A Fibronectin Matrix Is Required for Differentiation of Murine Erythroleukemia Cells into Reticulocytes

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Abstract. Erythroid differentiation of murine erythroleukemia (MEL) cells is far more extensive when the cells are attached to fibronectin-coated dishes than in suspension culture. Cells induced in suspension culture for 4 d become arrested at a late erythroblast stage and do not undergo enucleation. Incubation of cells in suspension beyond 4 d results in lysis. In contrast, cells induced by DMSO on fibronectin-coated dishes for 7 d differentiate into enucleating cells, reticulocytes, and erythrocytes. As determined by quantitative immunoblotting, cells induced in suspension culture accumulate ~33% of the amount of the major erythroid membrane protein Band 3 present in erythrocytes, whereas cells induced on fibronectin-coated dishes accumulate 80–100% of the amount present in erythrocytes. Both suspension-induced cells and cells induced on fibronectin-coated dishes accumulate ~90% of the amount of spectrin and ankyrin present in erythrocytes. As revealed by immunofluorescence microscopy during enucleation of MEL cells, both Band 3 and ankyrin are sequestered in the cytoplasmic fragment of the emerging reticulocyte. Enucleated and later-stage cells detach from the fibronectin matrix, due to the loss of the surface fibronectin receptor; this mimics the normal release of reticulocytes from the matrix of the bone marrow into the blood. Thus a fibronectin matrix provides a permissive microenvironment within which erythroid precursor cells reside, proliferate, migrate, and express their normal differentiation program.

MURINE erythroleukemia (MEL) cells have been used extensively as an in vitro model for erythroid differentiation (15, 22, 32). These Friend-virus-transformed cells grow in suspension culture. When treated with DMSO or certain other chemicals they undergo part of erythroid differentiation, such as induction of mRNAs for globin and the erythrocyte membrane protein Band 3 (2, 25, 40, 51). These cells, however, become arrested at a late erythroblast stage and generally do not enucleate (16, 32, 49, 51). Nor, as is shown here, do they accumulate more than a small fraction of the amount of Band 3 characteristic of erythrocytes.

Normally erythropoiesis occurs within the stroma of the bone marrow outside the marrow sinusoids (53, 54). Bone marrow–derived stromal cells are essential for the growth and differentiation of hematopoietic cells in vitro (11, 13). The bone marrow stroma consists mainly of fibroblasts, macrophages, endothelial cells, and extracellular matrix proteins, such as collagens, laminin, fibronectin, and proteoglycans (6, 12, 29, 43, 44, 57). Erythroid progenitors isolated from human bone marrow also proliferate, migrate, differentiate, and enucleate when plated in the presence of erythropoietin on a cultured monolayer of stromal fibroblasts established from the hematopoietic tissue of human fetal liver (47). But due to the complexity of such cell cultures, the role of stromal fibroblasts and their cell surface–associated extracellular matrix proteins in erythroid differentiation is poorly understood.

We began to suspect that attachment of erythroid cells to an extracellular matrix might be important for differentiation when we and others showed that uninduced MEL cells attach specifically to fibronectin-coated dishes and that this attachment is mediated by an ~140-kD cell surface fibronectin receptor (18, 35, 36). These cells do not attach to other extracellular matrix proteins, such as collagen types I, III, and IV, laminin, vitronectin, and cartilage proteoglycans (18, 35), that are present in the bone marrow stroma (6, 57). Importantly, erythroid differentiation of MEL cells in suspension culture is accompanied by the parallel losses of cellular adhesion to fibronectin and of the fibronectin receptor (36). We hypothesized that this loss reflects the behavior of erythroid cells in marrow: cells differentiate while attached to a fibronectin matrix, and loss of the receptor could trigger release of reticulocytes from the marrow into the blood (35).

As a test of this hypothesis, we have studied differentiation of MEL cells attached to fibronectin-coated dishes. We show that differentiation is far more extensive than in suspension culture. Most of the cells enucleate to become reticulocytes.

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1. Abbreviations used in this paper: Fn, fibronectin; MEL, murine erythroleukemia.
and accumulate the erythroid membrane proteins spectrin, ankyrin, and Band 3 in amounts comparable with those present in erythrocytes.

This system has allowed us to begin studying the remodeling of the plasma membrane that occurs during erythropoiesis. Throughout this period erythrocyte membrane proteins are preferentially synthesized (10, 24), and membrane proteins characteristic of immature erythroblasts, such as the transferrin receptor, are eliminated (34). Enclosure of the late erythroblast is one stage where major remodeling of the plasma membrane occurs. Most of the dialyglycoproteins and spectrin remain with the cytoplasmic fragment (reticulocyte) of the enucleating erythroblast, whereas a set of con-canavalin A (Con A)-binding glycoproteins are enriched in the plasma membrane surrounding the nucleus (17, 23, 42).

Exactly how erythrocyte-specific membrane proteins are segregated away from the membrane proteins that are destined to be lost remains an enigma. Here we show that Band 3 and ankyrin are also sequestered within the reticulocyte portion of the enucleating MEL cells. More importantly, we show that a fibronectin matrix provides a permissive microenvironmental niche in which MEL cells proliferate and express their full differentiation program.

Materials and Methods

Materials

Phenylmethylsulfonyl fluoride (PMSF), p-tosyl-L-lysinechloromethylketone (TLCK), N-tosyl-L-phenylalaninechloromethylketone (TPCK), poly-l-lysine (mol wt 34,000), and radioimmunoassay-grade BSA were all purchased from Sigma Chemical Co., St. Louis, MO. FITC-celite and Lissamine Rhodamine B-200 chloride-celite were purchased from Calbiochem-Behring Corp., La Jolla, CA. Protein A-Sepharose CL-4B and gelatin-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Rhodamine-conjugated goat anti–rabbit IgG was purchased from Zymed Laboratories Inc., San Francisco, CA. BSA for induction medium was purchased from Armour Pharmaceutical Co., Kankakee, IL. Imferon was from Immu-Cell, Newton, CT and was further purified by gel filtration chromatography on a Sephacryl 4B column. Ankyrin was purified from the Triton X-100–insoluble cytoskeletal shell of ghosts exactly as described by Bennett and Stenback (5).

To raise antibodies against spectrin and ankyrin, the concentrated proteins were denatured by boiling in Laemmli (28) sample buffer and separated by SDS-PAGE. Spectrin (α and β subunits) and ankyrin bands were excised from gels and washed extensively with a solution of 20% isopropyl alcohol and 10% acetic acid to remove residual SDS from the gel fragments. These fragments were washed extensively in PBS, homogenized in a small volume of PBS using a Polytron (Brinkmann Instruments Co., Westbury, NY) and centrifuged as above. The supernatant (urea-SDS extract) usually contained 10–15% of the total cellular spectrin and ankyrin, whereas the pellets did not contain these proteins, as judged by immunoblotting. The NP-40 and urea–SDS extracts were mixed, with an equal volume of Freund's complete adjuvant, and ~150–200 μg protein was injected subcutaneously into rabbits. After 4 wk, rabbits were boosted with immunogens emulsified in Freund's incomplete adjuvant every 3 wk and sera were collected on the eighth day after each boost. Antibodies against murine Band 3, a generous gift from Dr. Ron Kopito, was raised by immunizing rabbits with a synthetic peptide corresponding to the 12 COOH-terminal amino acid residues of Band 3 (26). All preimmune and immune antibodies used in this study were purified from sera using protein A-Sepharose CL-4B as an affinity matrix according to the manufacturer's instructions (affinity chromatography; Pharmaica Fine Chemicals). For direct immunofluorescence microscopy, anti–Band 3 and anti–ankylin IgGs were conjugated with FITC and Rhodamine B-200, respectively, according to Brandzaeg (8). Molar ratios of fluorochrome to protein in the purified conjugates of anti–Band 3 and anti–ankyrin IgG were 3.5 and 4.8, respectively.

Sample Preparation for SDS-PAGE

The unattached population of cells from fibronectin-coated dishes was recovered by gently swirling medium in the dishes. The attached cells were detached by incubation with 0.2% solution of trypsin in HBBS-Hepes buffer for 5 min at 37°C. All cells were washed twice with HBSS-Hepes buffer containing 50 mg/ml BSA and 150 μg/ml soybean trypsin inhibitor and once with HBBS-Hepes buffer alone. 10 million washed cells were suspended in 75 μl of Buffer A (5 mM Tris-HCl, 5 mM EDTA, 1% NP-40, 0.1 mM PMSF, 0.1 mM TPCK and TLCK, 0.1 M β-mercaptoethanol, pH 7.4), vortexed, incubated for 15 min at 4°C, and centrifuged in an Eppendorf centrifuge (Brinkmann Instruments Co.) for 15 min. The supernatant (NP-40 extract) was saved. The cytoskeletal proteins that sedimented with the nuclei were recovered by extracting the pellet with 35 μl of Buffer B (5 mM Tris-HCl, 5 mM EDTA, 8 M urea, 0.1% SDS, 0.1 M β-mercaptoethanol, pH 7.4) and centrifuging as above. This supernatant (urea–SDS extract) usually contained ~30% of the total cellular spectrin and ankyrin, whereas the pellets did not contain these proteins, as judged by immunoblotting. The NP-40 and urea–SDS extracts were mixed, with an equal volume of 2X Laemmli gel sample buffer (containing 8% SDS instead of standard 4% SDS), boiled for 3 min and stored at ~2°C. To prepare mouse erythrocyte ghosts, washed 10 times with HBSS-Hepes, ghosts were resuspended in 70 μl of Buffer A, 30 μl of Buffer B, and 100 μl of 2X gel sample buffer, and boiled and stored as above. All samples were analyzed by SDS-PAGE based on the discontinuous Tris–glycine buffer system of Laemmli (28); the gels contained 7.5% acrylamide and 0.2% N,N'-methylene bisacrylamide.
to verify that the distinct morphology exhibited by differentiated cells was not an artifact of staining. Stained cells were photographed using Kodalchrome ASA-64 film.

For localization of proteins by immunofluorescence microscopy, MEL cells were induced to differentiate in 16-chamber glass slides that were coated with fibronectin. The unattached populations of cells were washed with HBSS-Hepes and plated on poly-L-lysine-coated glass chamber slides. Unoccupied poly-L-lysine sites on the slide were blocked by washing the slide twice with HBSS-Hepes buffer containing 1% BSA. Cells that remained attached to fibronectin were also washed with HBSS-Hepes-BSA. In all cases, cells were fixed for 30 min at room temperature with 3% paraformaldehyde PBS and rinsed several times with HBSS-Hepes. The second rinse contained 50 mM glucose to quench any unreacted formaldehyde. The washed cells were then treated with appropriate preimmune or immune IgG (20 μg/ml) in HBSS-Hepes containing 1% BSA for 40 min at 4°C and then rinsed three times with HBSS-Hepes. The cells were then treated for 30 min with 100-fold diluted rhodamine-conjugated goat anti-rabbit IgG in HBSS-Hepes containing 1% BSA at 4°C, washed several times with HBSS-Hepes, and mounted in 90% glycerol containing 10 mM P-phenylenediamine. Direct immunofluorescence cells were processed exactly as above except that immune IgG conjugated to an appropriate fluorochrome were used. Cells were examined with Zeiss II fluorescence microscope (Carl Zeiss Inc.) equipped with epillumination. Fluorescence was excited with an OSMAN HBO 50-W bulb. Phase and fluorescence pictures were taken using an Ektachrome ASA-400 film. Staining for ankyrin did not require permeabilization of cells because paraformaldehyde-fixed cells permeabilized by exposure to 0.1% Triton X-100 for 2 min were indistinguishable from unpermeabilized cells in terms of the intensity and distribution of fluorescence.

Results

MEL Cells Undergo Erythroid Differentiation and Enucleation on Fibronectin-coated Dishes

MEL cells, growing in suspension culture, attach tightly and specifically to dishes coated with fibronectin, but not to dishes coated with other extracellular matrix proteins, such as collagen types I, III, and IV, laminin, vitronectin, and cartilage proteoglycans (18, 35). The remarkable specificity of MEL cell adhesion to fibronectin suggested to us that such interactions might play an important role in the erythroid differentiation process. To examine this possibility, cells in suspension culture or attached to Fn-coated dishes were induced by DMSO. After 4 d of induction, culture medium was replaced with fresh, DMSO-free medium and the cells were incubated for an additional 3 d. Initially, we examined the morphology of Wright/Giemsa-stained cells, noting the distinct cell types that were distinguished on the basis of overall size, size of the nucleus, and the location of the nucleus within the cell. Erythroid differentiation is normally accompanied by a dramatic decrease in cell size and condensation of the nucleus. Uninduced cultures consisted predominantly of large cells with a large nucleus (Fig. 1 and Table II). After DMSO induction for 4 d in suspension culture, most of the cells were hemoglobinized and <70% were small, containing a centrally located, condensed nucleus (Table II). Incubation of suspension-cultured cells beyond 5 d resulted in the lysis of the terminally differentiated population of cells and overgrowth of cells that were uncommitted to undergo differentiation. Enucleating cells (type IV) or reticulocytes (type V) were not detected in suspension-induced cells.

Attachment of MEL cells to fibronectin by itself did not induce erythroid differentiation, because these cells continued to proliferate and migrate on the Fn-coated dishes for several days. When growing, these cells exhibited impressive stress fibers, but after 4 d of DMSO treatment most of the cells lost stress fibers, became distinctly smaller in size, and

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### Table 1. Loss of Adhesion to Fibronectin during MEL Cell Differentiation on Fibronectin-coated Dishes

| Days of Induction | Number of cells Unattached | Number of unattached cells that reattached to Fn-coated dishes |
|-------------------|---------------------------|----------------------------------------------------------------|
| 0                 | 8 ± 4                     | 85 ± 5                                                          |
| 2                 | 18 ± 5                    | 80 ± 6                                                          |
| 4                 | 42 ± 7                    | 51 ± 4                                                          |
| 6                 | 60 ± 5                    | 32 ± 6                                                          |
| 7                 | 80 ± 7                    | 16 ± 3                                                          |

Cells were plated on Fn-coated dishes and induced by DMSO. The unattached and the attached populations of cells were recovered from these dishes and counted in a Coulter counter (Coulter Electronics Inc., Hialeah, FL). In this experiment, the unattached populations of cells were obtained after 2, 4, 6, and 7 d of differentiation were washed once with the induction medium and incubated on freshly prepared Fn-coated dishes at 37°C in a CO2 incubator. After 1 h, the unattached and the attached populations of cells were recovered and counted as above. In all cases, data presented are mean ± SEM obtained from three replicate dishes. ND = not done.

### Detection of Proteins by Western Blotting

Polypeptides were transferred from SDS-polyacrylamide slab gels to nitrocellulose for detection of Band 3, spectrin, and ankyrin with specific antisera (46). After electrophoresis, gels were soaked for 15 min in transfer buffer (20 mM Tris, 150 mM glycine, 0.03% SDS, 20% isopropanol, pH 8.3), and electrophoretically transferred to a nitrocellulose sheet (BA 85; Schleicher & Schuell, Inc., Keene, NH) in transfer buffer at 0.2 A for 12 h at 4°C. The sheets were then washed sequentially with 10% isopropanol, distilled water, and Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Protein-free sites on the nitrocellulose sheets were then blocked by incubating the sheets with Buffer C (20 mM Tris-HCl, 150 mM NaCl, 5% BSA, pH 7.5) for 2 h at 37°C. The sheets were then incubated with 100-fold diluted antiserum in Buffer C containing 0.2% NP-40 for 12 h at 4°C on a rocking platform, washed several times with Tris-buffered saline, incubated for 1 h in Buffer C at 37°C, and reacted with iodinated protein A (0.1 μCi/ml [35 μCi/μg protein A; Amersham Corp., Arlington Heights, IL]) in a buffer containing 0.2% NP-40 for 2 h at 24°C. The sheets were then washed with Tris-buffered saline, air dried, and exposed to XAR x-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 12 h at ~70°C. To determine the efficiency of protein transfer under the transfer conditions used here, ~10 μg of iodinated erythrocyte membrane protein was subjected to SDS-PAGE on a separate gel and electrophoretically transferred to nitrocellulose. Determining the radioactivity in Band 3 and spectrin polypeptide bands excised from the dried gel and the nitrocellulose sheet revealed that >85% of each of these proteins was transferred to nitrocellulose. To quantitate Band 3, spectrin, and ankyrin in MEL cells, we loaded precisely controlled numbers of erythrocyte ghosts and MEL cells side by side on the same gel and subjected them to immunoblotting. Band 3, spectrin, and ankyrin bands were localized on the nitrocellulose sheets, excised, and counted in a gamma counter. Because the amount of radioactivity associated with these standard erythrocyte proteins was linearly proportionate (over a sixfold range) to the number of erythrocyte ghosts added to the gel lane, calibration curves were constructed for each of these proteins to calculate its concentration in MEL cells.

### Microscopy

Cells washed three times with HBSS-Hepes were plated on poly-L-lysine-coated 16-chamber glass slides (50,000 cells/0.5 cm² square chamber; LABTEK, Miles Laboratories Inc., Elkhart, IN). All cells attached to the glass within 15 min at 4°C and no differences were observed between uninduced and induced populations of cells with respect to their adhesion to poly-L-lysine-coated glass. Cells were then stained with Wright/Giemsa and examined under a Zeiss II light microscope (Carl Zeiss Inc., Thornwood, NY). At least 200 cells per sample were counted in randomly chosen fields. These cells were classified as described in the legend to Table I. In some experiments, cells were also examined without staining under phase optics to verify that the distinct morphology exhibited by differentiated cells was
Figure 1. Photomicrographs of Wright/Giemsa stained uninduced and induced MEL cells. Cells were incubated in the presence or absence of DMSO on Fn-coated dishes as described in Materials and Methods. (a) Uninduced cells growing on fibronectin for 4 d. (b) Unattached population of cells recovered after 4 d of differentiation. (c) Cells that detached during 4-7 d of differentiation. Here, the arrows point to reticulocyte-like cells. Bar, 15 μm.
In suspension induction I I ili IV V VI

On fibronectin typical of mature erythrocytes. Type I cells always retain their ability to attach to Fn-coated dishes and a threefold increase in this population of cells occurs 6 d later. Large uninduced cells with a large nucleus; type II, small cells with a condensed nucleus located centrally; type III, same as type II but with an eccentrically located nucleus; type IV, enucleating cells; type V, enucleated cells with reticular cytoplasm that is characteristic of reticulocytes; and type VI, biconcave shaped cells in suspension culture reflect overgrowth of cells that were uncommitted to differentiate on day 4. Data presented are mean ± SEM obtained from three separate experiments.

Cells were stained with Wright/Giemsa stain and examined under the microscope. At least 200 cells were counted per sample and classified as follows: type I, large uninduced cells with a large nucleus; type II, small cells with a condensed nucleus located centrally; type III, same as type II but with an eccentrically located nucleus; type IV, enucleating cells; type V, enucleated cells with reticular cytoplasm that is characteristic of reticulocytes; and type VI, biconcave shaped cells.

Table II. Morphology of MEL Cells Induced in Suspension Culture and on Fn-coated Dishes

| Induction | Days of induction | Number of cell types in the total population |
|-----------|------------------|--------------------------------------------|
|           |                  | I   | II  | III | IV  | V   | VI |
| In suspension | 0              | 70 ± 8 | 25 ± 6 | <5  | 0   | 0   | 0  |
|            | 4              | 20 ± 7 | 70 ± 9 | <5  | 0   | 0   | 0  |
|            | 6              | 60 ± 6 | 30 ± 8 | <5  | 0   | 0   | 0  |
| On fibronectin | 0              | 75 ± 4 | 20 ± 8 | <5  | 0   | 0   | 0  |
|            | 4              | 18 ± 8 | 62 ± 6 | 15 ± 3 | <5 | 0   | 0  |
|            | 6              | 15 ± 6 | 19 ± 3 | 30 ± 4 | 16 ± 5 | 18 ± 4 | 0  |
|            | 7              | 18 ± 4 | 7 ± 4 | 16 ± 6 | 24 ± 6 | 28 ± 7 | 8 ± 3 |

Table III. Differentiation of MEL Cells into Reticulocytes Requires a Fibronectin Matrix during Both the Initial and Later Stages of Development

| Initial induction for 4 d | Cells subsequently plated on: | Percent cells in the total population |
|--------------------------|-------------------------------|-------------------------------------|
|                          | Enucleating cells | Reticulocytes |
| In suspension BSA-coated dishes | 0 | 0 |
| Fc-coated dishes | 0 | 0 |
| On fibronectin BSA-coated dishes | 7 ± 3 | 5 ± 3 |
| Fc-coated dishes | 32 ± 7 | 28 ± 5 |

Cells induced in suspension culture for 4 d and the unattached population of cells obtained after 4 d of differentiation on Fc-coated dishes were incubated for an additional 2 d in BSA-coated dishes (cells in suspension) and Fc-coated dishes. Cells were then stained with Wright/Giemsa stain and at least 200 cells were counted per sample. Data presented are mean ± SEM obtained from five replicate dishes.

Overcrowding of culture dishes with proliferating cells and lack of available attachment sites on the dishes may contribute to the partial detachment of cells from the dishes during the initial 4 d of differentiation. However, the majority of cells lose their capacity to adhere to fibronectin only after 6-7 d of differentiation (Table I). At which time most of the nonadherent cells in the culture lack the 120-140-kD cell surface fibronectin receptor found in the uninduced cells (data not shown; see reference 35). Thus, cells induced on Fc-coated dishes detach and can reattach to fibronectin during the initial 4 d of differentiation.

To determine whether differentiation of MEL cells into reticulocytes requires attachment to the fibronectin matrix during the initial or latter stages of development, we performed the following experiment. Cells in suspension culture or attached to Fc-coated dishes were induced by DMSO. After 4 d of induction, suspension-induced cells and the population of cells detached from the Fc-coated dishes were incubated for 2 additional days in suspension culture (i.e., BSA-coated dishes) or in Fc-coated dishes. As shown in Table III, cells that were initially induced for 4 d in suspension culture did not differentiate into reticulocytes, neither in BSA- nor Fc-coated dishes. This was not unexpected because most of the cells after 4 d of differentiation in suspension culture lack the fibronectin receptor and do not adhere to fibronectin (35, 36). The behavior of cells initially induced for 4 d on fibronectin was strikingly different; after 2 more days of incubation ~6% of these cells were enucleating or had enucleated in suspension culture, and ~30% were enucleating or had enucleated in Fc-coated dishes (Table III). Thus, as judged by morphology, full differentiation of MEL cells requires a fibronectin matrix throughout the 6 d of development.

Synthesis and Accumulation of Erythrocyte Membrane Proteins

Those observations suggested that the full developmental potential of MEL cells can be induced by a fibronectin matrix. To biochemically characterize the differentiation process, we measured by the Western blotting technique the levels of Band 3, spectrin, and ankyrin in cells induced either in suspension culture or on Fc-coated dishes. To quantitate
Figure 2. Immunoblot shows induction of Band 3 protein during MEL cell differentiation. The indicated number of erythrocyte ghosts (lanes 2–7), cells induced in suspension culture (lanes 9 and 10), and on Fn-coated dishes (lanes 13–18), and uninduced cells growing in suspension culture (lane 8) or on Fn-coated dishes (lane 12) were subjected to SDS-PAGE and immunoblotting as described in Materials and Methods. A sample of $^{125}$I-labeled erythrocyte ghosts was run as molecular weight marker (lane 1). No sample was applied to lane II. U, unattached cells; A, attached cells.

The amounts of these proteins in MEL cells relative to those in erythrocytes and to assess the variabilities in protein transfer and antibody reaction, known amounts of mouse erythrocyte ghosts were analyzed in parallel with the MEL cell samples on the same gel. The amount of $^{125}$I-labeled protein A associated with each of the erythrocyte standard protein was proportionate (over a sixfold range) to the amount of erythrocyte ghosts applied to the gel, shown here for Band 3 (Fig. 2, lanes 2–7), spectrin (Fig. 3, lanes 2–7), and ankyrin (Fig. 4, lanes 2–7). Table IV summarizes and quantifies the results of these immunoblots.

As shown in Fig. 2 (lanes 8–10) and Table IV, DMSO-induced differentiation of MEL cells in suspension culture for 4 d was accompanied by a fourfold increase in the steady-state level of Band 3 relative to uninduced cells. However, this amount of Band 3 corresponded to 33% of the Band 3 present in an equivalent number of erythrocyte ghosts (Table IV). Incubation of these cells for an additional 2 d resulted in a sharp decrease in the cellular content of Band 3 (Fig. 2, lane 10, and Table IV).

The steady-state level of spectrin in suspension cultured cells induced for 4 d nearly equaled the spectrin content in
in MEL Cells Induced in Suspension Culture and on FN-coated Dishes

Table IV. Accumulation of Erythrocyte Membrane Proteins in MEL Cells Induced in Suspension Culture and on FN-coated Dishes

| Induction | Days of induction | Band 3 | Spectrin | Ankyrin |
|-----------|------------------|--------|----------|---------|
| In suspension | 0                | 100    | 100      | 100     |
|           | 4                | 33     | 92       | 85      |
|           | 6                | 16     | 17       | 1       |
| On Fibronectin | 0                | 3      | 20       | 3       |
|           | 4 Unattached     | 54     | 90       | 91      |
|           | Attached         | 53     | 93       | 86      |
|           | 6 Unattached     | 76     | 95       | 91      |
|           | Attached         | 67     | 92       | 81      |
|           | 7 Unattached     | 81     | 98       | 97      |
|           | Attached         | 60     | 77       | 78      |

Note: Values presented are expressed as percentage of total radioactivity detected when equivalent number of erythrocyte ghosts were subjected to SDS-PAGE and immunoblotting.

Localization of Band 3 and Ankyrin in MEL Cells Differentiating on Fibronectin Matrix

During enucleation of the normal mammalian erythroblast, the erythrocyte membrane sialoglycoproteins and spectrin are retained by the reticulocyte (17, 42). To localize Band 3 and ankyrin in differentiating cultures of MEL cells on Fn-coated glass slides, cells were fixed with paraformaldehyde and incubated with the appropriate antibody. The bound antibody was then detected by fluorescence microscopy using rhodamine-conjugated goat anti–rabbit IgG. No fluorescence was detected when cells at any stage of development were incubated with either the preimmune IgG plus the secondary antibody or the secondary antibody alone (not shown). Band 3 was barely detectable in uninduced MEL cells (Fig. 5 b), a result consistent with the immunoblotting data (Fig. 2, lane 12, and Table IV). After 4 d of differentiation, cells expressed high levels of Band 3 and it was uniformly distributed throughout the cytoplasm in both the fibronectin-attached (Fig. 5 c) and unattached population of cells (not shown). Some cells exhibited punctate clusters of Band 3. After 7 d of differentiation, most of the Band 3 was sequestered in the cytoplasmic fragment of the emerging reticulocyte (Fig. 5 f).

By contrast, a low but detectable level of ankyrin was present in uninduced MEL cells (Fig. 6 b). As with Band 3, ankyrin was uniformly distributed in the cytoplasm after 4 d of differentiation (Fig. 6 d), and was sequestered in the cytoplasmic fragment of the enucleating cell after 7 d of differentiation (Fig. 6 f). To localize Band 3 and ankyrin in the same population of enucleating MEL cells, fixed cells were first incubated with FITC-conjugated rabbit anti–Band 3 IgG followed by rhodamine-conjugated rabbit anti–ankyrin IgG. Immunofluorescent micrographs shown in Fig. 7 demonstrate that both Band 3 (Fig. 7 b) and ankyrin (Fig. 7 c) become sequestered in the cytoplasmic fragment of the emerging reticulocyte during enucleation. Note that some enucleating cells totally lack Band 3 in the membrane surrounding their nucleus (upper two arrows in Fig. 7 b), whereas others exhibit a rim of fluorescence around the nucleus (Fig. 5 f). We
Figure 3. Immunoblot analysis of uninduced and induced MEL cells with anti-spectrin antibody. The indicated number of erythrocyte ghosts (lanes 2–7), cells induced in suspension culture (lanes 9 and 10) and on Fn-coated dishes (lanes 13–18), and uninduced cells growing in suspension culture (lane 8) or on Fn-coated dishes (lane 12) were subjected to SDS-PAGE and immunoblotting. A sample of 125I-labeled erythrocyte ghosts was run as molecular weight marker (lane 1). No sample was applied to lane II. Note that the antisera used here has a higher reactivity against α-spectrin than β-spectrin. U, unattached cells; A, attached cells.

believe that these two populations of enucleating cells represent cells at the two extreme stages of the enucleation process. In a separate experiment, distribution of spectrin in differentiating MEL cells was examined by immunofluorescence microscopy. As with Band 3 and ankyrin, spectrin was also sequestered within the reticulocyte portion of the enucleating cell (not shown). Thus, the membrane remodeling process observed in the enucleating MEL cell mimics the process that occurs during enucleation of a normal mammalian erythroblast; all three erythrocyte membrane proteins remains with the emerging reticulocyte.

**Discussion**

We have shown that a fibronectin matrix promotes complete terminal erythroid differentiation of MEL cells in vitro. Cells induced in suspension culture do not undergo enucleation, but become arrested at a late erythroblast stage. Whereas the amounts of spectrin and ankyrin accumulated by suspension-induced cells corresponded to those present in erythrocytes, these cells accumulated only about one-third of the Band 3 protein found in erythrocytes. In contrast, cells induced on Fn-coated dishes enucleated to become reticulocytes and accumulated amounts of Band 3, spectrin, and ankyrin comparable with those present in erythrocytes. During enucleation of MEL cells most of the Band 3 (Figs. 5 and 7) and ankyrin (Figs. 6 and 7) molecules were localized in the reticulocyte portion of the cell.

A major limitation of MEL cells induced in suspension culture has been their inability to undergo complete terminal erythroid differentiation. The most differentiated population of cells generally tend to lyse before the enucleation stage (32, 49, 51). Volloch and Housman (52) demonstrated that the lysis of these cells can be prevented by supplementing the culture medium with 5% BSA. We have confirmed that differentiation of MEL cells in the presence of BSA gives rise to cultures in which most of the cells are small, with a condensed nucleus, and contain high levels of hemoglobin, whereas differentiation in the absence of BSA gives rise to cultures in which most of the cells are large, with a large nucleus, and contain little hemoglobin (data not shown). However, this maneuver was insufficient to stabilize the most ma-
Figure 4. Immunoblot shows induction of ankyrin during MEL cell differentiation. The indicated number of erythrocyte ghosts (lanes 2-7), cells induced in suspension culture (lanes 9 and 10) and on Fn-coated dishes (lanes 13-18), and uninduced cells growing in suspension culture (lane 8) or on Fn-coated dishes (lane 12) were subjected to SDS-PAGE and immunoblotting as described in Materials and Methods. A sample of $^{125}$I-labeled erythrocyte ghosts was run as molecular weight marker (lane 1). No sample was applied to lane 11. U, unattached cells; A, attached cells.

We showed that this blockade in the complete differentiation of MEL cells can be overcome by inducing cells with DMSO on Fn-coated dishes. After 7 d of differentiation on Fn, well over half of the cells were enucleating or had enucleated and ~8% of the cells had differentiated into cells with a morphology characteristic of mature erythrocytes (Table II). Not all the cells responded to the effects of DMSO, because roughly 20% of the cells in this culture exhibited morphology typical of uninduced cells after 7 d (Table II). The problem of DMSO-resistant cells exists even in a cloned population of MEL cells, as has been previously reported (49, 52).

MEL cells do not attach to other extracellular matrix proteins, such as collagen types I, III, and IV, laminin, and vitronectin (18, 35). As expected, MEL cells do not differentiate into reticulocytes, upon induction by DMSO, on dishes coated with collagen types I and III (data not shown).
Figure 5. Distribution of Band 3 protein in MEL cells induced to differentiate on Fn-coated glass slides. Fixed cells were treated sequentially with rabbit anti-Band 3 antibody and rhodamine-conjugated goat anti-rabbit IgG. (a) Phase-contrast micrograph of uninduced cells attached to fibronectin. (b) Immunofluorescence micrograph of the same field as in a. (c) Phase-contrast picture of the Fn-attached population of cells after 4 d of differentiation. Note dramatic decrease in cell size. (d) Same field as in c, showing fairly uniform distribution of Band 3 over the cytosol. (e) Phase-contrast picture of the unattached population of cells after 7 d of differentiation. Here, arrows point to the enucleating cells. (f) Same field as in e, showing segregation of Band 3 away from the nucleus in enucleating cells. Exposure time to photograph b, d, and f was 16 s. Bar, 23 μm.

accompanied by a progressive decrease in cell adhesion (Table I). Cells that detached from the dishes between 1 and 4 d of differentiation were able to reattach to Fn (Table I). Many of these cells also differentiated into reticulocytes when incubated for an additional 2 d on Fn-coated dishes, but not in suspension culture (Table III). In contrast, cells that were initially induced for 4 d in suspension culture did not differentiate into reticulocytes when incubated for another 2 d on fibronectin (Table III). Thus, cells induced on Fn-coated dishes may detach and reattach to Fn throughout 6 d of dif-
Figure 6. Distribution of ankyrin in MEL cells differentiating on Fn-coated glass slides. Fixed cells were treated sequentially with rabbit anti-ankyrin antibody and rhodamine-conjugated goat anti-rabbit IgG. (a) Phase-contrast micrograph of uninduced cells attached to Fn. (b) Immunofluorescence micrograph of the same field as in a. (c) Phase-contrast picture of the Fn-attached population of cells after 4 d of differentiation. (d) Same field as in c, showing uniform distribution of ankyrin over the cytoplasm. (e) Phase-contrast picture of the unattached population of cells after 7 d of differentiation. Here, arrows point to the enucleating cells. (f) Same field as in e, showing segregation of ankyrin away from the nucleus in the enucleating cells. Exposure time to photograph b, d, and f was 25 s. Bar, 23 µm.

Differentiation, and importantly, an interaction with this matrix appears to be essential for full differentiation of MEL cells. Cells that detached from the dishes between 6 and 7 d of differentiation consisted mostly of enucleating cells, reticulocytes, and erythrocytes, whereas cells that remained attached to the dishes were uninduced cells and cells at intermediate stages of development (data not shown). This is consistent with our previous finding that, during induction of suspension MEL cells, loss of adhesion to fibronectin was correlated with the loss of cell surface fibronectin receptor (36). This mimics the release of reticulocytes from the bone marrow matrix into the circulation (35, 36).

The submembranous cytoskeleton of erythrocytes is composed of α- and β-spectrin dimers linked, at one end, to the
Figure 7. Localization of Band 3 and ankyrin in enucleating MEL cells by direct immunofluorescence microscopy. a, b, and c represent the same field: (a) phase-contrast picture of enucleating cells (arrows); (b) cells treated with FITC-conjugated rabbit anti-Band 3 IgG; (c) cells treated with rhodamine-conjugated rabbit anti-ankyrin IgG. Bar, 22 μm.

Synthesis of these membrane proteins is induced upon DMSO treatment of MEL cells (19, 35, 37, 41).

Cells induced in suspension culture for 4 d contained 33% of the amount of Band 3 present in erythrocytes, and incubation for an additional 2 d resulted in a sharp decrease in their Band 3 content (Table IV). This decline in Band 3 level probably reflects the lysis of the most mature cells in these cultures (Table II). On the other hand, cells induced on Fn-coated dishes continued to accumulate Band 3 throughout 7 d of culture, at which time these cells contained ~81% of the amount of Band 3 present in erythrocytes (Table IV). At what level the expression of Band 3 is regulated by the fibronectin matrix is not known. It is possible that Fn matrix prolongs the viability of the differentiating population of cells, thereby allowing these cells to accumulate more Band 3. It will also be important to determine whether Fn accelerates transport of Band 3 from the rough endoplasmic reticulum to the plasma membrane or stabilizes Band 3 protein or Band 3 mRNA. The amount of Band 3 mRNA does increase during at least the first 4 d of induction in suspension, in parallel with the increase in synthesis of Band 3 (25, 35), suggesting regulation at the level of mRNA accumulation.

After 4 d of differentiation, both suspension-induced cells and cells induced on Fn-coated dishes contained as much spectrin and ankyrin as those present in erythrocytes (Table IV). Thus, Fn does not influence the accumulation of spectrin and ankyrin during the first 4 d of differentiation. As with Band 3, the amount of spectrin and ankyrin decreased sharply in suspension-induced cells after an additional 2 d, whereas high levels of these proteins were maintained throughout 7 d in cells induced on Fn-coated dishes. Synthesis of spectrin and ankyrin in suspension-induced cells peaks on the fourth day of differentiation and declines thereafter (37; V. P. Patel, unpublished observations). Thus, the decline in the spectrin and ankyrin levels in suspension-induced cells between 4 and 6 d of differentiation probably results both from natural turnover of these proteins and the lysis of most mature cells in these cultures. We do not know, however, what proportion of these proteins are assembled into a cytoskeletal network similar to that in erythrocytes.

It is not clear how cells induced on Fn-coated dishes manage to maintain high levels of spectrin and ankyrin throughout 7 d of differentiation. Biogenesis of erythroid cytoskeletal proteins has been extensively studied in the avian erythroid cells (30, 33). In this system, the assembly of spectrin and ankyrin into the cytoskeleton is regulated at the posttranslational level, and the long-term stability of the assembled cytoskeleton is dependent on the Band 3 content of the cells (56). Soluble forms of spectrin (unassembled) do turn over more rapidly than the insoluble form of spectrin (assembled) in both avian cells (30, 55, 56) and in MEL cells (38; V. P. Patel, unpublished observations). We hypothesize that the extracellular fibronectin matrix promotes the accumulation of Band 3 in MEL cells and that this, in turn, stabilizes the assembled spectrin and ankyrin molecules. The cytoskeleton is thought to be a key determinant of membrane integrity in mature erythrocytes (1, 21, 31). Thus, the fragility of the suspension-induced cells, which accumulate only a small fraction of the Band 3 present in erythrocytes, may be a direct consequence of their inability to assemble a stable cytoskeleton. However, the “matrix effect” could be exerted at the level of protein 4.1, which binds spectrin to short...
oligomers of actin to form the erythrocyte cytoskeleton (9, 39, 50).

A key characteristic of the erythrocyte membrane is that all of the integral membrane and cytoskeletal proteins are cross-linked together, and the integral membrane proteins cannot diffuse laterally in the plane of the phospholipid bilayer (14, 20, 45). Several years ago Geiduschek and Singer (17) showed that most of the Con A–binding glycoproteins and spectrin remains with the reticulocyte portion of the enucleating mouse erythroblast, whereas a set of other Con A–binding glycoproteins are enriched in the plasma membrane surrounding the nucleus. Our fluorescent micrographs suggest that most Band 3 (Fig. 7 b), ankyrin (Fig. 7 c), and spectrin (not shown) remains with the cytoplasmic fragment of the enucleating MEL cells. Enrichment of these erythroid proteins could result from the displacement of the cytoplasm caused by the extrusion of the nucleus. Alternatively, the enucleation process could involve interactions of erythroid membrane proteins similar to those in mature erythrocytes. It is now possible to study this process because MEL cells can undergo full differentiation in vitro.

As noted earlier, within the bone marrow the differentiating erythroid cells are anchored within a three-dimensional matrix consisting of many fibrous proteins and proteoglycans. It is remarkable that an artificial two-dimensional matrix solely of Fn can support all of the essential aspects of erythropoiesis in vitro, and, indeed, that a tumor cell can be induced to differentiate quite normally on such a matrix.

Even stages of erythropoiesis earlier than those studied here (erythroblast to erythrocyte) can occur when cells are attached to a Fn matrix. Erythroid progenitors isolated from normal human bone marrow adhere specifically to dishes coated with Fn, and when these cells are plated on Fn-coated dishes in the presence of erythropoietin, they proliferate, migrate, differentiate, and remain associated with Fn until the enucleation stage (48). Koury et al. (27) have shown that Friend virus–infected mouse erythroblasts can differentiate into reticulocytes when they are cultured in the presence of erythropoietin in methylcellulose-containing medium. This culture system contains 30% FCS, which contains fibronectin. Moreover, cells are immobilized in these cultures due to the viscosity of methylcellulose. It would be important to determine whether adhesion of erythroid precursor cells to substrates other than fibronectin could also promote full differentiation. But MEL cells, as noted above, do not adhere to any other physiologically relevant substrates. Because MEL cells do not differentiate completely when plated in a fibronectin-free, methylcellulose-containing medium (V. P. Patel, unpublished observation), it appears that erythroid precursors prefer a fibronectin-rich microenvironment to express their full differentiation program.

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