Although establishment of the true therapeutic effect of verapamil in combination with adriamycin awaits the completion of this trial and others, the in vitro effects suggest that reversal, or at least modification, of adriamycin resistance is a clinical possibility.

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Loss of Adhesion of Murine Erythroleukemia Cells to Fibronectin During Erythroid Differentiation

Abstract. *Uninduced murine erythroleukemia cells specifically attached to fibronectin-coated dishes but not to dishes coated with laminin or type I or IV collagen. Dimethyl sulfoxide–induced differentiation of these cells caused a dramatic decrease in adhesion to fibronectin that was correlated with synthesis of the erythrocyte glycoprotein "band III," a membrane marker of the differentiated erythrocyte. Loss or modification of fibronectin binding sites on the cell surface during erythroid differentiation may cause the release of reticulocytes from the interstitial matrix of bone marrow into the blood.*

Differentiation of the mammalian erythrocyte from its nucleated erythroblast precursor takes 4 to 6 days and involves extensive remodeling of the cell surface membrane (*1*). At least two cell surface glycoproteins, "band III" (the anion-exchange protein) and glycophorin, are unique to erythrocytes (*2*) and are synthesized preferentially during erythropoiesis (*3, 4*). Spectrin, the principal structural protein of the submembrane cytoskeleton in erythrocytes (*5*), is also synthesized during erythropoiesis (*3, 6*). Erythroid differentiation also involves loss of glycoproteins characteristic of the erythroblast, such as the transferrin receptor (*7*).

We report here that erythroid differentiation by murine erythroleukemia (MEL) cells in vitro is also accompanied by loss of their ability to attach to the glycoprotein fibronectin. Fibronectin is a major fibrous component of the extracellular matrix in bone marrow (*8*) and is found on the surface of many types of cells and in the blood plasma. It and other extracellular matrix–associated proteins, like collagens and laminin, promote attachment and subsequent spreading of many cell types in vitro (*9*). Fibronectin also appears to be involved in the regulation of several differentiation pathways, including myogenesis, chondrogenesis, and adrenergic differentiation in explanted neural crest cells (*9*). We speculate that loss of fibronectin binding sites during erythroid differentiation may be involved in the release of erythrocytes from the interstitial matrix of bone marrow into the blood.

Murine erythroleukemia cells provide a valuable in vitro model for studying many aspects of erythroid differentiation (*10*). Treatment of these cells with dimethyl sulfoxide (DMSO) results in the initiation of a differentiation process in which they synthesize hemoglobin, spectrin (*6*), and band III protein (*11*). Although MEL cells normally grow in suspension culture, attachment to the surface of culture flasks can be mediated by serum fibronectin (*12*).

Exponentially growing MEL cells attached to fibronectin-coated dishes but not to dishes coated with bovine serum albumin (BSA) (Table 1). Attachment was dependent on the amount of fibronectin applied to the dishes: 50 percent adhesion was observed at 5 μg per 10-cm^2 dish and > 90 percent adhesion at 12 μg. Most of the added cells became firmly attached within 60 minutes of plating and most of the adhering cells retained their spherical shape; only 10 to 15 percent had spread by 60 minutes (Fig. 1a). Attachment of MEL cells was not promoted by collagen type I (50 to 500 μg per dish), collagen type IV (50 to 500 μg), or laminin (1 to 25 μg) (Table 1). This negative result cannot be attributed to possible inactivation of these proteins during the preparation of dishes, because dishes coated with collagens promoted attachment and spreading of Chinese hamster ovary (CHO) fibroblasts (Table 1) (*13*). Furthermore, CHO cells attached to dishes coated with fibronectin or laminin. We also examined the adhe-

Table 1. Adhesion of MEL, CHO, and HepG2 cells (*18*) to plasma fibronectin, collagen, and laminin. Attachment of cells to 35-mm petri dishes coated with proteins (*19*) in the indicated amounts was measured by incubating 4×10^5 cells at 37°C in a CO_2 incubator. After 90 minutes the medium was removed and unattached cells were counted in a Coulter counter; the dishes containing the attached cells were washed three times with the incubation medium and once with Hanks buffered salt solution before trypsinization (0.2 percent trypsin and 0.02 percent EDTA) to detach the cells. Detached cells were quickly resuspended in fresh incubation medium containing soybean trypsin inhibitor (25 μg/ml) and counted as above. Data are means ± standard errors for four replicate dishes.

Addition to dish	Attached cells (percent)		
	MEL	CHO	HepG2
None	5 ± 2.7	10 ± 4.9	7 ± 3.0
Fibronectin (12 μg)	90 ± 5.9	79 ± 5.0	25 ± 3.6
Laminin (12 μg)	10 ± 6.0	72 ± 4.0	85 ± 4.5
Collagen type I (200 μg)	4 ± 2.6	83 ± 5.6	20 ± 4.0
Collagen type IV (150 μg)	6 ± 3.7	90 ± 4.0	18 ± 5.6

sive properties of the human hepatoma line HepG2; only laminin promoted significant attachment (Table 1). These results demonstrate that MEL cells, which do not synthesize fibronectin by themselves (*12*), can specifically attach to culture dishes coated with fibronectin.

Adhesion of MEL cells to fibronectin was progressively lost when the cultures, in suspension, were induced to differentiate by DMSO (Fig. 2). The most dramatic decrease in cell adhesion was observed 4 days after the initiation of induction, when about 70 percent of the plated cells remained unattached. This decrease in adhesion was observed even when fibronectin was increased to 30 μg per 10-cm^2 dish. We were unable to examine adhesion beyond 4 days because of the extreme fragility of the end-stage cells of the differentiation pathway (*14*). Mature human and mouse erythrocytes from circulating blood do not adhere to fibronectin-coated surfaces. None of the adherent fraction of the induced cultures spread (Fig. 1b). Both unattached and attached cells from these cultures were viable, as shown by their exclusion of trypan blue and by their metabolic activity. Treatment of MEL cells with DMSO did not result in the induction of fibronectin synthesis, as judged by immunoblotting. It is conceivable that DMSO-induced cultures secrete proteases that can degrade fibronectin, causing an artifactural decrease in cell adhesion. This seems unlikely, however, since the culture supernatant derived from cells induced for 4 days supported normal attachment of uninduced MEL cells.

After 4 days of differentiation induced by DMSO, cells synthesized band III protein at a level six to eight times higher than that of uninduced cells (lanes 3 and 6 in Fig. 3). Thus band III protein synthesis can be considered a membrane marker of the differentiated state. At 4 days of differentiation, cells that attached to fibronectin did not synthesize any band III protein, while unattached cells synthesized this protein in abundance (lanes 8 and 10 in Fig. 3). Similar differences were also apparent in uninduced cells, although band III protein was synthesized at low levels by the attached cells (lanes 12 and 14). Since there was no significant difference in [^{35}S]methionine incorporation between the unattached and attached populations of cells induced for 4 days (unattached cells, 1.31 × 10^5 count/min per microgram of protein; attached cells, 1.26 × 10^5 count/min per microgram of protein), we conclude that both populations were equally active in protein synthesis. Thus synthesis of band III protein during differentiation is correlated with loss of cellular adhesion to fibronectin.

Our preliminary findings indicate that the fibronectin-adherent and -nonadherent populations of cells induced for 4 days do contain the same amount of hemoglobin, another protein whose synthesis is induced by DMSO. Since MEL cells become committed to the differentiation pathway asynchronously (*15*), it is possible that different cells in this cloned culture do express different markers of differentiation—globin and band III protein—to different extents. It will therefore be important to examine the adher-

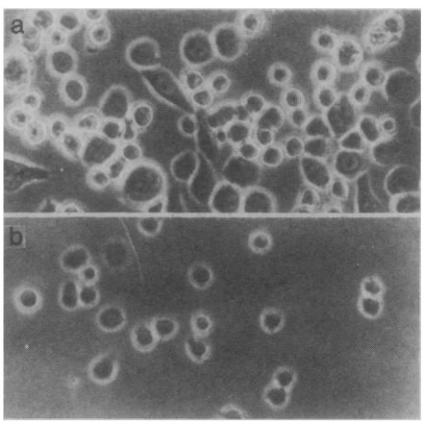

Fig. 1 (left). Phase-contrast photomicrograph showing uninduced MEL cells (a) and MEL cells induced for 4 days (b) attached to dishes coated with fibronectin (12 μg) after 60 minutes of incubation at 37°C. Conditions are described in the legend to Table 1.

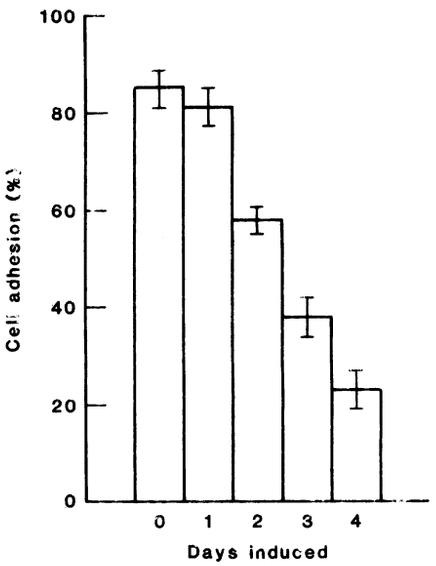

Fig. 2 (right). Fibronectin-promoted adhesion of MEL cells during DMSO-induced differentiation. Exponentially growing MEL cells were induced under culture conditions described by Volloch and Housman (*14*). Induction was initiated by incubating cells in DMEM supplemented with 13 percent (by volume) heat-inactivated fetal bovine serum, 5 percent (weight to volume) BSA; 1.8 m*M* Imferon, and 1.8 percent (by volume) DMSO at 37°C in a humid CO_2 incubator. Before the cell adhesion assay, cells were washed once by centrifugation and resuspended in the fresh induction medium. Attachment of these cells to dishes coated with fibronectin (12 μg) and BSA was measured as described in the legend to Table 1, except that the dishes were incubated for 60 minutes. Under these assay conditions, less than 5 percent of the plated cells attached to BSA-coated dishes. Data are means ± standard errors for four replicate dishes assayed per time point. Essentially similar results were obtained in two separate experiments.

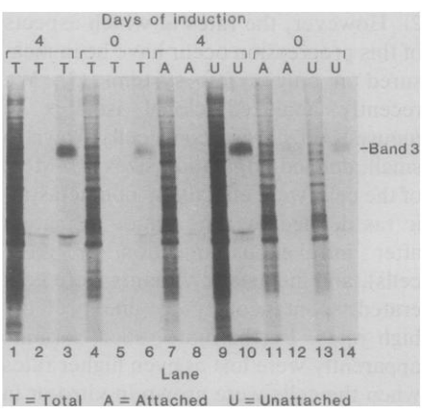

Fig. 3. Biosynthesis of band 3 protein in unattached and attached populations of MEL cells. Uninduced cells (lanes 4 to 6 and 11 to 14) and cells induced for 4 days (lanes 1 to 3 and 7 to 10) were incubated in 35-mm petri dishes coated (*19*) with 12 μg of fibronectin (lanes 7 to 14) or 20 mg of BSA (lanes 1 to 6) at 37°C in a CO_2 incubator. Each dish contained 4 × 10^5 washed cells suspended in 2 ml of methionine-free induction medium supplemented with [^{35}S]methionine (50 μCi/ml, 1230 Ci/mmole; Amersham). Induced cells were incubated in the presence of DMSO (1.8 percent). After 90 minutes, cells recovered from dishes coated with BSA (> 90 percent cells in suspension) or fibronectin as described in the legend to Table 1 were washed once in Hanks buffered saline solution supplemented with soybean trypsin inhibitor (25 μg/ml) and lysed in a detergent solution [10 m*M* sodium phosphate, 2 m*M* EDTA, 0.15*M* NaCl, 1 percent NP40, 0.5 percent sodium deoxycholate, and phenylmethylsulfonyl fluoride (*p*H 7.5) (50 μg/ml)]. Some 5 × 10^6 count/min (acid-precipitable) of each lysate was immunoprecipitated with 15 μl of antiserum to band 3 protein (lanes 3, 6, 8, 10, 12, and 14) or control serum (serum collected from rabbits before immunization with purified band III protein) (lanes 2 and 5) (*20*). Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. Efficiency of immunoprecipitation, estimated by using ^{125}I-labeled mouse erythrocyte membrane proteins as a source of band III protein, was 81 percent. A portion of each lysate (1 × 10^4 count/min) diluted in Laemmli gel sample buffer was also run on the same gel to assess the overall pattern of labeled proteins (lanes 1, 4, 7, 9, 11, and 13).

ent and nonadherent populations of cells for synthesis of spectrin and other erythrocyte-specific membrane proteins and for decreases in cell volume and condensation of nuclei to see which is correlated with the loss of fibronectin adhesion. It is possible that the appearance of band III protein is itself responsible for the loss of adhesiveness of the differentiated MEL cells. It will also be important to determine whether this phenomenon involves a decrease in the number of fibronectin binding sites on the surface of the differentiated MEL cells.

The relevance of our findings to erythropoiesis in bone marrow remains to be established. However, we note that fibronectin is found in close association with hematopoietic colonies in explant cultures of developing bone marrow (*15*). Bone marrow is also bathed by plasma, which contains the soluble form of fibronectin. It is known that fibronectin promotes cell attachment only when this glycoprotein is adsorbed as part of the substrate (*16*). The fibronectin matrix in bone marrow may anchor differentiating erythroid cells, preventing their premature release into the circulation. If this is true, loss or modification of fibronectin binding sites on the surface of the end-stage cells could underlie their release into the circulation.

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18. MEL cells were grown in suspension culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 13 percent (by volume) heat-inactivated fetal bovine serum. CHO and HepG2 cells were grown as monolayers in Ham's F-12 medium and DMEM, respectively, both supplemented with serum as above. The cells were detached with 0.2 percent trypsin (Gibco) and washed three times with Hanks buffered salt solution containing soybean trypsin inhibitor (50 μg/ml). All cells were washed once and assayed for attachment in their respective growth medium. Attachment of CHO and HepG2 cells was measured in the presence of soybean trypsin inhibitor (25 μg/ml).
19. Fibronectin was purified from human plasma as described by E. Engvall and E. Ruoslathi [*Int. J. Cancer* **20**, 1 (1977)], except that the gelatin-sepharose column was sequentially washed with 1*M* NaCl and 1*M* urea before fibronectin was eluted with 3*M* urea (J. Gardner, personal communication). The eluted fibronectin was ~ 98 percent pure, as measured by sodium dodecyl sulfate–gel electrophoresis. Fibronectin and laminin were applied to Falcon 1008 plastic petri dishes (diameter, 35 mm) essentially as described by E. Ruoslathi and E. G. Hayman [*FEBS Lett.* **97**, 221 (1979)] and by E. Engvall [*Methods Enzymol.* **70**, 419 (1980)]. Collagen type I (Flow) and collagen type IV fibers were reconstituted from solution onto the dishes by exposure to ammonium hydroxide vapors for a few minutes at 24°C, producing a firm adherent gel [T. Elsdale and J. Bard, *J. Cell Biol.* **54**, 626 (1972)]. Typically, dishes were coated with protein substrates just before the cell attachment assay.
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Dynamic Heterogeneity: Rapid Generation of Metastatic Variants in Mouse B16 Melanoma Cells

Abstract. *The ability of clonally derived lines of B16F1 and B16F10 melanoma cells to form experimental metastases in C57BL mice after intravenous injection was examined. Luria-Delbruck fluctuation analysis was applied to the results obtained with parallel subclones grown to small population sizes before testing for metastatic ability. The analysis demonstrated that variant cells capable of forming experimental metastases were generated in B16F1 cell populations at an effective rate of about* 1.3×10^{-5} *per cell per generation while in B16F10 cell populations the effective rate of production was about* 5×10^{-5} *per cell per generation. These results are consistent with a dynamic heterogeneity model of tumor progression. They suggest that the majority of cells in both lines are effectively nonmetastatic and that the higher metastatic ability of the B16F10 population may be due in part to a higher rate of generation of metastatic variants.*

The concept that generation of diversity in a tumor cell population leads to tumor heterogeneity and malignant progression has gained wide acceptance (*1*, *2*). However, the rates at which aspects of this progression occur have been measured in only a few systems (*3*). We recently analyzed clonal isolates of mouse KHT fibrosarcoma cells grown to small, defined population sizes (*4*). Most of the cells were effectively nonmetastatic (as defined by lung tumor formation after intravenous injection of KHT cells), and metastatic variants were generated spontaneously at unexpectedly high rates. Furthermore, such variants apparently were lost at even higher rates when the cells were grown in vitro or in vivo (*5*). These findings indicate that one feature of metastatic disease is a rapid turnover of metastatic variants and that the frequency of such variants in the population is determined by the effective rates of their generation and loss. This concept has been termed the dynamic heterogeneity model of tumor progression (*4*). In this study we examined whether such a model, which had been studied only in KHT fibrosarcoma cells, is also applicable to the widely studied murine B16 melanoma tumor. Specifically, we measured the rates of generation of metastatic variants in the relatively poorly metastatic B16F1 line and the more highly metastatic B16F10 line isolated by Fidler (*6*).

The rate of generation of metastatic variants was measured by growing clonal isolates to defined cell numbers before analysis. When variants are generated stochastically during the growth of a clone, a critical population size needs to be attained before there is a significant probability that variants will occur in that population. The critical population size is related to the rate of variant generation, as illustrated by the analysis of drug-resistant variants by Goldie and Coldman (*7*). For measurement of rapid rates the ability to monitor and grow cells to a predetermined small population size is crucial; thus we used an in situ monitoring procedure to identify clones grown to appropriate sizes for analysis (Fig. 1).

If the generation of metastatic variants