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Laminin-induced retinoblastoma cell differentiation: Possible involvement of a 100-kDa cell-surface laminin-binding protein

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ABSTRACT **Gene and protein expression of Y-79 retinoblastoma cells growing on poly(D-lysine) is switched from a photoreceptor-like to a conventional neuron-like pathway by the basement membrane glycoprotein laminin. Unlike other cell systems where laminin influences differentiation, Y-79 cells can neither attach to nor chemotactically respond to laminin. However, laminin increases attachment to poly(D-lysine). The laminin effects therefore seem to occur via an adhesion- and chemotaxis-independent mechanism. Moreover, these tumor cells do not exhibit high-affinity laminin binding, having only a single binding site of intermediate affinity. Laminin-Sepharose affinity chromatography of Y-79 cell surface proteins labeled with ^{125}I revealed a single major radiolabeled 100-kDa protein eluted by 20 mM EDTA, with an electrophoretic behavior different from that of integrins. No other proteins were eluted under more stringent conditions. This material, which we call LBM-100 (100-kDa laminin-binding molecule), may be a "differentiative" laminin-binding protein through which laminin influences gene expression and development independently of attachment.**

Laminin is a major basement membrane protein that promotes the adhesion, migration, and differentiation of a variety of cell types and is involved in tumor cell invasion. The ability of laminin to promote cell differentiation has been thought to be related to its potential for promoting cell adhesion (1–3). Tumor cells that use laminin as an attachment factor generally exhibit high-affinity surface receptors for laminin (4).

Human Y-79 cells are invasive tumor cells (5) derived from a multipotential stem cell of the neural retina (6, 7). These cells grow in suspension culture but will attach to a substratum coated with poly(D-lysine) (8). Polylysine has differentiative effects on some cell types (9), and another polycation, polyornithine, promotes extensive attachment and spreading of retinal neurons (10). Laminin affects the morphological phenotype of Y-79 cells (6–8, 11–13). Here we report that Y-79 cells do not show typical short-term interactions with laminin (e.g., adhesion and chemotaxis) but exhibit a profound long-term response to laminin, demonstrating changes in adhesion to poly(D-lysine), in gene expression, and in levels of protein production. Laminin binding and affinity chromatography studies indicate that these effects could be mediated through a single, intermediate-affinity, "differentiative" laminin-binding activity that is not directly involved in attachment.

MATERIALS AND METHODS

Cells. Y-79 cells were cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum, antibiotics, and glutamine. For certain experiments, cells were maintained in serum-free, chemically defined medium with insulin (5 μg/ml) transferrin (5 μg/ml), sodium selenite (5 ng/ml), and Zn^{2+} (0.5%). K-1735 melanoma and HT-1080 fibrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, antibiotics, and glutamine.

Attachment Studies. Plastic tissue culture dishes (Falcon) were coated by incubation with laminin (12.5 μg per 16-mm dish), poly(D-lysine) (5 μg/cm^2), or no substrate for 1 hr followed by saturation with bovine serum albumin (0.05%). When indicated, laminin (12.5 μg per dish) was coated onto poly(D-lysine)-treated dishes or added to the culture medium at the concentrations indicated. Cell suspensions (2×10^5 cells per dish in DMEM) were incubated with 0.05% albumin for 24 hr, and dishes were rinsed to eliminate unattached cells. Attached cells were detached and counted with a hemocytometer.

Immunocytochemistry. Cultured cells were permeabilized in 70% ethanol for 5 min to allow both extracellular and intracellular staining. Rabbit antibodies to neuron-specific enolase were used at 1:10 dilution for 30 min. Mouse monoclonal antibodies against neurofilaments (SMI 31, SMI 32, Sternberger–Meyer, Jarrettsville, MD) were used at 1:200 dilution. mouse monoclonal antibodies to choline acetyltransferase (Boehringer Mannheim) were used at 1:10 dilution. Samples were rinsed and fluorescein-conjugated goat anti-rabbit or rabbit anti-mouse IgG secondary antibodies were added for another 30 min.

RNA Blotting. Total cellular RNA (14) was electrophoresed through a 0.7% agarose/formaldehyde gel (20 μg per lane) and transferred to nitrocellulose (Northern blot) or was dot-blotted. *Bgl* II or *Hin*dIII restriction fragments from transcribed regions of a genomic clone for human interphotoreceptor retinoid-binding protein (IRBP; ref. 15) were radiolabeled with [α-^{32}P]dCTP by random priming and hybridized with the blots. These probes recognized a single band of 4.4 kilobases on Northern blots, as previously described for human Y-79 IRBP mRNA (16). Blots were hybridized with radiolabeled cDNA probes for β-actin for normalization.

Binding Studies. Laminin was radiolabeled with ^{125}I (New England Nuclear) by the chloramine-T method (17) to a specific activity of 5×10^6 cpm/μg. Binding of laminin to cells was measured as described (18, 19). Cells were incubated in 100 μl of binding medium with ^{125}I-laminin (50 ng/ml to 10 μg/ml) at 4°C for 3 hr. Cells were then rinsed with ice-cold binding medium and bound radioactivity was determined. Nonspecific binding was determined by adding unlabeled laminin in excess (0.5–1.0 mg/ml) to parallel samples and was subtracted from total binding.

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Abbreviation: IRBP, interphotoreceptor retinoid-binding protein.

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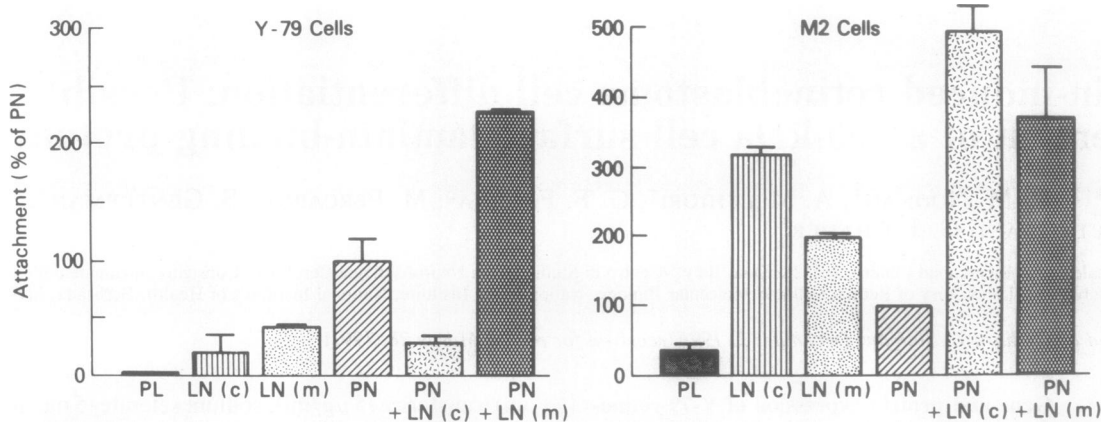

FIG. 1. Comparison of Y-79 retinoblastoma (*Left*) and K-1735 M2 melanoma (*Right*) cell attachment to laminin- and poly(D-lysine)-coated dishes over a 24-hr period. PL, plastic substratum; LN(c), laminin-coated substratum; LN(m), laminin added to the medium (12.5 μg/ml); PN, poly(D-lysine)-coated substratum; PN + LN(c), poly(D-lysine)-coated substratum overcoated with laminin; PN + LN(m), poly(D-lysine)-coated substratum with laminin added to the medium (12.5 μg/ml). Number of cells attached to PN dishes was arbitrarily set to 100%. Experiments were run in triplicate and repeated six times. Values given are for one representative experiment (mean ± SD of triplicates). Similar patterns were obtained when bacteriological plastic dishes were used.

Affinity Chromatography. Y-79 cells were surface-labeled with 250 μCi (1 μCi = 37 kBq) of ^{125}I by the lactoperoxidase method before extraction and chromatography on laminin-Sepharose columns (20). Briefly, radiolabeled live cells were washed in phosphate-buffered saline and then lysed in extraction buffer (25 mM *n*-octyl β-D-glucopyranoside/150 mM NaCl/100 mM Tris, pH 7.4/2 mM phenylmethylsulfonyl fluoride/1 mM $MnCl_2$). Lysates were incubated with 1.5 ml of laminin-Sepharose overnight at 4°C in extraction buffer and then transferred to a column. The column was washed twice with 4 column volumes of extraction buffer, until no detectable radioactivity was eluted. Bound material was eluted with 4 volumes of extraction buffer (without $MnCl_2$) containing 20 mM EDTA, followed by extraction buffer with EDTA and 0.5 M NaCl, and finally EDTA and 1.0 M NaCl. The fractions were collected, dialyzed against Tris/EDTA buffer and then distilled water, lyophilized, and subjected to SDS/7% PAGE.

RESULTS

Attachment Studies. Y-79 cells in serum-free medium are rounded and unremarkable in appearance (6). They attach poorly to uncoated plastic even after 24 hr (0.1–0.2% of seeded cells; Fig. 1); up to 500-fold more cells attach to poly(D-lysine)-coated dishes, however. When plated on a poly(D-lysine) substrate, cells flatten out, occasionally extending short, unbranched processes reminiscent of photoreceptor cell morphology in culture (6, 7). Laminin is not an effective Y-79 cell attachment substrate (0.5–1% of plated cells; Fig. 1). In fact, overcoating of poly(D-lysine) substrate with laminin actually inhibits attachment. Laminin also has little effect when simply added to the medium of Y-79 cells on uncoated plastic (Fig. 1). When laminin is added to cultures on poly(D-lysine), the cells show a more typical neuronal morphology, however, with numerous cells extending long thin branching "dendritic" processes (7, 13). When added to the medium of Y-79 cells on a poly(D-lysine) substrate, laminin markedly increases Y-79 cell attachment (Fig. 1). No differences in the attachment behavior were noted using either bacteriological or tissue culture plastic and trypsinized or untrypsinized cells. For comparison, we used K-1735 M2 melanoma cells, which interact with laminin in adhesion, migration, and binding studies in a "classical" manner (15). These cells, like other tumor cells, prefer laminin-coated substrates rather than either naked plastic or poly(D-lysine)-coated substrates for attachment (Fig. 1). Attachment of M2 cells to a laminin-coated substrate is 4 times higher than to poly(D-lysine)-coated dishes (70% of plated cells attached). Addition of laminin either as an overcoating or in the medium further increases attachment of M2 cells (to 100% of the seeded cells). Furthermore, laminin induces M2 cell attachment within 2 hr but has a significant effect on Y-79 cell adhesion only after 24 hr. Anti-laminin polyclonal antibodies can counteract laminin effects on both Y-79 and M2 cells (data not shown). These data show that Y-79 cells lack the ability to attach to laminin, whereas other cells attach rapidly under the same conditions.

Competition experiments were used to assess the effects of specific laminin peptides involved in cell attachment, CDPGYIGSR-amide (21) and CQAGTFALRGDNPQGC-amide (22), on the adhesion of Y-79 cells to poly(D-lysine)-coated dishes in the presence of laminin (Table 1). No inhibitory effects of the peptides were observed on Y-79 cells, whereas both peptides interfered with adhesion of the human fibrosarcoma cell line HT-1080 (Table 1). These data further substantiate that laminin does not serve as a "classical" attachment substrate for Y-79 cells.

Chemotaxis studies indicate that Y-79 cells are poorly migratory (1.9 ± 0.1 × 10^3 cells per filter), even on filters coated with poly(D-lysine). Addition of laminin increased migration about 2-fold. However, this effect is chemokinetic rather than chemotactic, as the same effect was noted when laminin was added to either the lower (4.6 ± 0.5 × 10^3 cells per filter) or both the upper and lower (5.6 ± 0.6 × 10^3 cells per filter) wells of the chamber. Thus, Y-79 cells can neither attach to nor respond chemotactically to laminin. Fibronectin was not active in this regard.

Immunocytochemistry. Y-79 cells grown on poly(D-lysine) stain lightly with antibodies to neuron-specific enolase (Fig.

Table 1. Effect of laminin peptides on cell attachment

Competitor	Attached cells, no. × 10^{-4}	
	HT-1080 cells	Y-79 cells
None	9.1 ± 1.2	9.2 ± 0.2
YIGSR (250 μg/ml)	6.2 ± 0.5*	9.2 ± 0.3
RGD (500 μg/ml)	5.5 ± 0.5**	8.6 ± 0.5

Cells were plated on poly(D-lysine) with laminin added to the medium at 12.5 μg/ml. Y-79 cells were plated on a poly(D-lysine) substrate. Competition with laminin peptides [CDPGYIGSR-amide (YIGSR) and CQAGTFALRGDNPQGC-amide (RGD)] was assessed after 24 hr. Significant difference from controls: *, $P = 0.005$; **, $P < 0.01$.

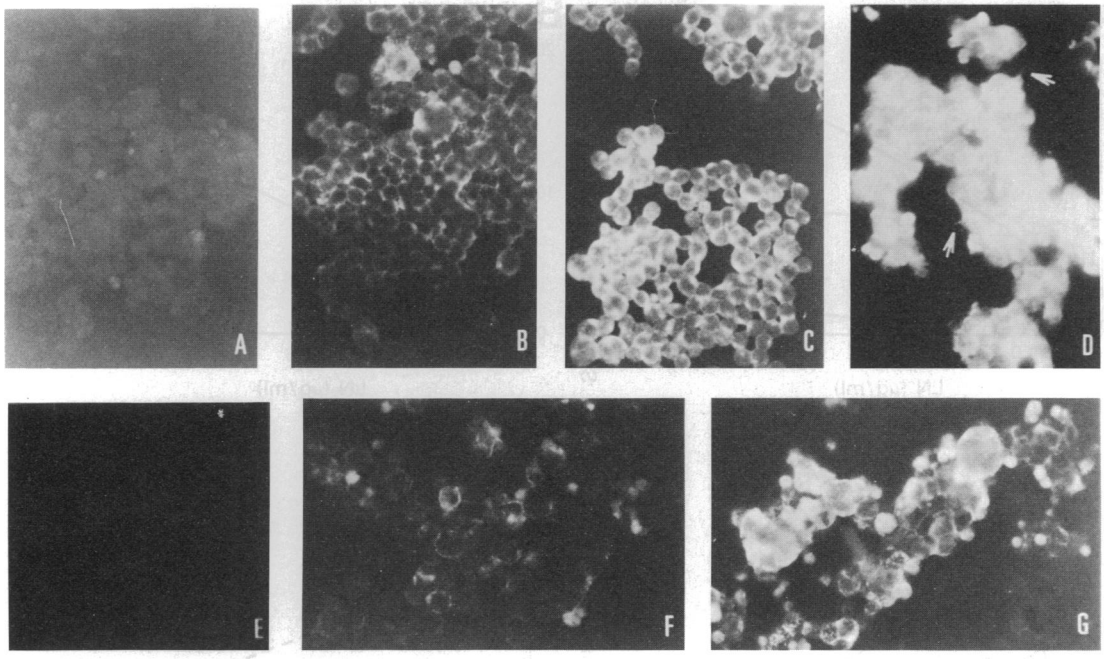

FIG. 2. Effect of laminin on protein expression as assessed by immunocytochemistry. (*A*) Nonimmune rabbit serum. (*B–D*) Neuron-specific enolase staining of Y-79 cells cultured on poly(D-lysine) for 4 days (*B*) or cultured on poly(D-lysine) for 2 days and treated with laminin at day 3 for 2 days (*C*), or at day 3 for 7 days (*D*). (*E*) Control, without primary antibody. (*F*) Neurofilament antibody (SMI 31 and SMI 32) staining of cells cultured on poly(D-lysine) for 8 days. (*G*) Neurofilament staining of cells grown on poly(D-lysine) and treated with laminin at day 5 for 3 days. Arrows indicate growing processes. (×260.)

2*B*). Laminin-treated cells show a much higher signal than the untreated controls; this is apparent after 2 days of treatment, prior to morphological differentiation (Fig. 2*C*), and is intense after 7 days of treatment, when processes are already visible (Fig. 2*D*). Antibodies to neurofilaments also show an increase in fluorescence with laminin treatment (Fig. 2*G*) as compared with controls (Fig. 2*F*). Monoclonal antibodies to another neuronal marker, choline acetyltransferase, also predominantly stain laminin-treated cells (data not shown). These results indicate that production of proteins specific for a conventional neuronal phenotype is increased upon laminin treatment of Y-79 cells grown on poly(D-lysine).

Northern Blotting. IRBP is synthesized specifically by photoreceptor neurons (23) and is a marker for early retinal differentiation (24). In agreement with immunological studies (25), Y-79 cells in suspension culture produced little IRBP message (Fig. 3, lane 1). Attachment to poly(D-lysine)-coated substrate, however, induced a substantial increase in IRBP mRNA (lane 2). Treatment of cells attached to poly(D-lysine) (lane 4) with laminin for 3 days decreased the level of IRBP mRNA by a factor of ≈2 (lane 3). A similar effect of laminin was observed when chemically defined, serum-free medium was used. Control β-actin expression was essentially unchanged by laminin (Fig. 3). Quantitation was performed by densitometric scanning and confirmed by dot-blot analysis; the experiment was repeated eight times. Paired *t*-test analysis of the normalized results indicated a significant augmentation of IRBP expression in cells plated on poly(D-lysine) ($P < 0.005$) and a significant reduction of IRBP expression by subsequent laminin treatment ($P < 0.001$).

We conclude that gene expression (IRBP), protein expression (neuron-specific enolase, neurofilaments, and choline acetyltransferase), and morphology (neurite extension) are all modified by laminin, reflecting a more conventional and neuron-like phenotype in the Y-79 cells.

Binding Studies. To this point, our data indicated that, although Y-79 cells do not attach to laminin and are not chemotactic to it, they are able to respond phenotypically to the presence of laminin and therefore must be able to interact with it in some manner. In binding assays, ^{125}I-laminin bound to the cells in a dose-dependent manner (Fig. 4 *A* and *B*). As laminin self-aggregates and precipitates at high concentrations, this binding cannot be saturated or give a precise measurement of the K_d. However, Scatchard analysis indicates an intermediate affinity binding of 0.1 μM (Fig. 4 *C* and *D*). This affinity is lower than that of the high-affinity receptor of low-metastatic K-1735 clone 10 cells (2–4 nM) or the

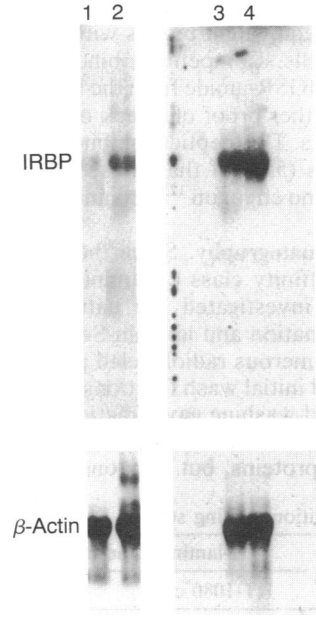

FIG. 3. Laminin effect on IRBP mRNA in Y-79 cells as assessed by Northern blotting. Lane 1, cells in suspension; lane 2, cells attached to poly(D-lysine)-coated substratum for 8 days; lane 3, cells attached to poly(D-lysine) and treated with laminin (12.5 μg/ml) at day 3 for 2 days; lane 4, cells attached to poly(D-lysine)-coated substrate alone for 5 days. β-Actin probe was used for normalization. Densitometric scanning of the blots was used for quantitation.

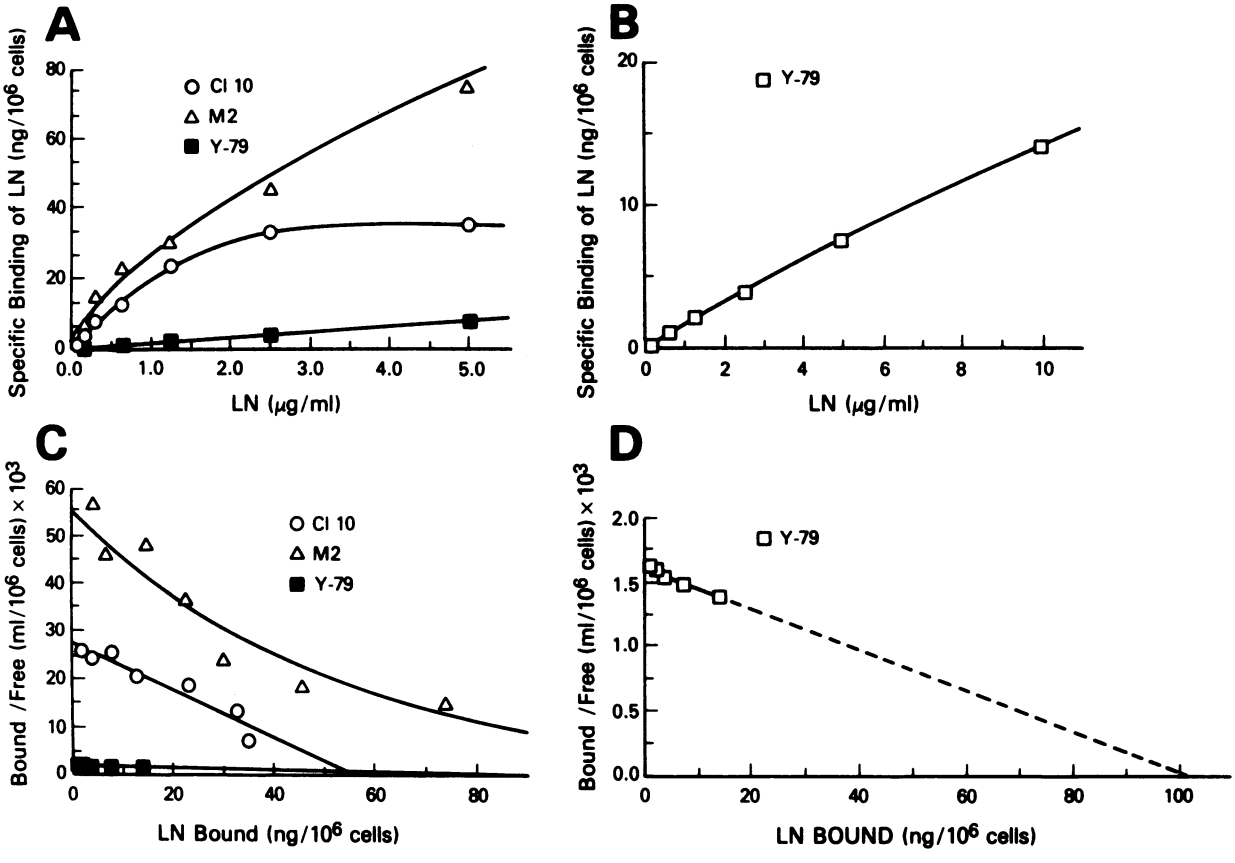

FIG. 4. Specific binding of ^{125}I-laminin (LN) to Y-79 retinoblastoma and K-1735 melanoma cells. (*A*) Binding to Y-79 cells (■), low-metastatic K-1735 clone Cl 10 melanoma cells (○), and highly metastatic K-1735 M2 melanoma cells (△). (*B*) Expanded view of binding to Y-79 cells. One more experimental point shown. (*C*) Laminin-binding affinities as evaluated by Scatchard analysis. (*D*) Expanded view of Scatchard analysis for Y-79 cell binding.

lower-affinity receptor of the highly metastatic melanoma variant M2 (8–10 nM, Fig. 4 *C* and *D*) (18). The affinity is, however, similar to or higher than those of most integrin receptors. Laminin attachment, chemotaxis, and binding studies all gave equivalent results with either trypsinized or untrypsinized cells. Competition binding studies using the peptide CDPGYIGSR-amide from the high-affinity domain of laminin gave further proof of a lack of high-affinity binding sites on Y-79 cells. This peptide inhibited ^{125}I-laminin binding to HT-1080 cells (55% of that caused by intact, unlabeled laminin) but had no effect on ^{125}I-laminin binding to Y-79 cells (Table 2).

Affinity Chromatography. Since the binding studies indicated a single affinity class of laminin-binding sites on the Y-79 cells, we investigated the nature of these sites by cell-surface iodination and laminin-Sepharose affinity chromatography. Numerous radiolabeled proteins were found in the unbound and initial wash fractions (Fig. 5 *Lower*, lanes 1 and 2); continued washing gave fractions with essentially no protein. Extraction buffer containing 20 mM EDTA eluted several distinct proteins, but only one of these was radiolabeled (lane 4), indicating that it was cell surface-associated. This band migrated at ≈100 kDa and was unaffected by reduction (data not shown). Further washing with higher salt eluted no other radiolabeled material (lanes 5 and 6). These data indicate that a single major cell surface laminin-binding component, which we call LBM-100, is present on Y-79 cells.

Table 2. Competition binding studies

Competitor	^{125}I-laminin binding, ng (% inhibition)	
	HT-1080 cells	Y-79 cells
None	9.09 ± 0.30	6.53 ± 0.18
Laminin	5.53 ± 0.23* (40)	3.65 ± 0.03* (44)
YIGSR	7.10 ± 0.25** (22)	7.15 ± 1.41 (0)

Cells (2×10^5) were incubated with 375 ng of ^{125}I-laminin in 100 μl of binding medium. Excess nonradioactive laminin or peptide [CDPGYIGSR-amide (YIGSR)] (250 μg/ml) was added to parallel samples. Significant difference from controls: *, $P = 0.01$; **, $P < 0.025$.

DISCUSSION

Adler and Hatlee (26) have reported that, in the normal developing retina, photoreceptor differentiation represents a constitutive ("default") pathway that precursor retinal cells follow in the absence of neuron-inducing (i.e., nonphotoreceptor) signals. Our studies suggest that attachment to poly(D-lysine) *in vitro* orients retinoblastoma cells to the default photoreceptor differentiation mode, whereas the presence of laminin (perhaps expressed transiently or spatially during the embryonic period) induces conventional neuronal (i.e., nonphotoreceptor) differentiation with decreased expression of photoreceptor proteins (IRBP), increased conventional neuronal proteins (neuron-specific enolase, neurofilaments, choline acetyltransferase), and neurite formation. Interestingly, laminin has been shown to induce the "transdifferentiation" of retinal pigment epithelial cells into neurons in amphibians (27).

Laminin effects on cellular differentiation have generally been assumed to be mediated through cell attachment to the matrix via laminin receptors. For example, adhesion to laminin is considered the basic element in neurite extension (1–3). Laminin receptors of the integrin family have been described which are involved in cell adhesion (28) and in neurite outgrowth in the neuron-like cell line PC12 (29). Receptors of the integrin family have affinities of about 1 μM

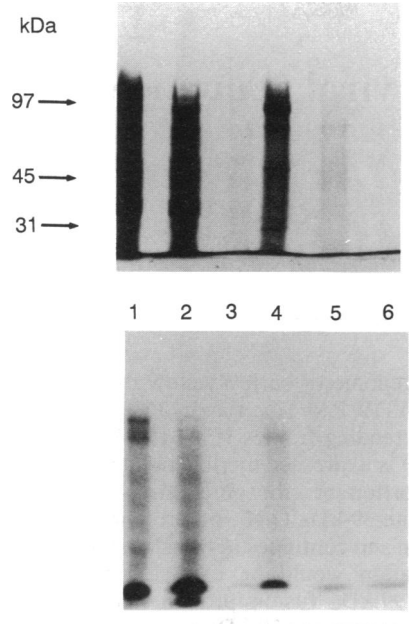

FIG. 5. SDS/PAGE of fractions from laminin-Sepharose affinity chromatography of cell lysates of surface-radioiodinated Y-79 cells. Cells were grown on poly(D-lysine). Coomassie blue staining (*Upper*) and autoradiogram (24-hr exposure) (*Lower*) are shown. Lane 1, unbound material; lane 2, first wash with extraction buffer; lane 3, second wash with lysis buffer; lane 4, elution with lysis buffer containing 20 mM EDTA without divalent cations; lane 5, elution with same buffer for lane 4 but with 0.5 M NaCl; lane 6, elution with same buffer as for lane 5 but with 1 M NaCl.

(30), about 1 order of magnitude lower than that exhibited by Y-79 cells for laminin. Integrins of higher affinity have been found, however (31). Laminin also binds to cells via other mechanisms (for review see ref. 32). Our data suggest that Y-79 cell interactions with laminin do not follow the classical pathway utilizing adhesion and chemotaxis receptors yet have profound effects on the cell phenotype. The different nature of this interaction is substantiated by the lack of effect on Y-79 cells of either the CDPGYIGSR or CQAGTFALRGDNPQGC laminin peptides.

The interactions of laminin with Y-79 cells appear to be mediated by an intermediate-affinity "differentiative" laminin-binding activity. Laminin-Sepharose affinity chromatography suggests that this binding is due to a single major laminin-binding molecule of 100 kDa (LBM-100). While the molecular structure of LBM-100 is still unknown, it does not appear to be a member of the known laminin-binding proteins (i.e., the 67-kDa complex or the integrins), because of its 100-kDa size on SDS/PAGE, which is unaffected by sulfhydryl reduction, and its failure to form heterodimers. It is possible that this binding site exists in other cell types where laminin affects differentiation. A 110-kDa cell surface fibronectin-binding protein with characteristics similar to LBM-100 has been reported to be associated with differentiation in hepatocytes (33). It will be interesting to examine other cell systems to determine whether adhesion to laminin can be separated from laminin-induced phenotypic changes in these cases.

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