Fibronectin–Plasma Membrane Interaction in the Adhesion of Hemopoietic Cells

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Abstract. Many hemopoietic cell lines were examined for their ability to adhere to culture dishes coated with extracellular matrix proteins. Adhesion assay was performed with murine and human leukemic cell lines representative of different stages of differentiation along both erythroid and myeloid lineages. All the hemopoietic cell lines tested adhered to fibronectin but not to laminin, types I, III, and IV collagen, serum-spreading factor, and cartilage proteoglycans. In addition to immortalized cell lines, immature erythroid and myeloid mouse bone marrow cells adhered to fibronectin. To define the fibronectin region involved in hemopoietic cell adhesion, proteolytic fragments, monoclonal antibodies, and synthetic peptides were used. Among different fibronectin fragments tested, only a 110-kD polypeptide, corresponding to the fibroblast attachment domain, was active in promoting adhesion. Moreover, a monoclonal antibody to the cell binding site located within this domain prevented hemopoietic cell adhesion. Finally, the tetrapeptide Arg-Gly-Asp-Ser, which corresponds to the fibronectin sequence recognized by fibroblastic cells, specifically and competitively inhibited attachment of hemopoietic cells to this molecule. The cell surface molecule involved in the interaction of mouse hemopoietic cells with fibronectin was identified as a 145,000-D membrane glycoprotein by adhesion-blocking antibodies. This glycoprotein was found to be antigenically and functionally related to the GP135 membrane glycoprotein involved in the adhesion of fibroblasts to fibronectin (Giancotti, F. G., P. M. Comoglio, and G. Tarone, 1986, *Exp. Cell Res.*, 163:47–62). On the basis of these data, we conclude that interaction of hemopoietic cells with fibronectin involves a specific fibronectin sequence and a 145,000-D cell surface glycoprotein. We speculate that this property might be relevant for the interaction of hemopoietic cells with the bone marrow stroma, which represents the natural site of hemopoiesis.

FIBRONECTIN is a major glycoprotein component of the extracellular matrix and is known to promote cell adhesion and to affect growth and differentiation (17). The molecular aspects of its interaction with fibroblasts have been defined in detail. The site responsible for binding with the cell surface receptor has been identified and shown to contain the active sequence Arg-Gly-Asp-Ser (28). Moreover, plasma membrane glycoproteins of ~140,000 D were identified in different fibroblast species as putative fibronectin receptors (3, 9, 13, 25, 30).

In this paper we have investigated the interaction of hemopoietic cells with fibronectin. The bone marrow stroma is thought to be required for the specific lodgment, proliferation, and differentiation of hemopoietic progenitor cells in vivo (41). The importance of bone marrow stroma is also demonstrated by in vitro cultures, in which the long term production of hemopoietic cells depends on the formation of a monolayer of bone marrow-derived stromal cells (5, 32). The bone marrow stroma consists of fibroblasts, adipocytes, and endothelial cells (38), as well as extracellular matrix proteins such as types I, III, and IV collagen, fibronectin, laminin, and proteoglycans (1, 16, 43). Since actively dividing hemopoietic progenitor cells are preferentially associated with the stromal layer of bone marrow cultures (4, 12), it is possible that certain components of this matrix play a role in the maintenance of hemopoiesis. This possibility is also suggested by the observation that inhibitors of matrix formation decrease stem cell production (44), whereas compounds that stimulate matrix production increase stem cell proliferation (34). Moreover, immunofluorescence studies of the developing bone have indicated that hemopoietic colonies are associated with fibronectin-rich areas of the bone marrow (40), suggesting a possible role of this protein in anchoring hemopoietic cells to the stroma. In accordance with this hypothesis, it was reported that murine erythroleukemia cells adhere to fibronectin in an in vitro assay (26).

In this paper we demonstrate that immature myeloid and erythroid cells can adhere to fibronectin. Adhesion occurs via the same cell attachment domain and active site of fibronectin that functions in fibroblast adhesion. Moreover, antibodies to a 145-kD plasma membrane glycoprotein, which is structurally and immunologically related to the GP135 molecule that mediates adhesion of fibroblasts to fibronectin, can prevent hemopoietic cell adhesion (9, 10).

Materials and Methods

Materials

Fibronectin was purified from human plasma by affinity chromatography on denatured collagen (gelatin), as already reported (36). Fibronectin fragments, corresponding to the Hep-1/Fib-1 (28 kD), Gel (40 kD), Cell (110 kD), and Fib-2 (20 kD) domains of the molecule, were kindly provided by L. Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genova). These were obtained by thermolysin digestion of the intact molecule and were separated by chromatography on hydroxylapatite column (42). Serum-spreading factor preparations consisted of fetal calf serum (Flow Laboratories, Inc., McLean, VA) adsorbed on Sepharose-coupled gelatin to remove fibronectin and on Affi-gel Blue (Bio-Rad Laboratories, Richmond, CA) to remove albumin (14). These preparations were shown to be devoid of fibronectin by radioimmunoassay (37) and effectively promoted attachment of murine fibroblasts in a dose-dependent manner also in the presence of fibronectin antibodies. Laminin was purchased from Bethesda Research Laboratories (Gaithersburg, MD), bovine dermal collagen (95% type I + 5% type III) was from Flow Laboratories (Vitrogen 100), and human placenta basement membrane collagen (type IV) and gelatin (swine skin denatured collagen) were from Sigma Chemical Co. (St. Louis, MO). Bovine nasal and chick sternal cartilage proteoglycans were a kind gift of M. Pacifici (University of Pennsylvania, Philadelphia). The cell attachment promoting fibronectin peptide Arg-Gly-Asp-Ser and the inactive one, Gly-Arg-Gly-Glu-Ser-Pro, were kind gifts of H. Richter (Max Planck Institute, Martinsreed) and E. Dejana (Istituto "M. Negri," Milano), respectively. Substance P (Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH_2), also used as control peptide, was purchased from UCB-Bioproducts S. A. (Braine-L'Alleud, Belgium).

Cells

Murine hemopoietic cell lines included Friend erythroleukemia clone 745 E and WEHI-3B D+ myelomonocytic leukemia cells (24). These were provided by G. B. Rossi (Istituto Superiore di Sanità, Roma) and W. Piacibello (Università di Torino, Italy), respectively. Human hemopoietic cells included the following leukemia cell lines: K-562 (undifferentiated), KG-1 (myeloblastic), HL-60 (promyelocytic), and U937 (monoblastic-monocytic) (7). KG-1 and K-562 were obtained from American Type Culture Collection (Rockville, MD), HL-60 were provided by C. Tarella (Ospedale Maggiore "S. Giovanni Battista," Torino, Italy), and U937 by F. Malavasi (Università di Torino, Italy). The KG-1 line was grown in Iscove's modified Eagle's medium supplemented with 20% fetal calf serum. All the other hemopoietic cell lines were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum. The Hep-G2 human hepatoma cell line, obtained from Dr. G. Actis (Ospedale Maggiore "S. Giovanni Battista," Torino, Italy), SR-BALB mouse fibroblasts (9), and baby hamster kidney (BHK)[1] cells are routinely cultured in our laboratory in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Murine bone marrow cells were prepared by flushing femur shafts with RPMI 1640 medium plus 5% fetal calf serum. Debris were sedimented at 1 *g*, and cells were washed three times with serum-free RPMI.

Antibodies

The anti-BHK serum was prepared by injecting rabbits with intact cells, as previously described (35). Before use, the antiserum was adsorbed on Sepharose-coupled hamster fibronectin to remove the corresponding antibodies (36). An IgG fraction of this antiserum was prepared by chromatography on Sepharose-coupled protein A (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ). Monospecific antibodies to GP135 were purified from the anti-BHK IgGs using the antigen immobilized on nitrocellulose filter, as previously described (10). Briefly, [^{35}S]methionine-labeled cell surface proteins were immunoprecipitated from 1×10^8 SR-BALB mouse fibroblasts with the anti-BHK serum, separated by SDS PAGE under nonreducing conditions, and electrophoretically transferred to nitrocellulose paper by Western blotting (see below). The nitrocellulose fragment corresponding to GP135 was localized by autoradiography and excised. After saturation with 5% bovine serum albumin (BSA), the nitrocellulose fragment was incubated with 20 mg/ml of anti-BHK IgGs. After extensive washing with phosphate-buffered saline (PBS), bound antibodies were eluted in 100 μl of a pH 3 buffer (28.5 mM citric acid, 29 mM potassium phosphate, 0.52% barbital, and 19 mM boric acid) containing 10 μg/ml of carrier BSA and immediately neutralized. Antibodies from 10 purification cycles were pooled and concentrated. A typical preparation consisted of ~50 μg of purified antibodies. Each batch was tested for specificity by immunoblotting on a crude membrane fraction of SR-BALB mouse fibroblasts, as previously described (10).

The monoclonal antibody f 33, directed toward the cell binding site of fibronectin (33), was kindly provided by V. Miggiano (Hoffmann-LaRoche, Basel) and S. Barlati (Università di Brescia, Italy). The monoclonal antibody MAB 52, reacting with the gelatin binding region of fibronectin, was isolated in our laboratory (37).

Adhesion Assay

Adhesion to coated dishes was measured using a previously described assay (9, 36). In experiments designed to compare the adhesion-promoting activities of different matrix proteins, culture wells were coated for 1 h at room temperature with PBS containing 25 μg/ml fibronectin, laminin, collagens, or proteoglycans. Serum-spreading factor preparations were diluted 1:200 (final protein concentration ~500 μg/ml). Fibronectin fragments were used at 25 μg/ml. In all other experiments, fibronectin was bound to the wells by taking advantage of its affinity for denatured collagen. In this case culture wells were coated with 2 mg/ml gelatin, rinsed with PBS, and further incubated with the indicated concentrations of fibronectin. As shown in the Results section, gelatin coating alone did not support adhesion of hemopoietic cells. Adsorption of the matrix proteins to the dishes was checked by measuring adhesion of the proper cell types. Adsorption of cartilage proteoglycans was evaluated by antibodies to the proteoglycan core protein (2). About 0.5 μg/cm^2 of protein was absorbed to the plastic surface when coating was performed with 25 μg/ml. Cells were treated for 1 h before harvesting with 20 μM cycloheximide and plated in serumless medium containing 20 μM cycloheximide and 1 μM monensin to prevent synthesis and secretion of cellular adhesive factors (9). After incubation for 1 h at 37°C in a humidified atmosphere containing 5% CO_2, unbound cells were removed by rinsing with PBS and adherent cells were fixed and stained with May-Grünwald Giemsa dye. To quantify adhesion, the light absorbance of the stained cells on the microtiter well surface was measured by an automated photometer (Titertek, Elfab Oy, Finland). We quantitated adhesion to Terasaki microtest wells (Falcon, Div. of Becton Dickinson, Oxnard, CA) by directly counting the cells on photomicrographs that cover the entire adhesion surface. To evaluate activity of the antibodies and peptides described above, these were added to the adhesion medium at the beginning of the assay.

Adhesion of fresh bone marrow cells was tested as described above. To quantify the adherent cells, these were detached by 0.01% trypsin plus 1 mM EDTA and counted. Cytospin smears were prepared from aliquots of both the adherent and nonadherent cell fractions, and morphological analysis was performed after fixation in methanol and staining with May-Grünwald Giemsa dye.

Antibody Adsorption Experiments

Affinity-purified GP135 antibodies were adsorbed on many cell surface antigens that were immobilized on nitrocellulose paper (10). For this purpose, whole membrane proteins (250 μg of proteins) from Friend cells were prepared, separated by SDS PAGE, and electrophoretically transferred to nitrocellulose filters as described below. GP145 isolated by immunoprecipitation and purified mouse plasma fibronectin were also bound to nitrocellulose by the same procedure. The filters were saturated with 5% BSA and cut in 2-mm slices. Each slice, with 5–10 μg of bound antigen, was incubated with 50 μl of medium containing 1 μg of affinity-purified GP135 antibodies. After 2 h at room temperature, the supernatant was collected, concentrated, and tested in adhesion assay.

Immunoprecipitation and Immunoblotting of Membrane Proteins

Membrane proteins were immunoprecipitated from metabolically labeled cells. Metabolic labeling was achieved by incubating Friend and WEHI-3B cells at a density of 5×10^5/ml in complete medium containing 50 μCi/ml of [^{3}H]6-D-glucosamine (20 Ci/mM, Amersham Corp., Arlington Heights, IL) or in methionine-free medium containing 40 μCi/ml of [^{35}S]methionine (800 Ci/mM, New England Nuclear, Boston, MA) for 15 h. Subconfluent monolayers of SR-BALB fibroblasts were incubated with 25 μCi/ml of [^{35}S]methionine. Cell surface proteins were immunoprecipitated from intact cells (9, 10, 36) by incubating the cell suspension with anti-BHK serum or control preimmune serum (10 μl with 2×10^6 cells) for 1 h at

1. *Abbreviation used in this paper*: BHK, baby hamster kidney.

Table I. Adhesion of Hemopoietic Cell Lines to Fibronectin

Cell line	Fn	Lam	SSF	Coll.I/III	Coll.IV	Gel	PG
Friend (proEb)	+	−	−	−	−	−	−
WEHI-3B D+ (myelo/mono)	+	−	−	−	−	−	−
K562 (undiff. blasts)	+	−	−	−	−	−	−
KG-1 (mybl/proM)	+	−	−	−	−	−	−
HL-60 (proM)	+	−	−	−	−	−	−
U937 (monoB/mono)	+	−	−	−	−	−	−
BHK (fibroblasts)	+	+	+	±	±	−	−
Hep-G2 (hepatoma)	−	+	−	+	+	±	−

Cells were plated on culture dishes coated with fibronectin (Fn), laminin (Lam), collagen types I and III (Coll.I/III), collagen type IV (Coll.IV), serum-spreading factor (SSF), chick sternal cartilage proteoglycan (PG), and gelatin (Gel), as described in details in the Materials and Methods section. Cell adhesion was evaluated after 1 h at 37°C. +, indicates adhesion of >70% of the plated cells. ±, indicates adhesion of 30–50% of the plated cells. −, indicates that <10% of the plated cells attached. proEb, proerythroblasts. myelo, myelocytes. mono, monocytes. undiff. blasts, undifferentiated blasts. mybl, myeloblasts. proM, promyelocytes. monoB, monoblasts.

4°C with gentle agitation. Unbound antibodies were then removed by washing, and cells were extracted for 20 min at 0°C by Triton X-100 (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5% Triton X-100 plus 2 mM phenylmethylsulfonyl fluoride, 6 μM pepstatin, and 100 μM leupeptin as protease inhibitors). After centrifugation at 10,000 *g* for 30 min, soluble immunocomplexes were recovered by adsorption on protein A-Sepharose beads (Pharmacia Fine Chemicals). After washing, bound material was eluted by boiling beads in 1% SDS and analyzed by SDS PAGE. This was carried out in 5–10% acrylamide slab gels using the procedure described by Laemmli (18). When necessary, gels were processed for fluorography as described by Laskey et al. (19), dried, and placed in contact with Kodak-X-Omat SO-282 film. Molecular weight markers (under reducing conditions) were: myosin (200,000), phosphorylase B (93,000), BSA (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,000).

Immunoblotting analyses were performed on a crude membrane fraction. For this purpose, Friend cells were broken by Dounce homogenization in hypotonic buffer (20 mM Tris-HCl, pH 7.4). After removal of nuclei by low gravity sedimentation, membrane fragments were collected by centrifugation at 100,000 *g*. The membrane pellet was solubilized by boiling in SDS, and proteins were separated by SDS PAGE and transferred to nitrocellulose paper by Western blotting, according to the procedure of Towbin et al. (39). Immunostaining of blotted membrane proteins with the anti-BHK or the GP135 affinity-purified antibodies was performed as described previously (10). Due to the limited amount of affinity-purified GP135 antibodies available, immunostaining with these antibodies was performed on a strip 2-mm wide covering the entire length of the lane that was cut out of the nitrocellulose paper. The strip was incubated with 100 μl of buffer containing 50 μg/ml antibodies in a humidified chamber. After extensive washing, bound antibodies were revealed by ^{125}I-labeled protein A.

Results

Adhesion of Hemopoietic Cells to Fibronectin

The ability of a number of different hemopoietic cell lines to interact with various matrix components was evaluated by an adhesion assay (9, 36). Lines tested included human and murine leukemic cells representative of different lineages and levels of differentiation (Table I). Cells were plated in serumless medium on dishes precoated with a saturating concentration (25 μg/ml) of fibronectin, laminin, serum spreading factor, types I, III, and IV collagen, gelatin, and cartilage proteoglycan. Most of these components are known to be present in the bone marrow stroma (1, 16, 43). To block synthesis and secretion of cellular adhesive factors, cells were treated with cycloheximide and monensin (see Materials and Methods section). All the hemopoietic cell lines tested adhered to fibronectin but failed to attach to the other extracellular matrix proteins (Table I). Adhesion parameters were studied with two murine cell lines representative of both the erythroid (Friend) and the myeloid (WEHI-3B) lineage. The number of adherent cells reached a plateau level by 90 min at 37°C, and the extent of adhesion was proportional to the amount of fibronectin used to coat the dishes. Both Friend (Fig. 1) and WEHI-3B cells responded to a threshold dose of 1.5 μg/ml fibronectin and showed maximum adhesion on dishes coated with 25 μg/ml fibronectin. Attachment was always followed by a degree of spreading, which varied for the different cell lines. Fig. 2 shows the results obtained with Friend and WEHI-3B cells.

To verify whether adhesion to fibronectin was a peculiar property of leukemic cells adapted to in vitro culture, mouse bone marrow cells were tested. A significant proportion of the nucleated cells (45 ± 7.4%) adhered to fibronectin (Table II). Morphological examination indicated that immature myeloid and erythroid cells were preferentially recovered in the adherent fraction. On the other hand, most of fully differentiated cells did not attach to the coated dish. A negligible number of bone marrow cells adhered to dishes coated with laminin or gelatin. It was thus concluded that adhesion to fibronectin is a general property of immature hemopoietic cells.

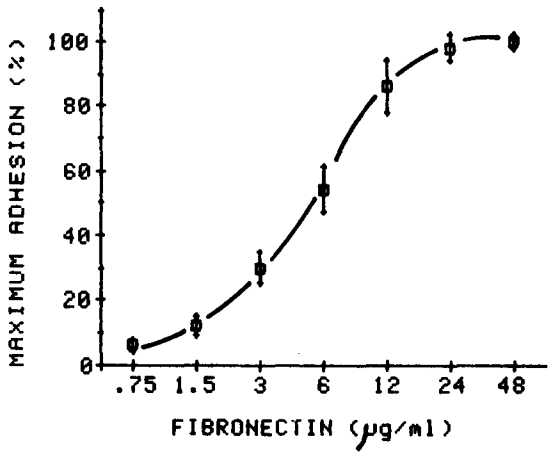

Figure 1. Adhesion of Friend cells to dishes coated with different amounts of fibronectin. Friend cells were plated on dishes coated with 2 mg/ml gelatin followed by the indicated concentrations of fibronectin. After 1 h at 37°C, attached cells were fixed, stained, and counted as specified in the Materials and Methods section. Adhesion values are expressed as percentage of the maximum adhesion and represent the mean of three different experiments.

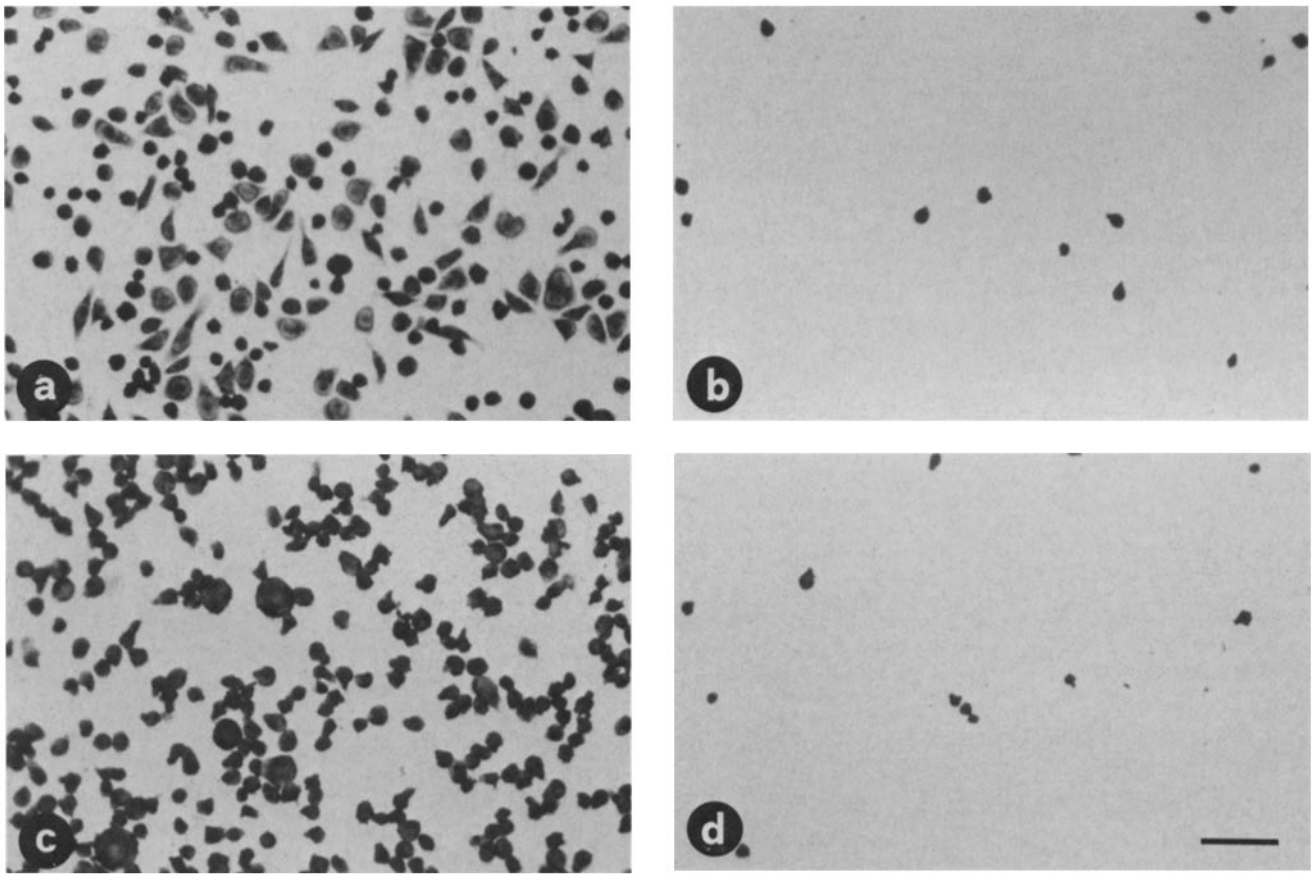

Figure 2. Adhesion of Friend and WEHI-3B cells to fibronectin and laminin. Friend (*a* and *b*) and WEHI-3B (*c* and *d*) cells were plated on dishes coated with 25 μg/ml fibronectin (*a* and *c*) or laminin (*b* and *d*). After 1 h at 37°C, attached cells were fixed and stained with May-Grünwald Giemsa. Bar, 25 μm.

Interaction of Hemopoietic Cells with the Cell-attachment Site of Fibronectin

To assign the hemopoietic cell adhesion promoting activity to a specific fibronectin domain, we have evaluated the adhesion of Friend and WEHI-3B cells on a number of purified fibronectin fragments. These were obtained by thermolysin digestion of the intact molecule (42) and consisted of a 110-kD fragment containing the cell-binding region, a 40-kD peptide spanning the collagen binding site, and two fragments of 28 and 20 kD with affinity for heparin and fibrin, respectively. As shown in Fig. 3, Friend cells adhered on dishes coated with the purified 110-kD cell binding domain, but failed to adhere significantly to the other fibronectin fragments. The extent of adhesion and spreading on the 110-kD domain was comparable to that observed on the intact fibronectin molecule. Similar results were also obtained with WEHI-3B cells. These data indicate that the 110-kD domain is involved in the adhesion of hemopoietic cells. Characterization of the interaction of hemopoietic cells with fibronectin was extended using a monoclonal antibody (MAB f33) that recognizes the cell binding site of fibronectin and prevents fibroblast adhesion (33). As shown in Fig. 4, *a* and *b*, this antibody blocked the adhesion of both Friend and WEHI-3B cells to fibronectin, while the control antibody MAB 52, which recognizes the gelatin binding domain of fibronectin (37), had no effect. Inhibition with the monoclonal antibody f33 was dose dependent and occurred up to a dilution of 10 μg/ml.

A further analysis of the interaction of hemopoietic cells with fibronectin was performed using the synthetic cell attachment promoting peptide Arg-Gly-Asp-Ser (28). This peptide, added in solution to the adhesion medium together with hemopoietic cells at the beginning of the assay, could compete with substratum-bound fibronectin in a dose-dependent manner (Fig. 4, *c* and *d*). Maximal inhibition of cell adhesion was achieved with 3 mM tetrapeptide. Equivalent concentrations of an inactive esapeptide Gly-Arg-Gly-Glu-Ser-Pro (29) or of an unrelated peptide had no effect.

These results suggest that the same site of fibronectin involved in fibroblast adhesion is also involved in the adhesion of Friend and WEHI-3B cells.

Identification of the Membrane Glycoprotein That Mediates Hemopoietic Cell Adhesion to Fibronectin

To identify the membrane component involved in hemopoietic cell adhesion to fibronectin, we used an antiserum (anti-BHK) that inhibits adhesion of hamster and murine fibroblasts to fibronectin (9, 35, 36). When the anti-BHK serum was added to Friend and WEHI-3B cells at the beginning of the adhesion assay, a dose-dependent inhibition was observed (not shown).

Membrane proteins of [^{35}S]methionine-labeled Friend and

Table II. Adhesion of Bone Marrow Cells to Fibronectin

Cell type	Adherent cells
	%
Total bone marrow	45 ± 5.4
Blasts	36 ± 18.3
Promyelocytes	61 ± 4.3
Myelocytes	73 ± 6.3
Metamyelocytes	80 ± 8.6
Granulocytes	25 ± 3.8
Proerythroblasts	78 ± 22.4
Basophilic erythroblasts	70 ± 2.6
Polychromatophilic erythroblasts	58 ± 3.7
Acidophilic erythroblasts	25 ± 6.4
Erythrocytes	17 ± 3.2

4×10^6 murine bone marrow cells were plated on a 50-mm dish coated with 25 μg/ml fibronectin. After 90 min at 37°C, unbound cells were collected by rinsing, while adherent cells were detached by trypsin plus EDTA. Cells from both fractions were counted and examined for their morphology after May-Grünwald Giemsa staining. The percentage of adhesion for each morphological class was calculated according to the formula $A \times B/C \times 100$, in which A is total no. of adherent cells, B is percentage of the given cell type in adherent fraction, and C is no. of cells of a given type plated. Data are the mean ± SD from three different experiments and include values for the granulocytic and erythroid lineages.

WEHI-3B cells were immunoprecipitated with the anti-BHK serum and separated by SDS PAGE under nonreducing conditions. As shown in Fig. 5, anti-BHK antibodies specifically recognized a major protein of 145,000 D on the surface of both Friend and WEHI-3B cells, together with minor components (lanes *c* and *g*). The 145,000-D component could also be labeled with [^{3}H]glucosamine (Fig. 5, lane *e*) and is thus indicated as GP145. This glycoprotein was likely to contain intrachain disulfide bonds since it acquired a lower electrophoretic mobility when separated under reducing conditions (Fig. 5, lanes *c'* and *e'*). This peculiar electrophoretic behavior is common to the GP135 surface glycoprotein recognized by the same antiserum on mouse fibroblasts and implicated in the adhesion of these cells to fibronectin (9, 10).

These data suggested a structural and functional relationship between hemopoietic cells GP145 and fibroblast GP135. To further investigate this point, we used monospecific antibodies purified by affinity on nitrocellulose-bound GP135 (10). The purification of these antibodies from the anti-BHK serum is described in the Materials and Methods section. Immunoblotting experiments on a crude membrane fraction of Friend cells indicated that purified antibodies reacted selectively with GP145 (Fig. 6, lane *c*). The two molecules are thus antigenically related.

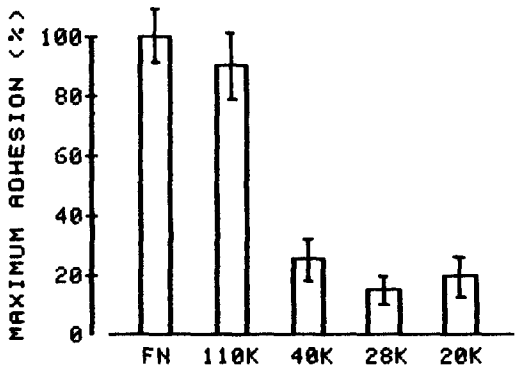

Figure 3. Adhesion of Friend cells to proteolytically derived fibronectin fragments. Friend cells were plated on dishes coated with 25 μg/ml of fibronectin (*FN*) or the Cell (*110K*), Gel (*40K*), Hep-1/Fib-1 (*28K*), and Fib-2 (*20K*) domains of fibronectin (see reference 23 and Materials and Methods section). After 1 h at 37°C, attached cells were fixed, stained, and counted as specified in the Materials and Methods section. Adhesion values are expressed as percentage of the maximum adhesion and represent the mean of three different experiments.

To investigate the role of GP145 in the adhesion of hemopoietic cells to fibronectin, we took advantage of the affinity-purified GP135 antibodies. When added together with the cells at the beginning of the adhesion assay, these antibodies inhibited fibronectin-mediated adhesion of both Friend and WEHI-3B cells, while preimmune IgG had no effect (Fig. 7). Inhibition was dose dependent and reached a value of 80% at 40 μg/ml. Since GP135 antibodies recognized only GP145 among membrane proteins of hemopoietic cells, the simplest interpretation of this result is that GP135 antibodies block cell adhesion by directly interacting with GP145. This interpretation was confirmed by adsorption experiments. Nitrocellulose-bound membrane antigens of Friend cells, including GP145, other unrelated membrane polypeptides, and fibronectin, were used to adsorb affinity-purified GP135 antibodies (see Materials and Methods section for details). Adsorption with GP145 removed virtually all (87% ± 4) the adhesion-preventing activity. On the contrary, adsorption with equivalent amounts of either fibronectin or membrane polypeptides unrelated to GP145 was uneffective and removed <10% (± 3) of the activity. By the above-described results, we conclude that the membrane glycoprotein GP145 mediates adhesion of hemopoietic cells to fibronectin.

Discussion

In this paper we report that myeloid and erythroid hemopoietic cells interact with and adhere to fibronectin. None of the cell lines tested adhered to laminin, types I, III, and IV collagen, or proteoglycans which, together with fibronectin, represent the major matrix components of the bone marrow stroma (1, 16, 43). It is generally accepted that contiguity of hemopoietic cells with the bone marrow stroma is necessary for the maintenance of hemopoiesis (5, 12, 38). The in vitro interaction of hemopoietic cells with fibronectin, reported in this paper, might reproduce a molecular mechanism by which hemopoietic cells are anchored to the bone marrow stroma, near the proper signals for their proliferation and differentiation (6). Blood cells are normally released to the circulation only when fully differentiated. In this respect, note that few end-stage cells (erythrocytes and granulocytes) adhered to fibronectin. It has also been reported that dimethylsulfoxide-induced differentiation of murine erythroleukemia cells is accompanied by loss of their ability to attach to fibronectin (26). Thus, in the erythroid (27) and granulocytic lineages, loss of fibronectin-binding ability during differentiation may be the underlying cause for release of end-stage cells into the circulation.

We have found that the same site of fibronectin that functions in fibroblast and platelet adhesion is also involved in the adhesion of hemopoietic cells. In fact, a monoclonal antibody to the fibroblast binding site of fibronectin (33) also

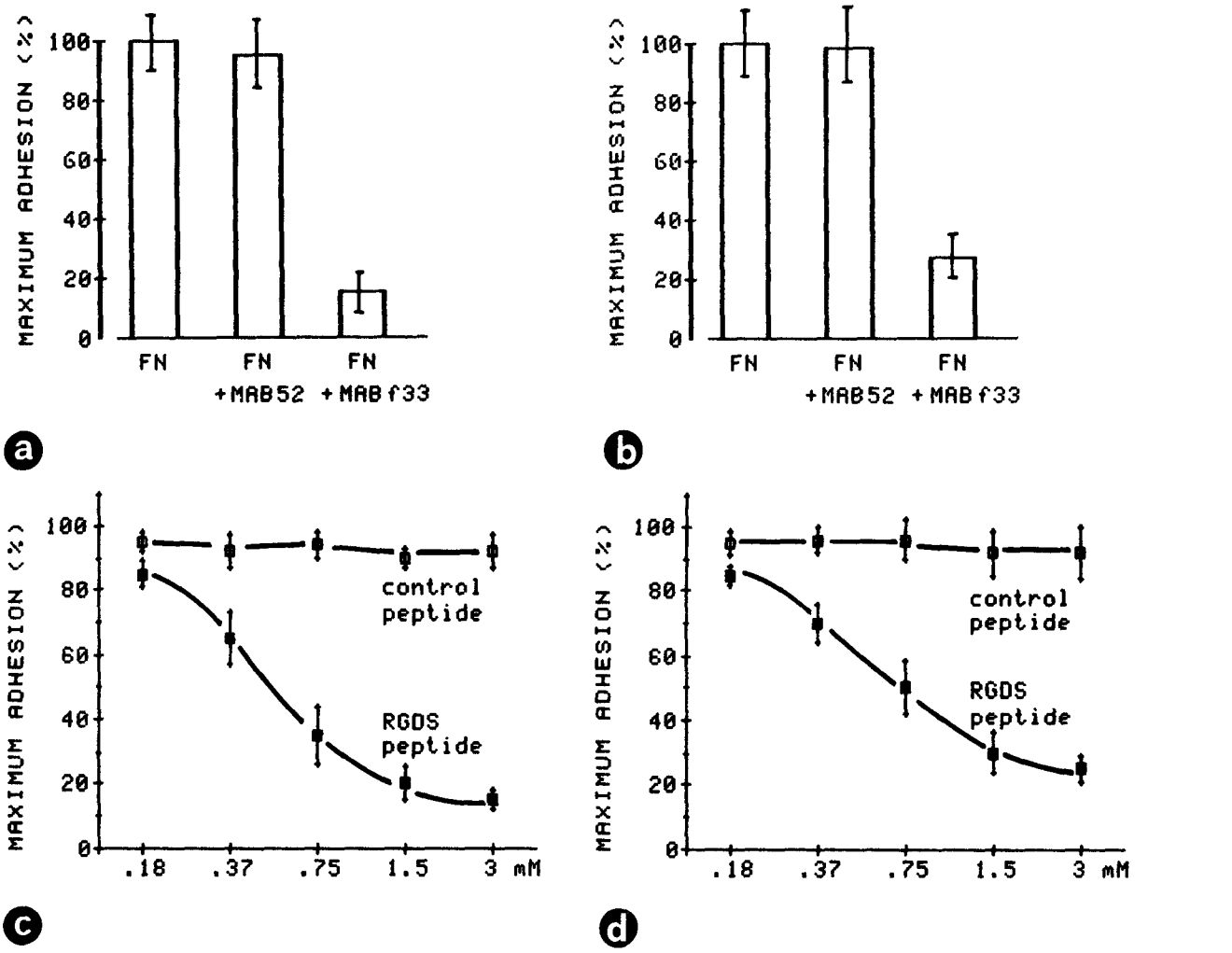

Figure 4. Identification of the fibronectin site involved in the adhesion of hemopoietic cells. (*a* and *b*) Friend (*a*) and WEHI-3B (*b*) cells were plated on dishes coated with 2 mg/ml gelatin followed by 25 μg/ml fibronectin in the presence of fibronectin monoclonal antibodies of known specificity. The monoclonal antibody f33 is directed against the cell binding site and was previously shown to inhibit fibroblast adhesion to fibronectin. The monoclonal antibody MAB 52 is directed against the gelatin-binding domain. Both antibodies were used at 20 μg/ml. (*c* and *d*) Friend (*c*) and WEHI-3B (*d*) cells were plated on dishes coated as specified above. The peptide Arg-Gly-Asp-Ser (*RGDS*), corresponding to the sequence of the cell attachment site, or a control peptide (substance P) was added at different concentrations at the beginning of the assay. After 1 h at 37°C, attached cells were fixed, stained, and counted as specified in the Materials and Methods section. Adhesion values are expressed as percentage of the maximum adhesion and represent the mean of three different experiments.

prevented the adhesion of hemopoietic cells. Moreover, the tetrapeptide Arg-Gly-Asp-Ser, which provides the recognition signal for fibroblasts (28) and platelets (11), competed with substratum-bound fibronectin for the hemopoietic cell surface.

A membrane glycoprotein of 145 kD (GP145) appears to be an important effector of the interaction of murine hemopoietic cells with fibronectin, since affinity-purified antibodies that reacted with this molecule blocked their adhesion. Hemopoietic cell lines have the distinctive property to adhere selectively to fibronectin. By contrast, other mesenchymal cell lines adhere to more than one extracellular matrix molecule, including fibronectin, laminin, vitronectin (serum-spreading factor), and collagens, presumably through different cell surface receptors (15, 20, 22, 23, 31). This suggests that GP145 may be regarded as a fibronectin-specific mediator of cell adhesion.

The data presented in this paper indicate that GP145 of hemopoietic cells is related to a cell surface adhesive glycoprotein, GP135, of mouse fibroblasts (9, 10). GP135 was identified as a critical component because its selective cleavage from the cell surface led to loss of fibroblast adhesion to fibronectin (9). The fibroblast GP135 (9, 10) and the hemopoietic cells GP145 displayed a decreased electrophoretic mobility in the presence of reducing agents, suggesting the presence of intrachain disulfide bonds in both molecules. In addition, GP135 monospecific antibodies did react with GP145 and blocked the adhesion to fibronectin of both fibroblasts (10) and hemopoietic cells, indicating that the two glycoproteins are both antigenically and functionally related. The two proteins, however, are not identical, as they displayed different molecular weights. This can be attributed to a different extent of glycosylation of the same polypeptide backbone. Alternatively, the two molecules may have related but distinct primary sequences, being coded by two different genes.

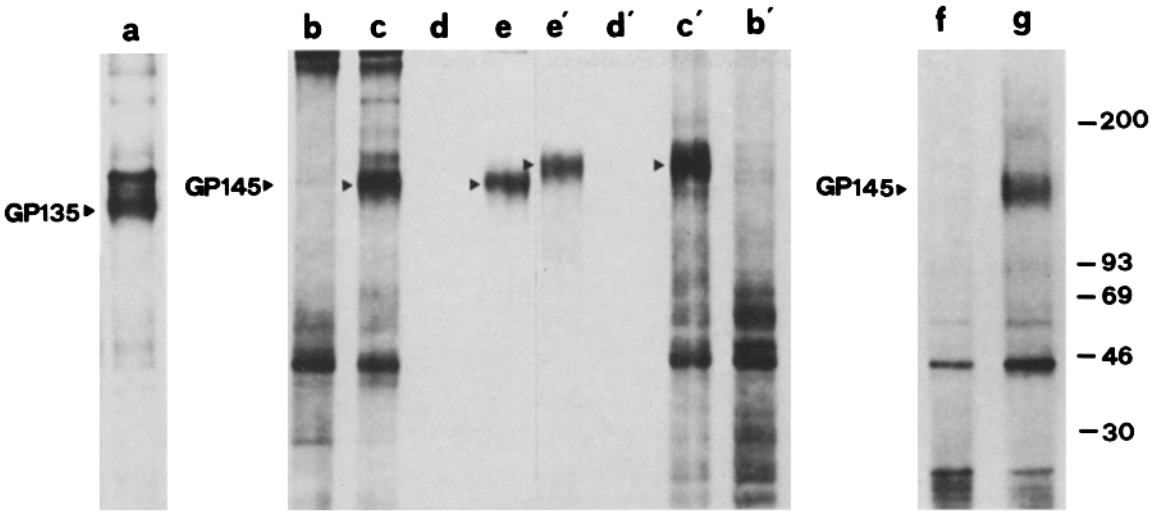

Figure 5. Membrane proteins recognized by the anti-BHK serum on the surface of Friend and WEHI-3B cells. Lane *a*, membrane proteins of [^{35}S]methionine-labeled SR-Balb fibroblasts were immunoprecipitated with the anti-BHK serum and separated under nonreducing conditions for comparison. Lanes *b–e*, membrane proteins of Friend cells, labeled with [^{35}S]methionine (*b* and *c*) or [^{3}H]glucosamine (*d* and *e*), were immunoprecipitated with anti-BHK (*c* and *e*) or preimmune serum (*b* and *d*) and separated by SDS PAGE under nonreducing conditions. Lanes *e'–b'*, the same samples separated under reducing conditions. Lanes *f–g*, membrane proteins of [^{35}S]methionine-labeled WEHI-3B cells were immunoprecipitated with anti-BHK (*g*) or preimmune serum (*f*) and separated under nonreducing conditions. The positions of both hemopoietic cell GP145 and fibroblast GP135 are indicated for reference. Migration of molecular weight markers ($\times 10^{-3}$) is shown on the right.

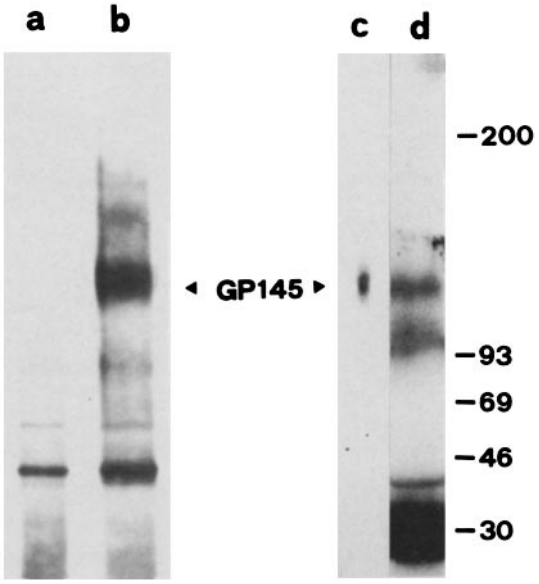

Figure 6. Antigenic relationship between GP145 of hemopoietic cells and the fibroblast GP135. Proteins from a crude membrane fraction of Friend cells were separated by SDS PAGE under nonreducing conditions and, after transfer to nitrocellulose paper, were reacted with 50 μg/ml of affinity-purified antibodies to fibroblast GP135 (*c*) or anti-BHK immunoglobulins (*d*). To use a minimal amount of antibodies, a 2-mm wide strip was excised from the nitrocellulose filter for incubation with affinity-purified GP135 antibodies. Bound antibodies were revealed by ^{125}I-labeled protein A. To localize the position of GP145, membrane proteins immunoprecipitated with anti-BHK (*b*) or preimmune serum (*a*) from the surface of [^{35}S]methionine-labeled cells were separated in the same gel and transferred to nitrocellulose for reference. Migration of molecular weight markers ($\times 10^{-3}$) is shown on the right.

The GP145 molecule may resemble other 140-kD glycoproteins involved in adhesion to fibronectin, which show altered SDS PAGE mobilities in the absence of reducing agents. These were identified by different experimental approaches on human MG-63 osteosarcoma cells (30), Chinese hamster ovary cells (3), as well as chick myoblasts and fibroblasts (13, 25). More recently, the membrane glycoprotein GPIIIa was identified as the fibronectin-binding site on human platelet surface (8). This molecule occurs in a complex with the glycoprotein GPIIb at the surface of activated platelets and its electrophoretic mobility is affected by disulfide bond reduction (21) with a pattern similar to that reported here for the GP145 of hemopoietic cells and elsewhere for the fibroblast GP135 (9, 10).

Even if it is not yet known whether all these membrane glycoproteins are structurally related, clearly a set of membrane glycoproteins with similar properties mediate the interaction of fibroblasts, platelets, and hemopoietic cells with fibronectin.

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Note Added in Proof: While this paper was under editorial processing, V. Patel and H. F. Lodish (*J. Cell Biol.*, 1986, 102:449–456) have reported that undifferentiated murine erythroleukemia cells express a fibronectin receptor

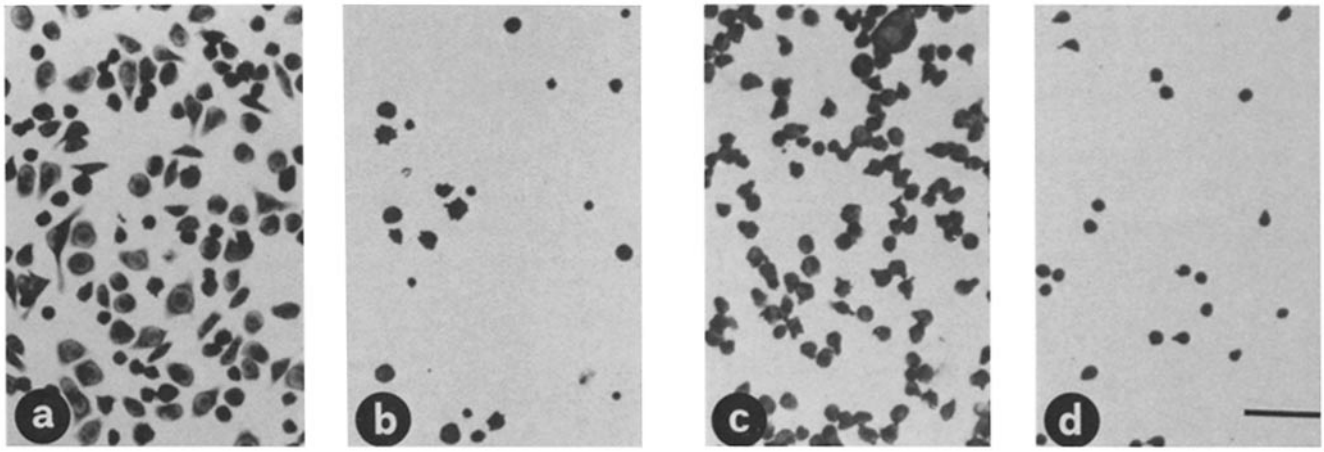

Figure 7. Inhibition of Friend and WEHI-3B cell adhesion by affinity-purified antibodies to GP135. Friend (*a* and *b*) and WEHI-3B cells (*c* and *d*) were plated on dishes coated with 2 mg/ml gelatin followed by 25 μg/ml fibronectin. At the beginning of the assay, 40 μg/ml affinity-purified GP135 (*b* and *d*) or control immunoglobulins (*a* and *c*) were added to the wells. After 1 h at 37°C, attached cells were fixed and stained with May-Grünwald Giemsa. Bar, 25 μm.

with properties in common with those reported here for the GP145 of myeloid and erythroid cells.

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