly, 92 kDa gelatinase in HL-60 was detectable after 6 hours of incubation with TPA and TNF-α and maximal activity was achieved after 72 hours of incubation (79). Additionally, Petrides has also shown that TNF-α-evoked secretion of 92 kDa gelatinase was completely abolished by inhibiting protein synthesis at the level of transcription with actinomycin D or translation with cycloheximide and he suggests that TNF-α stimulates HL-60 cells to produce gelatinase by de novo synthesis requiring continuous synthesis of RNA and protein rather than release of presynthesized granules (79). Because in this study KG-1 cells were cultured in serum free media without any differentiating or stimulatory factors to collect conditioned media for zymography, it might have taken longer for the basal secretion of type IV gelatinases.

In the Matrigel-based assay, however, I found KG-1 cells to be invasive within 2 hours of incubation and type IV collagenases were detectable in the media collected after 2 hours from the upper compartment (Fig. 5). This may have been due to the interaction of KG-1 cells with the components of Matrigel enhancing the production of type IV collagenases. One such component of Matrigel is laminin. Liotta has shown that the type IV collagenolytic activity of human melanoma cells A2058 increased 200-300% after addition of laminin to their conditioned media and this induction of type IV collagenases was more pronounced (580%) when a fragment of laminin which binds to the cell surface laminin receptor was used (120). Similarly, Bracke et al. suggested that laminin could facilitate ECM breakdown by binding plasminogen, tissue type plasminogen activator and complement factors thereby "concentrating" proteolytic activities, and they have shown that invasion of precultured embryonic chick heart fragments by MO_4 cells (Kirsten Murine Sarcoma virus transformants of a continuous fetal C_3H mouse cell line) can be inhibited by using a flavonoid [(+)-catechin] which binds to laminin and not to fibronectin or type IV collagen (121). It could be interesting therefore to look at the effect of Matrigel components on the production of type IV collagenases by adding them to the cell cultures of KG-1 cells.

I confirmed in zymography studies the production of 92 and 72 kDa gelatinases by polymorphonuclear leukocytes (PMNL). Murphy and Duran have shown previously that PMNL produce these enzymes and possibly use them for penetration through ECM during their migration through different tissue compartments in the body (71,69). These gelatinases, however, unlike those of KG-1 cells, were detectable within 2 hours in PMNL

conditioned media and in greater amounts (Fig. 6). This could be due to the fact that PMNLs are fully mature and differentiated cells and reports have shown that production of type IV collagenase/gelatinases is induced by differentiating immature cells to highly differentiated mature forms (12). In addition, Borregaard has shown that there are special granules in granulocytes that contain only gelatinases and the concentration is much higher than in the granules that contain both gelatinases and lactoferrin (70). He also shows that stimulation of neutrophils results in a 15-25% release of total cell gelatinase and even after that distinct gelatinases are found in the neutrophils as evidenced by subcellular fractionation (70).

Studies of MMP expression in tumors showed that these proteases are an important component of the invasive phenotype of many malignant tumors including breast, prostate, colon, lung, ovarian and thyroid cancers (122). For example, Liotta and Garbisa have examined several murine tumor cell lines and have shown that the cell line with the highest incidence of *in vivo* metastasis exhibited the greatest level of type IV collagen degrading activity (123). The present study with KG-1 and primary leukemic cells also suggests the role of type IV collagenases/gelatinases in the invasive property of leukemic cells (Fig. 9, Table 3 and 4), since the *in vitro* invasiveness of KG-1 cells was reduced considerably by inhibitors of MMPs. This reduction in percentage of invasiveness was detected after incubation with both synthetic (1,10-phenanthroline; up to 75%) and natural (rh-TIMP-2; up to 79.5%) inhibitors of MMPs (Table 3) and incubation with the monoclonal antibody against 72 kDa type IV gelatinase (Table 4). These results are in accord with other studies which have similarly shown that the invasive and metastatic ability of tumor cells can be

modified by the inhibition of type IV collagenases/gelatinases (87,92,130). For example, with 1,10-phenanthroline, Mignatti totally blocked the invasion of B16 tumor cells into the amnion (92). Watanabe has shown that the invasiveness of promonocytic U-937 cells was significantly inhibited by TIMP-1 and EDTA, both inhibitors of MMPs. The U-937 cells in these experiments, however, were seeded onto the Matrigel after 48 hours treatment with TPA (79). Other studies indicate an inverse correlation between TIMP expression and efficiency of tumor cell invasion and metastasis suggesting a major role for TIMPs in regulating the degradation of ECM by MMPs (88,90). Khokha genetically modified Swiss 3T3 cells to synthesize an RNA (antisense RNA) complementary to the mRNA encoding TIMP, which induced a reduction in TIMP levels and caused the previously noninvasive cells to become invasive when assessed by amnion invasion assay (89). Based on this knowledge and the results obtained in the present investigation, we can infer that TIMPs play an important inhibitory role in regulating the migration of leukemic cells. However, TIMPs have been found to have a growth promoting activity known as 'erythroid potentiating activity' (EPA) (135,136) and Avalos showed that a protein identical to TIMP-1 enhanced colony formation by the erythroleukemia cell line K-562 by an autocrine interaction with a cell surface receptor (135). This EPA role of TIMPs needs to be further evaluated in leukemia.

Moreover, the percentage of inhibition of *in vitro* invasion with anti 72- monoclonal antibody (51.6%) was lower than that achieved by 1,10-phenanthroline (up to 75%) (Table 3 and 4). It may be because 1,10-phenanthroline inhibits all species of MMPs and in my studies I have not investigated the effect of specific inhibitors of other MMPs (such as

stromelysins) beside MMP-2. I have also not tested the effect of anti 92- antibody (because of its unavailability) on the *in vitro* invasion of KG-1 cells. It will be interesting to investigate whether a combination of anti 92- and 72- antibodies results in a higher percentage inhibition of the invasiveness of KG-1 cells. Other MMPs such as stromelysins may also be involved in this process of invasion by KG-1 cells. For example, stromelysins (stromelysin-1, stromelysin-2 and PUMP-1) degrade type IV collagen and probably other collagen types which have interrupted triple helices and can degrade the non-collagenous components of ECM such as fibronectin and different proteoglycans (62,124). Hence, testing different substrates (besides gelatin) with zymography could provide more information about the production of other MMPs by leukemic cells and their role in *in vitro* invasion. Interestingly, inhibitors of serine proteinases were able to inhibit the invasiveness of KG-1 cells up to 95% showing the involvement of these enzymes in the process of *in-vitro* invasion (Fig. 9 and Table 3). Such an inhibitory effect of the serine proteinase inhibitor, benzamidine, on the invasion and chemotaxis of human ovarian carcinoma line, OVCAR-3 has been shown in the Matrigel-based assay (98). Serine proteinases, however, may not be detected in gelatin zymograms because gelatin is not the natural substrate for these enzymes. Because serine proteinases have been implicated in the activation of enzymes including MMPs (14), the higher percentage of inhibition in the Matrigel-based assay achieved with serine proteinase inhibitors than inhibitors of MMPs may be due to the inhibition of degradation of Matrigel components other than type IV collagen, and inhibition of the activation of MMPs.

The next part of the study was to test the *in-vitro* invasiveness and type IV collagenase production by leukemic blasts from patients diagnosed with AML. I found that the leukemic cells from 10 patients with AML of different FAB classifications had *in-vitro* invasive potentials ranging from 1.4% to 11.5% (Table 6). Except for the peripheral blood of AML patients no. 2 (M2) and 3 (M4) and the bone marrow of AML patients no. 4 (M2), 5 (M1) and 9 (M1), the invasiveness of AML cells was not significantly different from KG-1 cells. Primary AML cells were also found to produce 92 and 72 kDa gelatinases (Fig. 10, 13-17) except for cells from the peripheral blood of AML patient no. 5 (M1) (Fig. 14) and AML patient no. 6 (M5) (Fig. 15) which did not produce 92 kDa and 72 kDa gelatinases respectively. The gelatinolytic activities of approximately 82 kDa and 62 kDa for KG-1 (Fig. 13) and 62 kDa for patients nos. 3, 4, 5, 7, 8 and 9 (Figs. 13, 14, 16 and 17) were also detected in the zymograms. These activities probably represent the activated forms of 92 and 72 kDa gelatinases respectively. These results indicate that primary leukemic cells possess the capability of converting the pro-gelatinases to their active forms since activation of the proenzyme form is required for initiation of matrix degradation and acquisition of the invasive phenotype (122). The activated form of 92 kDa was, however, undetectable compared with the activated form of 72 kDa in the conditioned media of primary leukemic cells (Figs. 13, 14, 16 and 17). MMP-9 or 92 kDa gelatinase has been shown to be activated *in vivo* by plasmin (resulting from the action of u-PA on plasminogen). By contrast, 72 kDa gelatinase can be activated *in vitro* and *in vivo* by an autolytic process as shown by Stetler-Stevenson (68) or, as Sato showed, via interaction with MT-MMP (66, 67). Hence in AML, blast cells might be using the

plasminogen-plasmin system to activate pro-MMP-9 whereas MMP-2 might be activated by either of the two processes mentioned above. It is of interest to investigate whether primary leukemic cells express MT-MMP and whether MMP-2 can be isolated in a complex with MT-MMP from the membranes of leukemic cells; such studies are under way in Dr. Janowska's laboratory. The results also indicated that for both KG-1 and primary leukemic cells the secretion of the 72 kDa form was much more pronounced than its activated form of 62 kDa (Figs.12, 13, 16, 17). This is probably because a) the activation of progelatinases occurs in close association with the membrane (65, 67, 122), b) the presence of active forms on the membrane is transient before they are released from the membrane, this release is, however, followed by proteolytic degradation (14) and c) these cells might be producing TIMPs, which bind to activated MMPs and inhibit their activity (82, 84). The comparable activities of both pro and active forms of MMP-2 in conditioned media of PB cells from patients nos. 4 and 5 (Fig. 14) could be due to the presence of more than one mechanism of MMP-2 activation. In the three samples tested, i.e., from AML patient no.1 (M1;100% blasts), 2 (M2;96% blasts) and 3 (M4;100% blasts) (Figs. 12 and 13), expression of these gelatinases was found within 2 hours indicating that unlike in KG-1 cells the basal levels of type IV collagenases/gelatinases are higher in primary AML blast cells. No significant difference was found between the mean percentage invasiveness of the peripheral blood blasts from patients with FAB classification M1 (patients nos. 1, 5, 9 and 10), M2 (patients nos. 2 and 4) and M5 (patients nos. 6 and 8) (Table 6). In AML-M5 (monocytic leukemia) the monocytic blasts are more mature (large cells, usually moderately, occasionally strongly basophilic,

scattered fine azurophilic granules, nucleus round to convoluted with one or several nucleoli) as compared with M0 and M1 (lack granules, condensed chromatin and prominent nucleoli) and, in AML-M5 patients usually have gum and skin infiltrations. In this study, blast cells from two patients diagnosed with AML M5 did not secrete type IV collagenases/gelatinases at higher levels and M5 blasts were not more invasive *in vitro* than leukemic cells belonging to other FAB subtypes. However, the number of patients within each group was too small for significant comparison and more samples from each FAB subtype need to be studied. The results also indicate a difference (from 2 to 16.8) in the gelatinolytic activities of secreted 92 and 72 kDa gelatinases by blasts from the five AML patients tested (nos. 6 to 10) (Table 7) as examined by SigmaScan/Image measurement. No statistically significant correlation was seen between the percentage of *in vitro* invasiveness of these cells and the level of gelatinolytic activity detected (Table 7). A possible explanation for the lack of such correlations could be that, according to three-step theory of invasion and metastasis, proteolytic degradation is a major step in this complex process but is not the only one, the others being adhesion and migration (46,13). Therefore the role of adhesion molecules has to be investigated along with type IV collagenase production when making such correlations between secreted gelatinolytic activities and the *in vitro* invasive potential of leukemic cells. In addition, the number of samples in each FAB subtype was too small i.e., two M5 (patients nos. 6 and 8) and M1 (patients nos. 9 and 10) samples and one M0 (patient no.7) sample. Therefore more samples in each FAB subtype needs to be studied for such correlations. The analysis of intensity of gelatinolytic activities by Sigma Scan did not provide a very accurate

measurement of such activities because 92 kDa gelatinolytic activity is seen on zymograms with both peripheral blood and bone marrow cells from patient no. 9 and bone marrow cells of patient no.10 (Fig.17), but these activities are not calculable by Sigma Scan (Table 7).

Furthermore, in the *in vivo* dissemination of leukemic cells other factors such as physical disruption of the hematopoietic microenvironment as a result of leukemic cell proliferation, the release of soluble factors from leukemic cells or from infiltrating inflammatory cells and cell-cell contact between invading leukemic cells and surrounding marrow stromal fibroblasts might play an important role. In a recent report, Matrisian has demonstrated the expression of MMPs by stromal cells surrounding the tumor and their role in the progression of colorectal cancer (125). It has been demonstrated that the adherent stromal layers from some AML patients, established in LTMC at presentation prior to any therapy, are functionally abnormal (126). Therefore, the role of the leukemic stroma has to be carefully studied in conjunction with studies of leukemic blasts. Though we are far from understanding the mechanisms of *in vivo* leukemic dissemination, studies of primary AML cells implicate type IV collagenases in this process.

In the final step of my study I tested the *in-vitro* migration potential and secreted gelatinases of normal bone marrow $CD34^+$ cells. Several difficulties are associated with the separation of $CD34^+$ cells; for example, the CD34 molecule is expressed in low copy numbers in comparison with other cell surface markers and $CD34^+$ are rare cells in a heterogeneous cell background (78). In the MACS system of separation, cells are labelled with very small superparamagnetic microbeads (diameter approx. 60 nm) which show

extremely low non-specific binding combined with fast attachment to the target cells. This system provided us with a 90% pure population of marrow $CD34^+$ cells. These were found to be 2.3 ± 0.6% invasive in the *in-vitro* invasion assay and did not secrete 92 and 72 kDa type IV collagenases (Table 8, Fig. 19 and 20). The percentage invasiveness observed with $CD34^+$ cells was not significantly different when compared with that of noninvasive HEL cells, mixed with 10% $CD34^-$ cells, which were 2.2 ± 0.6% invasive (Table 8). This indicates that the percentage of invasiveness obtained with $CD34^+$ cells could be due to the contamination with $CD34^-$ cells. After Wright staining, $CD34^-$ cells were found to be a population of myeloblasts, myelocytes, metamyelocytes, lymphocytes, monoblasts and erythroblasts. However, because the number of $CD34^+$ cells that could be isolated from the bone marrow samples was low and we studied only two marrow samples, further studies need to be carried out to understand the migratory ability of $CD34^+$ cells.

In conclusion, in this thesis I have demonstrated that:

i) a Matrigel-based assay, which I standardized, can be used to study the invasive potential of leukemic cells;

ii) undifferentiated and unstimulated leukemic cell lines and primary leukemic cells from the peripheral blood and bone marrow of AML patients are invasive *in vitro*.

iii) myeloblastic KG-1 and primary leukemic cells from 10 patients diagnosed with AML produce type IV collagenases/gelatinases;

iv) type IV collagenases/gelatinases are involved in the *in vitro* invasiveness of the leukemic cells as inhibition of these enzymes by inhibitors, including 1,10-phenanthroline, TIMP-2 and anti 72 kDa antibody, causes inhibition of the invasiveness of these cells;

v) normal undifferentiated and immature hematopoietic cells, identified by the presence of the CD34 antigen, are most probably unable to migrate through the Matrigel and do not produce type IV collagenases.

These data support the hypothesis that leukemic cells, unlike normal hematopoietic cells in the very early stages of maturation and development, secrete type IV collagenases/ gelatinases, which enable them to cross basement membrane barriers. The present study, therefore, contributes to a better understanding of the dissemination of leukemic blasts in AML.

6.0 FUTURE DIRECTIONS

Although this study suggests a putative role for type IV collagenases in the dissemination of leukemic blasts in AML, the ability to cross Matrigel or basement membrane barriers cannot be attributed to the secretion of type IV collagenases alone. To gain a better understanding of how these blasts disseminate from the marrow and enter the peripheral blood, the effect of other factors thus has to be carefully evaluated:

i) Studies on the role of integrin mediated interactions between neoplastic cells and components of the extracellular matrix in the process of invasion and metastasis have provided interesting new perspectives in tumor biology. For example, the inhibition of *in vitro* invasiveness of murine melanoma cells by RGD peptide, which is the binding motif present in the ECM proteins and is recognized by most integrins, indicates the importance of integrins in invasion and metastasis (47). Additionally, adhesion of hemopoietic progenitors to the bone marrow microenvironment via integrins such as $\alpha 4\beta 1$ may be important for the development of these cells whereas detachment from the components of the basement membrane, by downregulation of integrins, may be an important step in the onset of the invasion cascade of leukemic cells (24). For example, Dr. Janowska's group has suggested that in some cases of AML the malignant phenotype of leukemic blasts may be associated with downregulated transcription of the $\alpha 4$ integrin subunit (131). The use of anti-integrin antibodies can provide a promising perspective on the role of integrins in leukemic dissemination.

ii) An important function carried out by the invasive tumor cells is the production of matrix degrading enzymes which enable them to cross the barriers of ECM and basement membrane by proteolytic degradation of the components of these barriers (14,15). Since this study has shown the relationship between the secretion of type IV collagenases and the *in vitro* invasiveness of myeloblastic KG-1 and primary leukemic cells from AML patients and also the inhibition of *in vitro* invasiveness of KG-1 cells by specific inhibitors on serine proteinases, it would be important to investigate the production of other proteases such as serine, cysteine and aspartic and metalloproteinases other than type IV gelatinases by leukemic blasts and their role *in vitro* invasion. Such studies with leukemic cells will need to be carried out in conjunction with normal hematopoietic cells at the same stages of differentiation and maturation.

iii) The recent report by Petrides showing regulation of MMP-9 in promyelocytic HL-60 cells by TNF-α in an autocrine manner (79) indicates how the leukemic cells might be using different cytokines to their advantage. It would be important to look at the effects of various cytokines on the production of matrix degrading enzymes and *in vitro* invasion. This could be achieved by administration of cytokines or anti-cytokine antibodies to the leukemic cultures and evaluating whether this has an augmenting or inhibiting effect on the production of matrix degrading enzymes. Such studies may provide a better insight into mechanisms that govern regulation of abnormal hemopoiesis and dissemination of leukemic blasts in AML.

iv) Altering the ratio between MMPs and TIMPs in favor of the inhibitor has been shown previously to inhibit tumor cell invasion and metastasis (87-90). However, the exogenous

addition of TIMPs is just the first step in gathering the body of experimental proof needed to indicate that the molar balance between proteases and protease inhibitors dictates the invasive and metastatic phenotypes of tumor cells. In addition, inhibition of invasion and metastasis by TIMPs can identify the class of enzyme involved in dissemination, but it cannot specifically identify the individual metalloproteinase. Therefore it will be necessary to alter the expression of a single MMP and test for phenotypic change. Likewise, the potential use of TIMPs for therapeutic treatment to reduce metastasis in patients has to be approached with caution and has first to be evaluated for its potential effects on normal MMP functions.

v) Recently, membrane associated MMPs are under extensive study to determine their role in invasion and metastasis. For example, Sato has identified a membrane-type metalloproteinase (MT-MMP) which is present only on membranes and is not secreted into the media. He found that expression of MT-MMP in human fibrosarcoma HT-1080 and mouse fibroblast NIH 3T3 cells increased the number of invasive cells more than two-fold compared with control cells and that the invasion was in turn sensitive to TIMP (66). The role of membrane associated MMPs in invasion by leukemic cells thus needs to be studied; such studies are under way in Dr. Janowska's laboratory.

vi) Other hemopoietic cells may also influence the process of invasion. For example, a recent report indicates that platelets augment the basal level of 92 kDa gelatinase production by mammary tumor cells thereby increasing the latter's invasiveness through the ECM (127). This indicates the importance of cell-cell interaction in the process of

invasion and metastasis. Similar studies with leukemic cells could therefore reveal important information about the dissemination of leukemic cells.

vii) A recent report shows that in addition to collagens type I, III, IV and V, which are strongly expressed in bone marrow, collagen type VI proved to be the strongest adhesive collagen component for the hematopoietic cells and cell lines such as HL-60 and U-937 (32). Hence leukemic stroma needs to be studied in comparison with normal stroma to obtain a better understanding of the role of different stromal proteins in leukemic dissemination.

viii) It will be interesting to investigate the potential utility of serum levels of MMPs as a marker of leukemic dissemination and organ infiltration in AML. For example, in sera from lung and breast cancer patients, significantly elevated levels of MMP-2 were found compared with normal sera and a significant difference was found between enzyme levels in the presence versus the absence of distant metastases (128,129).

ix) Although the possibility of using MMP antagonists as tools for novel therapeutic approaches may exist, their use for cancer therapy poses two major problems. First, because no tumor-specific proteinase has been found, any proteinase antagonist will interfere with several physiological functions. Second, the enzyme involved in tumor invasion may be inaccessible to large biological molecules such as antibodies. This means that low molecular weight inhibitors have to be engineered which can be delivered efficiently to tumor sites. Based on the complexity and heterogeneity of the mechanisms that determine tumor invasion and metastasis, a more detailed understanding of

proteinases, receptors, activators and inhibitors will be required before we can exploit our knowledge of such antagonists for clinical purposes in leukemia.

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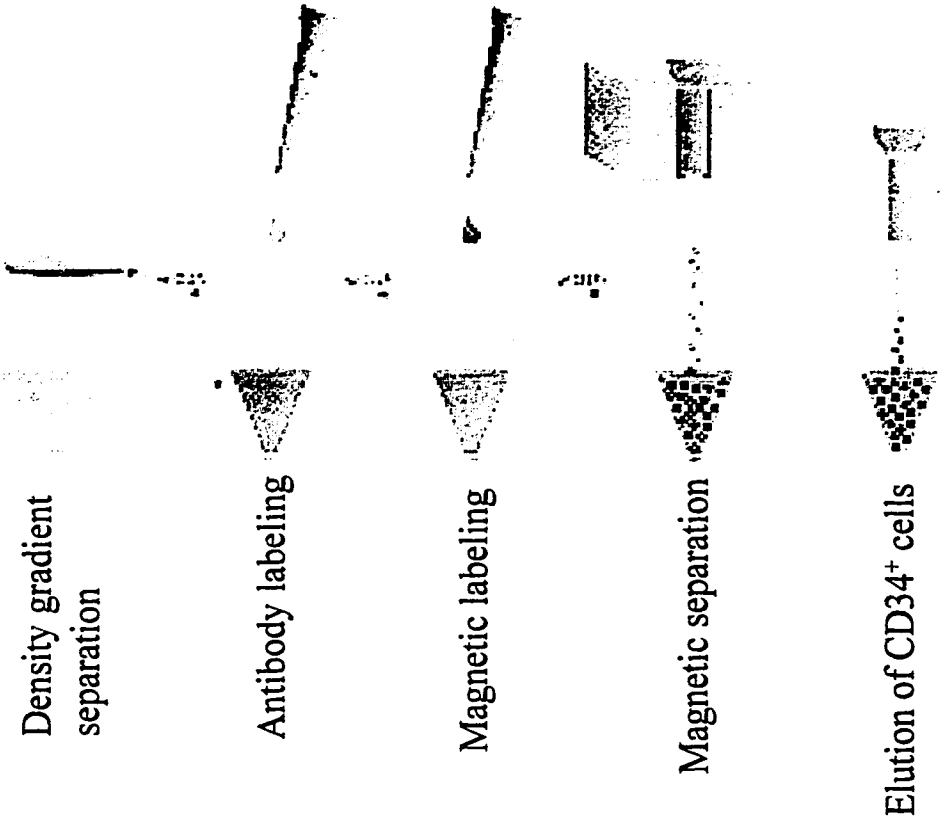
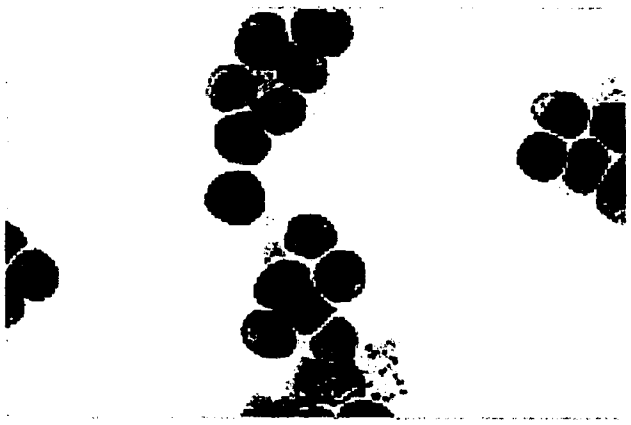
Appendix 1

The schematic presentation of the steps involved in isolation of CD34$^+$ cells from the bone marrow using MiniMACS system and the slide of Giemsa stained CD34$^+$ cells, shown on the following page, are taken from the 'Instruments and Reagents for Magnetic Cell Sorting' catalogue (1995); distributed by 'CytoBio Technics Inc. Oakville, ON.

Appendix 1. *Schematic presentation of isolation of CD34 cells from normal bone marrow using MiniMACS and CD34 kit.*





CD34+ cells isolated from human bone marrow using MiniMACS and CD34 Isolation Kit.
Giemsa stained.