

DIFFERENTIATION-LINKED SECRETION OF UROKINASE AND TISSUE PLASMINOGEN ACTIVATOR BY NORMAL HUMAN HEMOPOIETIC CELLS

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Leukemic cells from patients with acute myeloid leukemia (AML)¹ and chronic myeloid leukemia (CML) secrete plasminogen activators either of the urokinase (uPA) or the tissue (tPA) type, and in a recent study (1) it has been shown that the species of enzyme released by AML cells predicted the response to chemotherapy. Patients whose cells secreted only tPA failed to respond to combination chemotherapy, whereas those whose cells released uPA alone or a combination of uPA and tPA could be induced to achieve remission.

A number of other functional properties of myeloid leukemic clonogenic cells have also been shown to correlate with the response of the individual to induction chemotherapy (2–4). Generally speaking, these properties are those that characterize normal hemopoietic cells at various stages of differentiation and maturation. Poor responses are usually seen when leukemic cells display features of the early progenitor phenotype (3, 5–7). It seemed likely, therefore, that secretion of the two species of plasminogen activator by hemopoietic cells might also be differentiation linked and that the association between uPA secretion and favorable therapeutic outcome reflected the tendency of early cells to release tPA, whereas later cells secrete uPA.

In this paper we record the results of a series of experiments in which normal human bone marrow was fractionated by equilibrium density gradient centrifugation (8) into cells at different stages of differentiation (7, 9, 10). Correlation of cell density, growth characteristics in semisolid agar, and plasminogen activator release supports the notion that the species of plasminogen activator synthesized is a function of differentiation/maturation.

Materials and Methods

Bone Marrow Cells. Bone marrow aspirates from the posterior iliac crest were obtained from healthy donors with informed consent and the approval of the ethical practices

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¹*Abbreviations used in this paper:* AML, acute myeloid leukemia; CFUgemm, granulocyte/erythrocyte/megakaryocyte/macrophage CFUs; CFUgm, granulocyte/macrophage CFUs; CML, chronic myeloid leukemia; NSE, nonspecific esterase; PHA-LCM, PHA-stimulated lymphocyte-conditioned medium; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

committee of the Royal Free Hospital. Leucocyte-rich supernatants were obtained by sedimentation at unit gravity as previously described (6).

Cell Separation by Equilibrium Density Centrifugation. Cells were separated on the basis of differences in buoyant density in continuous Ficoll/Isopaque gradients. The method is essentially that of Loos and Roos (8), in which high- and low-density solutions consisting of different concentrations of Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and Isopaque (Nyegaard and Co., Lidingo, Sweden) in Tris-HCl and Ringer's solution are used to generate the gradients. The heavy and light solutions used for these experiments had osmolalities of 285 ± 3 mmol/kg and were pH 7.4. The density range used was ~ 1.04 to 1.09 g/cm³. Density gradients of 15 ± 0.5 ml were generated by continuous mixing of low- and high-density solutions in a gradient maker. This was dispensed into 14-mm diameter polycarbonate tubes (MSE Scientific Instruments, Crawley, Sussex, United Kingdom) through a $0.22\text{-}\mu\text{m}$ acrodisc filter. Cells from bone marrow leucocyte-rich supernatants were washed in supplemented McCoy's 5A medium (see below) to remove plasma, were recovered by centrifugation at 400 g for 6 min, and were then resuspended in low-density solution (0.5 to 0.8 ml) and gently layered on top of the gradient. An aliquot of these cells was retained so that the unseparated cell population could be cultured. Gradients were loaded with $\sim 7 \times 10^7$ nucleated cells (loads in excess of 10^8 cells decrease the resolution of the separation).

The preparation of the cells, generation, and loading of the gradient all took place at room temperature ($\sim 22^\circ\text{C}$). Gradients were spun at $2,600$ g for 25 min at 22°C . Preliminary experiments indicated that 25 min was sufficient time for equilibrium to be reached. 12–13 fractions were collected by gentle aspiration with syringes and filling tubes (Kwills; S & N Everet Ltd., London, United Kingdom).

The density of each fraction was calculated from the refractive index using a calibration curve obtained by weighing known volumes of various mixtures of heavy and light solutions at 22°C and then relating this to refractive index. Refractive index of this material has a linear relationship to density. The gradients were linear over the major part of the range and density increments of the fractions were ~ 0.004 g/cm³. Cells were recovered from the fractions by dilution with 4 ml of supplemented McCoy's 5A tissue culture medium containing 15% FCS, followed by centrifugation (400 g, 6 min) and resuspension in appropriate medium (McCoy's 5A for granulocyte/macrophage (CFUgm) CFU cultures, Iscove's modified Dulbecco's medium for granulocyte/erythrocyte/megakaryocyte/macrophage (CFUgemm) CFU cultures, RPMI 1640 containing 3% FCS for plasminogen activator assays). For morphological analyses cells were suspended in PBS, pH 6.8, supplemented with 10% FCS and were recovered by cytosedimentation at unit gravity and either stained with May-Grunewald-Giemsa or cytochemically for nonspecific or chloroacetate esterase as previously described (11).

Culture Methods for CFUgm and CFUgemm. CFUgm were cultured using the double-layer agar technique of Pike and Robinson (12). Cells from the various density fractions were cultured in 0.3% agar overlays. Not all fractions were cultured at the same cell density, as fractions that were expected to produce large numbers of colonies were diluted to avoid crowding and overlap of colony growth. The range of cell densities cultured was from $\sim 1 \times 10^4$ to 2×10^5 cells per overlayer. Feeder layers supplying the growth stimulus, granulocyte/macrophage colony-stimulating activity (gmCSA) were prepared containing 10^6 peripheral blood leucocytes per milliliter. Since we have previously demonstrated (11) that feeder layer activity has an effect on estimation of buoyant density distribution of CFUgm (higher levels of gmCSA recruit additional low-density progenitors to form colonies), each experiment was performed in triplicate using feeder layers from three different donors and the results were those obtained on the feeder layer stimulating the largest number of clones. Previous studies (13) in our laboratory have shown that this best-of-three approach ensures maximal stimulation of clone formation (13). Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air for 7 d.

For caseinolytic plaque assays, replicate cultures were made containing 10^5 low-density (~ 1.063 g/cm³) bone marrow cells and were examined at various time intervals. Gels were

cut in half and removed from the culture dish into a bath containing PBS, pH 6.8. The half gels were then placed, overlayer down, on to a microscope slide. After removal of the feeder layer, the overlayer gel was dehydrated by covering it with filter paper (Whatman 50 hardened; Whatman Ltd., Maidstone, Kent, United Kingdom) and additional absorbent paper weighted down with a glass sheet. After 10–15 min, the paper layers were carefully removed leaving a thin agar film on the slide. This was not allowed to dry before coating with the agar casein mixture for the caseinolytic plaque assay (see below).

The culture method for CFUgemm is a modification of that of Fauser and Messner, (14) the details of which have been published elsewhere (9). 1-ml cultures containing 10^4 to 2×10^5 cells in Iscove's modified Dulbecco's medium (Gibco, Paisley, Scotland) with 5×10^{-5} M 2-ME, 30% human citrated plasma, and 0.25% agar were prepared in 35-mm dishes (Falcon Labware, Oxnard, CA). The growth stimuli were erythropoietin and conditioned medium obtained by the method of Aye et al. (15) from PHA-stimulated leucocytes (PHA-LCM). PHA-LCM was used at 2.5% (vol/vol), the optimal concentration for the batch used, and erythropoietin was used at 1 U/ml. Cultures were scored after 14 d of incubation.

Agar gels were removed from the dishes, dehydrated using Whatman No. 50 filter paper as described above, and were air dried and stained from hemoglobin-containing cell using *O*-dianisidine (Helena Laboratories, Beaumont, TX) as previously described (16). Mixed colonies containing an erythrocytic component are easily identifiable by the deep russet colour of hemoglobin containing *O*-dianisidine-positive cells.

Cytochemical Staining. In situ staining of granulocyte/macrophage colonies for nonspecific and chloroacetate esterase was performed as previously described (17). The same technique was also applied to the caseinolytic plaque assay preparations in which cells were suspended directly in the RPMI agar-casein mixture. Staining for chloroacetate esterase defines cells of the neutrophil granulocyte lineage and staining for the nonspecific esterase (α -naphthylbutyrate esterase) defines cells of the monocyte/macrophage lineage.

Plasminogen Activator Assay. Marrow cells that had been separated on density gradients were incubated with RPMI 1640 containing 3% FCS at 4×10^6 cells/ml for 24 h at 37°C in a humid atmosphere and 5% CO₂ in air. Medium was harvested by centrifugation and stored at –80°C before analysis for plasminogen activator activity.

Harvest fluids were assayed by measuring plasminogen-dependent release of soluble radioactive fibrin degradation peptides from insoluble ¹²⁵I-fibrin-coated multiwell tissue culture plates (Linbro; Flow Laboratories, Inc., Irvine, Scotland) as previously described (1, 18). Results were calculated in terms of urokinase units by reference to urokinase standards assayed simultaneously. The molecular species of plasminogen activators present in harvest fluids were identified as uPA or tPA using specific inhibitory antibodies to these enzymes. Harvest fluid samples were incubated for 1 h at 4°C with purified rabbit antibody and assayed for residual activity using the ¹²⁵I-fibrin assay. All procedures have previously been described in detail (19).

Caseinolytic Plaque Assay. To obtain an estimate of plasminogen activator production by individual cells or individual clones grown in agar culture a caseinolytic plaque assay was used. Agar culture gels containing clones were dehydrated as described. They were immediately overlaid with a prewarmed solution of RPMI 1640 containing 0.75% agar, and 1.2% solution of commercial instant nonfat dry milk powder (Carnation Co., Johannesburg, South Africa) and 200 µg/ml of purified human plasminogen. This suspension was run between two prewarmed glass microscope slides to achieve a film of uniform depth (~1 mm). The preparations were allowed to set at room temperature, the top microscope slide was gently removed, and the gels were incubated at 37°C in a humidified atmosphere and were inspected at intervals for the development of plaques of lysis.

Cells in suspension were washed and mixed with the agar/casein/plasminogen mixture to give a final concentration of 2×10^6 cells/ml. This suspension was run between two slides and processed as above.

Cells or clones of cells responsible for the production of caseinolytic plaques could be

identified by dehydrating and staining the specimens for dual esterase as previously described.

Results

Marrow samples from 11 normal subjects were fractionated by equilibrium density centrifugation and cells from each fraction were examined to determine the type and rate of plasminogen activator that they produced. Essentially similar results were obtained in all cases.

Representative results from two experiments are presented in Fig. 1 in two ways. In the first (*A1* and *B1*) the rate of enzyme synthesis by all of the cells in each fraction is plotted as a function of gradient density. In the second (*A2* and *B2*) the rate of synthesis has been corrected for the number of cells in each fraction and is expressed in terms of milliunits of enzyme/ 10^7 cells/24 h. It is evident, from both graphic presentations, that the low-density cells (~ 1.045 – 1.065 g/cm³) synthesized exclusively tPA. More mature, higher-density cells (~ 1.07 – 1.085 g/cm³) released a mixture of tPA and uPA. It can be seen, moreover, from the profiles plotted in Fig. 1, *A1* and *B1*, that the cells that produced tPA comprised two populations. When corrected for cell number only one peak of tPA production (density ~ 1.063 g/cm³) was observed, indicating that although these cells can be divided into two populations on the basis of their density, they constituted a single population on the basis of the rate of enzyme production per cell.

The number of cells varied markedly along the gradient with the majority of nucleated cells fractionating at a density of ~ 1.07 g/cm³. Dashed lines indicate fractions that were pooled because of inadequate cell numbers for the assay (see Materials and Methods).

A summary of the results of the 11 density gradient experiments is presented in Table I. The bimodal distribution in the tPA-producing cell populations was observed in 7 of 11 cases. In 5 of these, the 1.072 -g/cm³ peak contained the greater number of tPA-producing cells and the 1.063 -g/cm³ peak the lesser number. In the other two cases the 1.063 -g/cm³ peak was greater than the 1.072 -g/cm³ peak.

In contrast, the uPA was produced by cells that fractionated in either a single peak or one that had a low broad profile. The modal density of uPA-producing cells could be estimated in five experiments since in three analyses there was a broad profile with no obvious peak, in another experiment no uPA was present, and in two others maximum uPA was produced by cells in the last fraction of the gradient. The mean density for the five experiments in which this could be estimated suggests that uPA-producing cells have a modal density of ~ 1.076 g/cm³. In the sample with no urokinase activity, a second bone marrow specimen from the same individual was tested and this also showed no uPA activity. However, neutrophil/granulocytes from the peripheral blood of this donor did produce uPA.

Fig. 2*a* shows representative density distribution profiles for the various myeloid progenitor cell populations and for neutrophil leucocytes (band forms and polymorphs) obtained from normal marrow. To obtain sufficient cells for

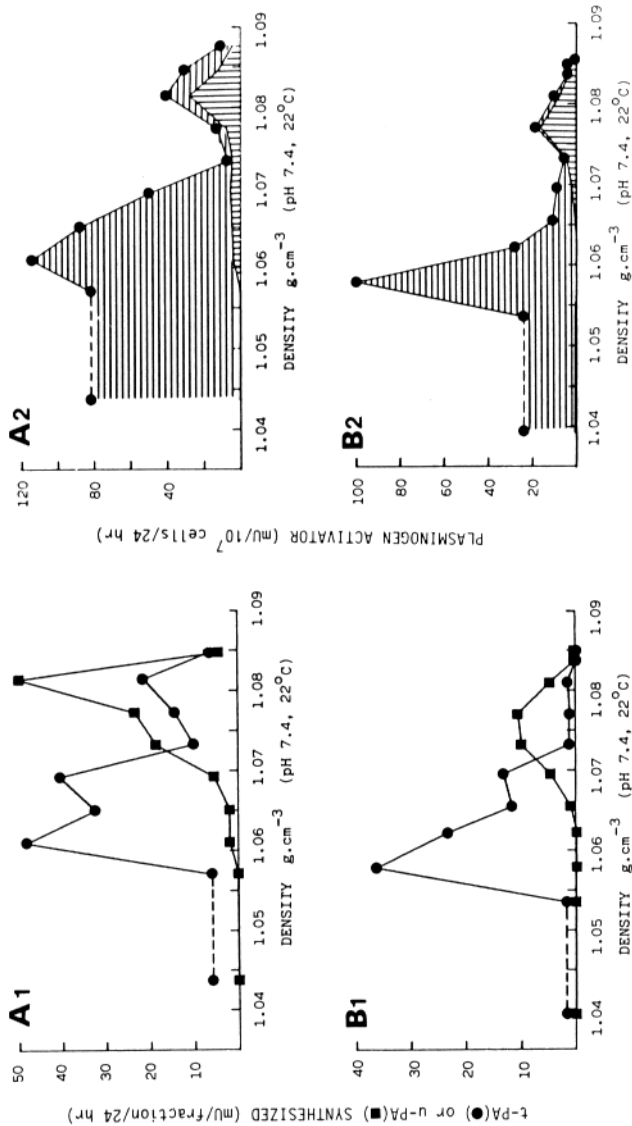


FIGURE 1. (A1 and B1) Equilibrium-density distribution profiles of two normal marrow specimens showing tPA (●) and uPA (■) expressed as units of enzyme per fraction per density increment. Cells producing tPA have a relatively lower density than those producing uPA. In both cases the tPA peaks were biphasic. (A2 and B2) Density-distribution profiles of the same marrow specimens showing the relationship between total units of plasminogen activator per 10⁷ cells produced in 24 h, and buoyant density. (Vertical hatching) uPA; (horizontal hatching) tPA. Dashed lines represent adjacent fractions that have been pooled.

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TABLE I
Buoyant Densities of the Three Major Plasminogen Activator-secreting Cell Populations

Exp.	Tissue plasminogen activator		Urokinase
	Peak 1	Peak 2	
	<i>g/cm</i> ³	<i>g/cm</i> ³	<i>g/cm</i> ³
1	1.0611 (Maj)	1.0694 (Min)	1.0815
2	1.0579 (Maj)	1.0696 (Min)	1.0771
3	1.0643 (Min)	1.0750 (Maj)	1.0800
4	1.0621 (Min)	1.0700 (Maj)	1.0743
5	1.0610 (Min)	1.0760 (Maj)	*
6	1.0623 (Min)	1.0695 (Maj)	*
7	1.0660 (Min)	1.0708 (Maj)	None
8	1.0640		≥1.0730
9	1.0610		≥1.0734
10	1.0630		1.0670
11	1.0672		*
Mean	1.0627 ± 0.0026	1.0715 ± 0.0028	1.0760 ± 0.0057

In 7 individuals the tPA-containing peak was biphasic with the first component at ~1.063 *g/cm*³ and the second component at 1.072 *g/cm*³. In four instances only one peak of tPA was obtained. The uPA-containing enzyme peak was found at a density of 1.076 *g/cm*³. The major (Maj) and minor (Min) contribution to the total tPA is indicated in parenthesis.

* Low urokinase levels with no definite peak.

the various types of analyses a pair of matched gradients was spun on the same rotor head and the recovered cells from one gradient were used for the CFUgemm cultures and cells from the second gradient were used for both CFUgm cultures and morphological analysis. As previously established (9), CFUgemm, the most primitive progenitor type, are the least dense with a modal density of 1.059 *g/cm*³. Cells forming granulocyte/macrophage clones are the progeny of CFUgemm and are a heterogeneous group of progenitor cells. These are usually classified according to clone size with CFUgm producing clones of 40 or more cells. Cluster-forming cells in day 7 cultures have been shown (7) largely to represent the progeny of CFUgm. Morphological analyses in previous studies (9) have shown that CFUgm correspond to small mononuclear cells and cluster-forming cells correspond to the morphological categories of myeloblasts, promyelocytes and myelocytes. The first and second tPA-producing cell populations in terms of their modal densities most closely resemble CFUgm and cluster-forming cells, respectively. Fig. 2*b* shows density distribution profiles for myeloblasts, promyelocytes, and myelocytes. The modal density for myeloblasts lies between the modal densities for peak 1 and peak 2 tPA-producing cells. Promyelocytes and myelocytes have largely overlapping profiles with modal densities of 1.069 and 1.072 *g/cm*³, respectively. These populations may thus correspond to the second peak of tPA, which has a modal density of 1.072 *g/cm*³. The uPA-producing cells with a modal density of 1.076 *g/cm*³ (Table I) could correspond to neutrophil/granulocytes or the subsidiary cluster-forming cell population at that density.

Since density-distribution profiles do not provide direct evidence for the cellular species secreting each enzyme type, we therefore attempted to assess

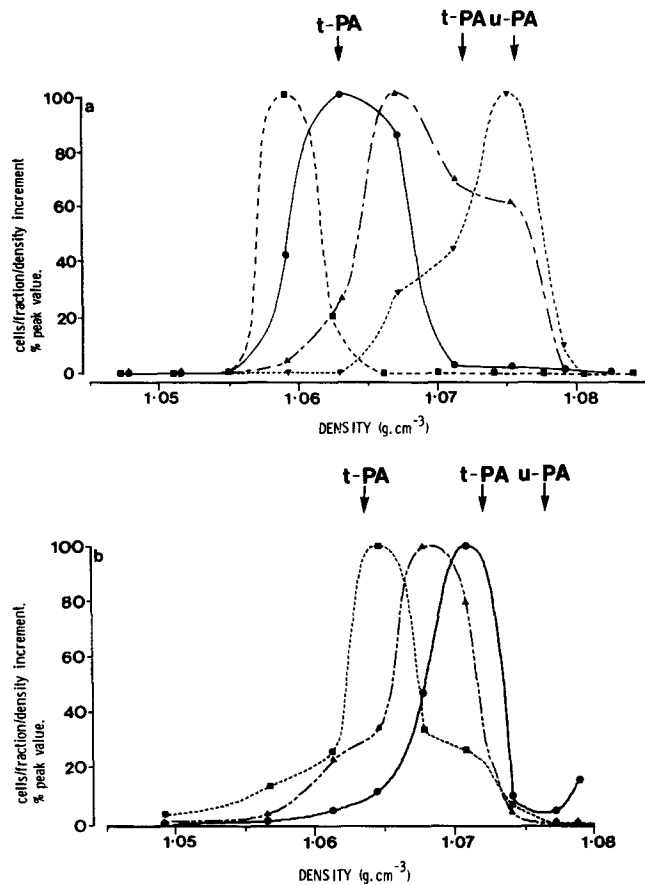


FIGURE 2. (a) Representative density distribution profiles for the various myeloid progenitor cell populations and for neutrophil leucocytes (band forms and polymorphs) fractionated from a normal marrow specimen. Multipotent progenitors (CFUgemm, ■); granulocyte/macrophage colony-forming cells (CFUgm, ●); cluster forming cells (▲); neutrophil leucocytes (▼). The location of the two tPA- and the uPA-producing cell populations are indicated. (b) Representative density distribution profiles of myeloblasts (■) promyelocytes (▲) and myelocytes (●) fractionated from a normal marrow specimen. The location of the two tPA- and the uPA-producing cell populations are indicated.

directly the type of plasminogen activator produced at various differentiation stages. To do this, fractionated low-density bone marrow cells ($<1.063 \text{ g/cm}^3$) were cultured in semisolid agar for varying periods of time. These cultures were overlaid with an agar indicator layer containing casein, plasminogen, and inhibitory antibodies specific for uPA or tPA. Plaques of caseinolysis indicated plasminogen activation. Fig. 3 shows plasminogen-dependent caseinolysis by day 3 and day 11 clones. The plaques of lysis surrounding the clones are evident in this figure as dark areas on an opaque background, and representative plaques are indicated by arrows. Fig. 3, *a1* and *b1* show lysis zones in a plasminogen-containing gel by day 3 and day 11 clones, respectively. No lysis was noted in the absence of plasminogen (Fig. 3, *a2* and *b2*). The failure of uPA antibody to inhibit plaque formation by day 3 clones (Fig. 3 *a4*) and the prevention of plaque formation by anti-tPA antibody (Fig. 3 *a3*) demonstrates that the enzyme secreted by these early progenitor cells was tPA. Inspection of the culture dishes showed small clones of two to six cells.

The production of tPA was followed by a period of secretion of both enzyme species from days 6–9 (data not shown), but by day 11 large neutrophil and/or

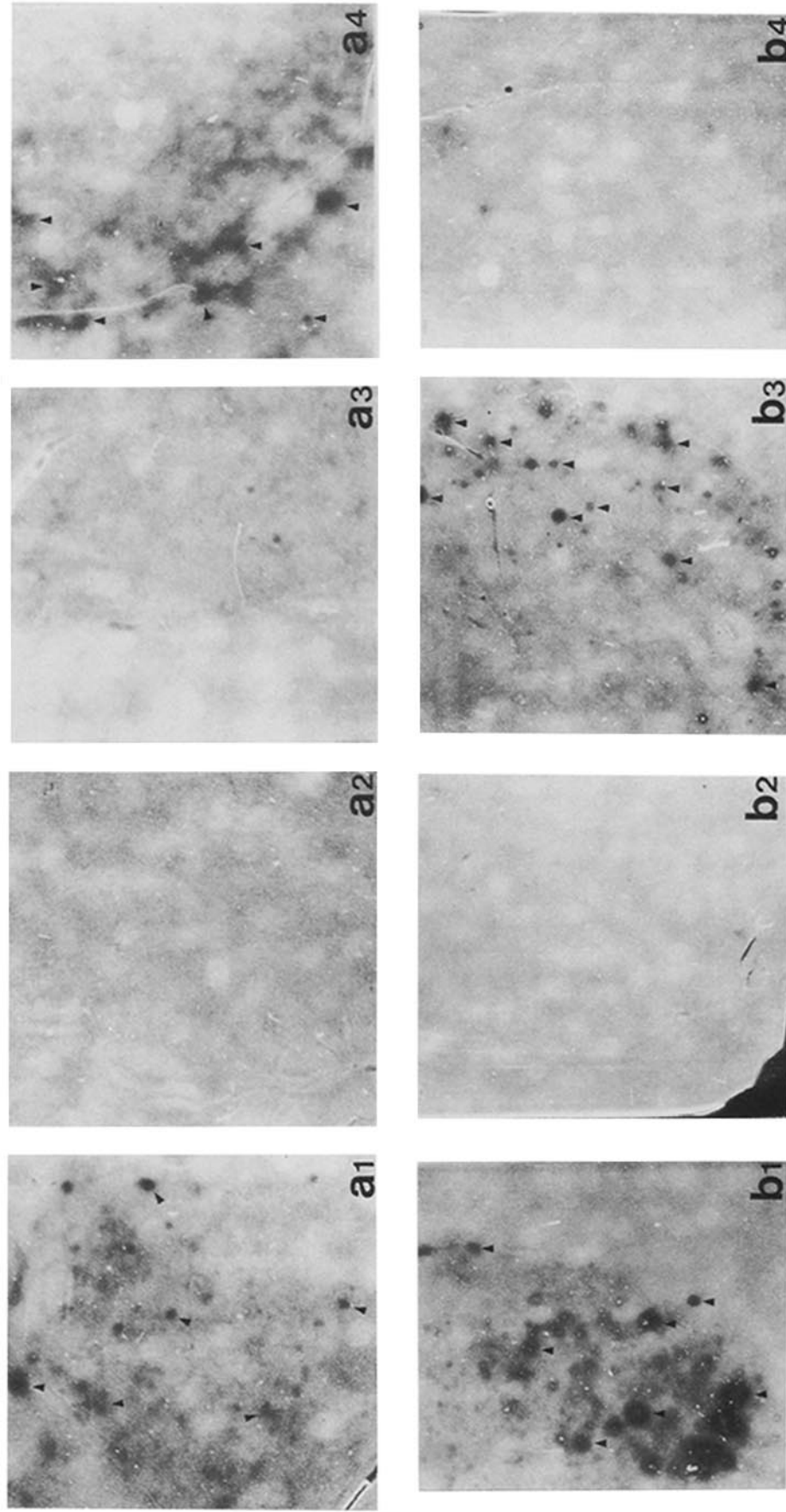


FIGURE 3. Plasminogen-dependent caseinolysis by normal cultures of low-density marrow cells ($<1.063 \text{ g/cm}^3$) cultured for (a) 3 d or (b) 11 d. Arrowheads indicate areas of lysis. (a1, b1) plasminogen-dependent lysis; (a2, b2) no visible plaques when plasminogen was omitted from gel; (a3, b3) effect of tPA antibody on plasminogen dependent lysis; (a4, b4) effect of uPA antibody on plasminogen-dependent lysis. (a) $\times 4$ (b) $\times 3$.

macrophage colonies were present and all of the secreted enzyme was uPA. In contrast to the results obtained at day 3, anti-uPA antibody abolished plaque formation (Fig. 3 *b4*), whereas anti-tPA antibody did not (Fig. 3 *b3*). This experiment demonstrates directly the relationship between progenitor differentiation stage and the species of enzyme secreted.

CFUgm differentiate both to the neutrophil/granulocyte and macrophage lineages. We therefore examined macrophages to determine whether the differentiation to this lineage is also associated with the production of uPA. Previous experiments have shown that macrophages do not have a clearly defined density-distribution profile, and differentiation in the monocyte/macrophage lineage is the exception to the usual rule associating increasing density with differentiation (10). We therefore examined the enzyme secretion of macrophages derived from low-density ($<1.063 \text{ g/cm}^3$) normal marrow cells cultured for 37 d in suspension culture (with 5% vol/vol PHA-LCM, as the growth stimulus). After this period of time the cultures contained abundant large macrophages; Fig. 4 shows plasminogen-dependent caseinolysis by these cells. Fig. 4 (*a1*) shows plaques of lysis in a plasminogen-containing gel. Morphological and cytochemical analysis of these gels showed that the large nonspecific esterase (NSE)-positive cells were centrally placed in plaques of lysis, whereas small NSE-negative cells also present in these cultures were not observed to be surrounded by lysis plaques. No lysis was observed in the absence of plasminogen (Fig. 4 *a2*). All plaques of lysis were inhibited by anti-uPA antibody (Fig. 4 *a4*), indicating that macrophages produce uPA.

Discussion

The results showed that the secretion of tPA and uPA by hemopoietic cells was a differentiation-linked property with tPA being produced by primitive progenitors and uPA being secreted by more differentiated cells.

It has been shown that a variety of human cells release plasminogen activators of either the tPA or the uPA type (19, 20–22). Apart from the involvement of tPA in the fibrinolytic system (23, 24), the physiological role of these two enzymes is obscure. The enzymes are produced by a wide variety of cells, including macrophages (25) and neutrophil polymorphonuclear leucocytes (1, 26), where they may be involved in a number of processes including proteolysis of inflammatory exudates, generation of chemotactic peptides, and processes that require regulated local proteolysis (27). A variety of neoplastic cells, including leukemic cells, also secrete these enzymes (1, 19, 28–30). Enzyme secretion by leukemic cells could reflect either normal hemopoietic stem cell behavior or aberrant gene expression. The results presented in this paper suggest that the former is the case.

Tissue plasminogen activator was secreted by two populations of bone marrow cells, one with a mean modal density of 1.063 g/cm^3 and the other 1.072 g/cm^3 . The first population corresponds to the modal density for CFUgm. We cannot exclude the possibility that other progenitor types also produce tPA. Several committed progenitor populations for other hemopoietic lineages have largely overlapping profiles with CFUgm (9). CFUgemm have a lower density than

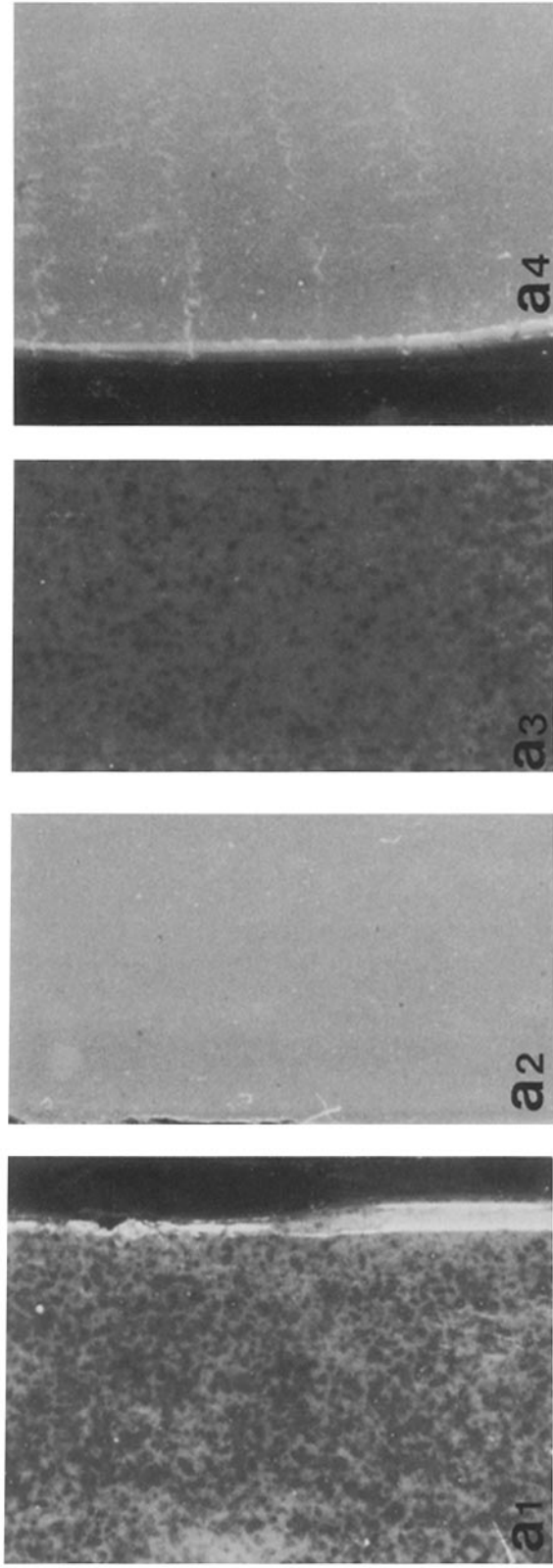


FIGURE 4. Plasminogen-dependent caseinolysis by macrophages. (a1) plaques of lysis in plasminogen containing gel; (a2) no visible plaques when plasminogen was omitted from gel; (a3) plaques of lysis in the presence of tPA antibody; (a4) no plaques of lysis in the presence of uPA antibody. X 4.

CFUgm and are also far less numerous than CFUgm and would not therefore contribute greatly to the tPA peak of density 1.063 g/cm^3 . CFUgm are committed progenitor cells from the granulocyte/macrophage series operationally defined in this assay system as cells producing colonies of 40 or more cells. Cells producing clusters of 2/39 cells have a broad density profile spanning 1.072 g/cm^3 (see Fig. 2*a*), and have been shown to represent the progeny of the CFUgm (7). They include the recognizable neutrophil granulocyte precursor stages of myeloblasts, promyelocytes, and myelocytes. Analysis of the density distribution of these cells suggests that myelocytes and possibly promyelocytes are responsible for the second tPA peak (see Fig. 2*b*).

The contribution to the total tPA by the two populations of cells varied, the lower density population being the major or sole source of enzyme in 6 of 11 experiments. In four experiments no enzyme was detected in the higher density range (i.e., 1.072 g/cm^3), all the tPA being contributed by cells of lower density (1.063 g/cm^3).

This variability is unlikely to represent differences in the proportions of the progenitor cell types. The levels of closely related progenitors have been shown to correlate highly with each other (31). As secretion of this enzyme is modulated by a wide variety of agents (29, 32–36), this variability may be due to changes in the level of enzyme secreted per cell.

Urokinase was secreted by cells with a modal density between 1.067 and 1.082 g/cm^3 in five experiments (see Table I). The density profiles of neutrophil granulocytes in normal marrow in Fig. 2*a* and promyelocytes and myelocytes in Fig. 2*b* suggest that these cells were responsible for this peak of uPA. It thus appears that either promyelocytes and myelocytes produce both types of enzyme or that the switch from tPA to uPA production occurs over a range of maturation stages.

The modal densities for uPA-secreting cells in the different experiments could indicate variations in the level of the bone marrow granulocyte reserve, which is labile and readily released into the blood. Thus the relative contributions of promyelocytes, myelocytes, and neutrophils to uPA production could vary markedly.

There was considerable heterogeneity in the amount of uPA produced, from undetectable levels to $300 \text{ mU}/10^7 \text{ cells}/24 \text{ h}$. Of 11 marrows, one contained no uPA secreting cells. This observation was confirmed in a second marrow from the same individual. This is somewhat surprising, since bone marrow samples contain neutrophils and peripheral blood neutrophils from this donor-secreted uPA. The reason for the failure to demonstrate any uPA in the bone marrow from this donor is obscure.

The density distribution profiles do not provide direct evidence for the cellular species secreting each enzyme type. We, therefore, cultured low-density marrow cells in semisolid agar for varying periods of time. Developing clones initially produced tPA (day 3), followed by a period of secretion of both enzyme species (days 6–9). By day 11 only uPA was secreted (see Fig. 3). Macrophages derived from low-density ($<1.063 \text{ g/cm}^3$) normal marrow cells cultured for 37 d also

secreted uPA exclusively (see Fig. 4). This demonstrates directly the relationship between progenitor stage and species of enzyme secreted.

The functional properties of AML clonogenic cells have been shown (2, 3) to have predictive value in that clone size in semisolid agar culture and the sensitivity of clonogenic cells to gmCSA are both related to response to therapy. In normal marrow progenitor cell populations clone size decreases and sensitivity to gmCSA increases as cells differentiate along the granulocyte/macrophage pathway (7). This relationship in normal cells and the observation that the two properties were linked in AML (3) led to the suggestion that the heterogeneity of AML phenotypes reflects the accumulation of clonogenic cells at various stages of the differentiation pathway and that this was related in some way to response to therapy (5, 7). Studies on self-renewal capacity, which is relatively high in cells from patients with a poor prognosis, have been consistent with this hypothesis (4). The results obtained in this study showing that early normal progenitor cells secrete tPA, together with the previous observation (1) that patients with AML whose cells secrete tPA alone failed to respond to induction chemotherapy, provide additional strong evidence for this association.

Response to therapy is not associated with the morphology of AML cells, except in the case of acute promyelocytic leukemia. Previous reports (3, 5-7) documenting a correlation between response to therapy and AML cell phenotypes have studied AML clonogenic cells rather than the whole AML cell population. The present study indicates that tPA is secreted by progenitor (clonogenic) cells; the secretion of this species of plasminogen activator may thus indicate that an early clonogenic cell population has accumulated. Why the accumulation of primitive precursors should determine a poor response of the patient to chemotherapy is unclear. The accumulation of primitive cells in the poor prognosis group might indicate a deficient intrinsic capacity of these cells for differentiation. Alternatively, stimuli that normally induce differentiation may be lacking. Patients deficient in gmCSA (a proliferation and differentiation stimulus) also show a poor response to therapy (37), suggesting that both intrinsic and extrinsic contributions to cellular differentiation may influence the response to chemotherapy.

While it is apparent that neutrophils and macrophages may require uPA for their role in inflammation, the reason why primitive hemopoietic cells produce tPA is less readily apparent. The production of proteases by progenitor cells may be necessary to provide a local proteolytic mechanism for generating biologically active peptides. Alternatively, the proteases generated might alter cell surface receptors to various cytokines, either on the cells secreting them or on adjacent cells, in such a manner that would affect their growth and differentiation capacity. In addition, stem cells are endowed with the capacity for migration and implantation in specific hemopoietic sites and this process could also require proteolytic enzyme secretion and its modulation.

Summary

Previous studies (1) have shown that the response of patients with acute myeloid leukemia to induction chemotherapy can be predicted by the species of plasmin-

ogen activator that their cells secrete. Patients whose cells secreted tissue plasminogen activator (tPA) only failed to respond to combination chemotherapy. Individuals whose leukemic cells display features of the early progenitor phenotype also respond poorly to therapy. This suggested that the two species of plasminogen activator secreted by leukemic cells might be produced by normal cells at distinct stages of differentiation. These results indicate that the secretion of the two enzyme types is a differentiation-linked property of normal cells with tPA being produced by granulocyte/macrophage progenitors and urokinase by more differentiated cells and by mature neutrophils and macrophages.

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