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Laminin potentiates differentiation of PCC4uva embryonal carcinoma into neurons

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Summary

The embryonal carcinoma PCC4uva differentiates into neurons in response to treatment with retinoic acid and dbcAMP. We used this *in vitro* model system to study the effects of laminin on early neural differentiation. Laminin substrata markedly potentiate neural differentiation of retinoic acid and dbcAMP-treated cultures. Only laminin induced more rapid neural cell body clustering, neurite growth and neurite fasciculation as compared to type IV collagen, type I collagen, and fibronectin substrata. Exogenous laminin substrata promoted greater cell attachment, cellular spreading and growth to confluence than type IV collagen, type I collagen, fibronectin and glass substrata. Laminin-induced effects were inhibited by addition of laminin antibodies or the synthetic laminin-derived peptide Ile-Gly-Ser-Arg-NH_2 (YIGSR-NH_2). Treatment with YIGSR-NH_2 also inhibited neural differentiation in the absence of exogenous laminin substrata, whereas synthetic peptides containing the RGD sequence and a control peptide YIGSK-NH_2 showed no inhibitory effects. These results are consistent with the hypothesis that specific interactions between an early differentiating cell population(s) and extracellular laminin are required during neural differentiation.

Key words: laminin, neurons, embryonal carcinoma.

Introduction

Development of the nervous system from embryonic stem cells involves induction, cell migration, neurite outgrowth and synapse formation within a complex and dynamic extracellular environment. It is clear that cell–cell and cell–extracellular matrix (ECM) interactions influence neural development and neuronal regeneration (for reviews, see Carbonnetto, 1984; Dodd and Jessell, 1988).

The presence of laminin during early development correlates with morphogenic events such as avian neural crest migration (Douband and Thiery, 1987) and neurite projection, which suggests a role for laminin in development of neurites along specific pathways (Rogers *et al.* 1983; Carbonetto, 1984; Bronner-Fraser, 1986; Liesi *et al.* 1985*a*,*b*; Riggott and Moody, 1987). *In vitro*, exogenous laminin substrata promote neurite outgrowth, neuronal survival and neural crest migration (Baron van Evercooren *et al.* 1982; Rogers *et al.* 1983, 1986; Manthorpe *et al.* 1983; Edgar *et al.* 1984; Adler *et al.* 1985; Davis *et al.* 1985*a*,*b*; Smalheiser *et al.* 1984; Liesi *et al.* 1984; Tomaselli *et al.* 1987; Kleinman *et al.* 1988; Bilozur and Hay, 1988).

Laminin influences cell adhesion, growth, morphology, differentiation and migration in many cell types (Kleinman *et al.* 1985). Laminin molecules derived from the EHS tumor are composed of three polypeptide chains, designated A ($400\times10^3 M_r$), B1 ($210\times10^3 M_r$) and B2 ($200\times10^3 M_r$), that are arranged as a cross (see Martin and Timpl, 1987, for review). Not all laminin chains are expressed in the cross-shaped configuration. For example, only B1 chains are expressed in two-cell stage mouse embryos; by the four- to eight-cell stage, embryos also synthesize B2 chains, and 16-cell embryos synthesize all three chains (Leivo *et al.* 1980; Leivo, 1983; Cooper and MacQueen, 1983). Hunter and colleagues (1989) recently described a distinct laminin chain that is localized in rat synapses.

The functions of laminin are associated with specific domains. Macromolecular interactions occur through domains that bind collagen type IV, heparan and entactin (Rao *et al.* 1982; Terranova *et al.* 1980; Martin and Timpl, 1987). A domain that promotes neurite outgrowth has been localized to the long arm of the cross-shaped molecule, and a cell attachment site has been localized to the region where the A and B chains intersect (Edgar *et al.* 1984; Engvall *et al.* 1986; Rao *et al.* 1982; Timpl *et al.* 1983). Evidence suggests that a site in the B1 chain interacts with a $67\times10^3 M_r$ laminin receptor (Terranova *et al.* 1983; Graf *et al.* 1987*b*).

Several polypeptide sequences appear to mediate cell–laminin interactions (Timpl *et al.* 1983; Aumailley *et al.* 1987; Goodman *et al.* 1987; Kleinman *et al.* 1987; Gehlsen *et al.* 1988), including a $67\times10^3 M_r$ laminin receptor (Rao *et al.* 1983; Lesot *et al.* 1983; Malinoff and Wicha, 1983; Terranova *et al.* 1983; Barsky *et al.* 1984; Graf *et al.* 1987*a*,*b*; Kleinman *et al.* 1988). One cell–laminin interaction involves a peptide sequence, Tyr-Ile-Gly-Ser-Arg (YIGSR) derived from the B1 chain. Further, this peptide will elute the $67\times10^3 M_r$ laminin binding protein from laminin affinity columns (Graf *et al.* 1987*a*,*b*). The peptide

inhibits the invasiveness of B16F10 melanoma cells (Iwamoto *et al.* 1987), neural crest cell migration within basement membrane gels (Bilozur and Hay, 1988), and mesenchymal cell migration within hydrated laminin gels (Davis *et al.* 1989).

The objective of this study was to investigate the influence of laminin on the earliest events of neural differentiation. PCC4uva embryonal carcinoma stem cells differentiate into neurons in response to treatment with retinoic acid and dibutyryl cyclic adenosine monophosphate (Sweeney *et al.* unpublished). Treatment of PCC4uva with 10 μM retinoic acid and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) for 5 days induces rapid neural differentiation. Approximately 80% of cells have neuronal morphology and express the type III β-tubulin isotype by 6 days in culture. These neurons also express neurofilaments and the neuron-specific microtubule-associated proteins, MAP2 and Tau.

This report shows that culturing PCC4uva stem cells on laminin-coated surfaces had profound effects on the early events associated with neural differentiation. Two effects were consistently observed: (1) a greatly increased degree of spreading and confluence at very early times after seeding; and (2) a strikingly accelerated rate of neural differentiation. In contrast, substrata coated with other ECM components did not promote neural differentiation.

Materials and methods

Cell culture and treatment

PCC4uva stem cells were cultured as described (Sweeney *et al.* unpublished). Undifferentiated stem cells were cultured using the methods described by Lo and Gilula (1980). Briefly, undifferentiated stem cell cultures were grown in high-glucose Dulbecco's modified Eagle's medium without sodium pyruvate (Gibco Laboratories Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 100 μg ml^{-1} gentamicin in 60 mm tissue culture dishes (Corning) in a humidified incubator regulated to provide 10% CO_2/90% air mixture at 37°C. Stem cells were maintained in the undifferentiated state by passage every 36 hours into 60 mm tissue culture dishes seeded at 6.0×10^6 cells/dish. To obtain neuronal cultures, 1×10^5 stem cells in 1 ml of medium were plated onto glass coverslips (18 mm×18 mm, no. 1, Corning, Corning, NY) in 35 mm dishes (Corning). Subculture densities were measured with a hemacytometer (Reichert Sci. Instr., Buffalo, NY). Beginning 24 h after subculture, the medium, supplemented with 10 μM retinoic acid and 3 mM dbcAMP, was replaced daily (Sigma Chemical Co., St Louis MO).

Preparation of retinoic acid and dibutyryl cyclic adenosine monophosphate solutions

A stock solution of retinoic acid (0.01 M) was prepared in 95% ethanol. The working solutions for each retinoic acid concentration was 100×, prepared from stock. The solution was replaced every 2 weeks. All procedures were conducted under a red safelight (Eastman Kodak Co., Rochester, NY). A 0.1 M solution of dbcAMP in serum-free DMEM was prepared weekly.

Purification of ECM proteins and preparation of substrata

Laminin and type IV collagen were prepared from Engelbreth-Holm-Swarm (EHS) sarcoma tumors as described (Kleinman *et al.* 1982; Timpl *et al.* 1982). Fibronectin was prepared from human plasma using affinity chromatography on gelatin and heparin (Engvall and Rouslahti, 1977). Rat tail collagen was prepared as described (Bornstein, 1958). Bovine serum albumen (BSA) was purchased from Sigma Chemical Company (St Louis, MO).

Laminin, FN, type IV collagen and type I collagen were diluted in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) to the concentrations indicated in figures and the figure legends. Poly-L-lysine was diluted to 100 μg ml^{-1} in water. Samples (1 ml) of solutions were air dried onto 35 mm tissue culture dishes containing 18 mm×18 mm coverslips. The dishes were then blocked with 2% BSA in DMEM for 12 hours and washed once with DMEM before plating cells.

Preparation of synthetic peptides

The peptides YIGSR-NH_2 and PDGSR, which are identical to sequences found in the B1 chain of laminin, and GRGDN, which is similar to a sequence from the A chain of laminin (Sasaki *et al.* 1988), were synthesized at the National Institutes of Health (NIDR, Bethesda, MD) as described (Graf *et al.* 1987*b*). The amide form of YIGSR was used because it has been demonstrated that it is approximately twice as effective in promoting cell adhesion as the non-amidated form (Graf *et al.* 1987*b*). The synthetic peptides YIGSK-NH_2, PFGGS, GRGDS and GRGDTP were synthesized at the University of Virginia. The YIGSK-NH_2 peptide was used as a control peptide because it is a conservative modification in which the positively charged epsilon amino lysine group is substituted for the positively charged guanidinium side group of arginine. The peptides were dissolved in DMEM and added at 5 mg ml^{-1} to achieve the desired concentrations. Appropriate volumes of FBS (Hyclone, Logan, Utah) were added with the peptide solution to maintain the concentration of FBS at 10%.

Fluorescence microscopy and photography

Cell cultures were observed with a Leitz Ortholux microscope using epifluorescence and differential interference contrast (DIC). Leitz Fluotar 16/0.45 and 40/0.75 DIC objectives were used. A Leitz Vario Orthomat camera and Kodak Tri-X film developed to 1600 ASA with Diafine developer were used for photography. For color photography, 3M 1000 developed by E6 processing was used.

Antibodies

Neurons were identified using a monoclonal antibody, TuJ1, which recognizes a type III neuron-specific β-tubulin isoform (Sullivan and Cleveland, 1986; Moody *et al.* 1988; Lewis and Cowan, 1988; Callamo *et al.* 1989). Laminin antibodies have been characterized previously (Little *et al.* 1989). Non-immune rabbit IgG was purified on a protein A–Sepharose column (Pharmacia Inc.). Rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG were used as secondary antibodies (Cooper Biomedical, West Chester, PA). The antiserum to the 67×10^3 M_r laminin receptor has been described (Graf *et al.* 1987*b*).

Measurement of cellular attachment, spreading and confluence

Two parameters, cell attachment (number of cells μm^{-2}) and culture confluence (total surface area covered by cells), were determined by examining 25 randomly chosen fields (3.0×10^5 μm^2). Cellular spreading (surface area covered by an individual cell) was estimated by measuring the area covered by the central most cell in 25 randomly chosen fields (3.0×10^5 μm^2). Cultures on coverslips were fixed for 10 min with 3% paraformaldehyde in PBS, followed by staining with Toluidine Blue for 1 min. The cultures were dehydrated through graded ethanol solutions and mounted on a slide with Krystalon (Harelco, Gibbstown NJ).

A Leitz Orthoplan microscope equipped with an Olympus Planapo 10/0.40 (culture confluence assay) or a Leitz Fluotar 40/0.75 DIC objective (cell attachment cellular spreading assays) was used. Images were digitized using a DAGE 68 NEWVICON camera with external gain and black level control, Gould IP8900 image processing hardware, and a DEC PDP11/70 computer. Cellular spreading and culture confluence were measured using GRYWIN, which performs image segmentation by grey level (Biomedical Image Processing Center, University of Virginia). Cell attachment was measured using a similar program, GRNCNT, also based on grey level image segmentation (Biomedical Image Processing Center).

Statistical analysis

Cell attachment, cellular spreading, and culture confluence were compared using analysis of variance (Statistical Analysis Systems, Cary, North Carolina) in a general linear model (Searle, 1971). *t*-statistics on least-square means, using pooled variances from the analysis of variance models, were used to test hypotheses. The level of significance for *t*-tests was $P<0.001$ unless stated otherwise.

Radiolabeling and immunoprecipitation

Cultures were labeled for 3 h with 100 μCi of [^{35}S]methionine (100 μCi ml^{-1}) in methionine-free, high-glucose, Dulbecco's modified Eagle's medium without sodium pyruvate, supplemented with 10 % fetal bovine serum and 100 μg ml^{-1} gentamicin. Cultures were suspended in a radioimmunoprecipitation (RIPA) buffer containing 1 % Triton X-100, 1 % sodium deoxycholate, 0.5 % sodium dodecyl sulfate, 0.1 M Tris–HCl, pH 8.0, and 0.02 M phenymethylsulfonyl chloride, for 30 min at 4 °C, then centrifuged for 5 min at 10 000 *g*. The pellet was discarded, and 300 μl samples of the supernatant were pre-incubated with 50 μl of a 10 % protein A–Sepharose/RIPA buffer suspension for 15 min at 4 °C. The beads sedimented at 10 000 *g*, the supernatant was transferred to a new tube and mixed with 10 μg of affinity-purified rabbit anti-mouse laminin IgG. The solution was tumbled gently for 1 h at 4 °C, then incubated with 100 μl of protein A–Sepharose for an additional 1 h. The beads were collected by centrifugation, washed in RIPA buffer, extracted with SDS sample buffer at 100 °C, and electrophoresed in 5 % SDS–polyacrylamide slab gels according to the method of Laemmli (1970).

Results

Expression of laminin by stem cells and differentiating neurons

PCC4uva embryonal carcinoma showed faint cytoplasmic staining and punctate surface immunoreactivity when cultures were probed with an affinity-purified polyclonal antibody to laminin (Fig. 1A,B). Following treatment with retinoic acid and dbcAMP for 48 h, bright patches of immunoreactive laminin were associated with primitive neuroepithelial-like cells throughout the cultures (Fig. 1C,D). At this time differentiating neurons had spread and extended broad processes. After 8 days of culture, immunoreactivity was associated with neuronal cell bodies, occasional epithelial-like cells, and fascicles of neurites (Fig. 1E,F).

PCC4uva stem cells and differentiating neurons (48 h after drug treatment) were examined by immunoprecipitation using laminin antibodies. Lysates from [^{35}S]methionine-labeled cultures (3 h) were electrophoresed on 5 % SDS–polyacrylamide gels (Fig. 2). Stem cells and differentiating neurons synthesized three laminin chains. Stem cells produced immunoreactive, radiolabeled polypeptides that migrated with a slightly lower apparent molecular weight than EHS tumor laminin A and B chains (lane a). More radiolabeled laminin chain polypeptides were immunoprecipitated from 48-h cultures (lane b) than from stem cell cultures. A control reaction that made use of non-immune purified rabbit IgG showed no detectable bands under identical conditions of analysis (lane c).

Immunolabeling with laminin receptor antibodies

PCC4uva stem cells cultured on laminin-coated surfaces showed faint immunoreactivity with a polyclonal antibody that recognizes the 67×10^3 M_r laminin binding protein. However, 48 h after treatment with retinoic acid and dbcAMP, differentiating neurepithelial-like cells show strong immunolabeling (Fig. 3B). The immunoreactive material appears to be associated with the undersurface of cells made permeable with detergent. After 7 days in culture, PCC4uva-derived neurons and other cells expressed the 67×10^3 M_r laminin binding protein (Fig. 3D). When cells were not made permeable prior to immunolabeling (Fig. 3F), the 67×10^3 M_r binding protein was primarily distributed along neurites and cell regions in contact with the substratum, rather than regions of cell–cell contact.

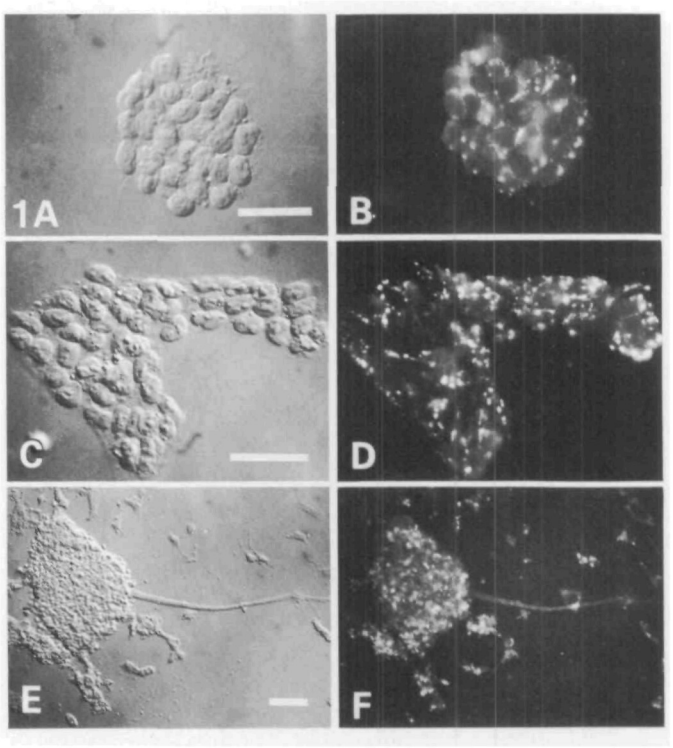

Fig. 1. Laminin is present in differentiating PCC4uva cultures. Differential interference contrast (DIC) and immunofluorescent micrographs show induced cultures that were probed with affinity-purified laminin antibodies. Cultures were fixed, made permeable and immunolabeled as described in Materials and methods. Stem cells (24 h) show punctate immunoreactivity (A,B). At 48 h cells are less rounded, the immunolabeling is more intense, and covers a greater area of cell culture (C,D). After 8 days in culture (1 day, untreated, retinoic acid and dbcAMP treatment for 5 days, then DMEM$^+$ alone for 2 days) large patches of immunoreactivity are apparent under epithelial-like cells. Detectable fluorescence is also associated with fascicles of neurites. In this and in all subsequent experiments, the cultures were grown using the drug treatment regimen describe (Sweeney *et al.* unpublished). Bars, 25 μm.

Influence of ECM on cell attachment, spreading and confluence

Extracellular matrix (ECM) substrata were tested for their capacity to influence the rate and extent of neural differentiation in the PCC4uva system. Attachment of stem cells to laminin substrata was significantly greater than to all other substrata tested (Fig. 4). In addition to promoting attachment, laminin substrata had a marked effect on cell morphology. Cells seeded on glass and examined by interference microscopy after 18 h display a small tightly bunched morphology with no intercellular space evident (Fig. 5A). Cells seeded on laminin substrata spread, separated and formed distinct margins with broad lamellipodia and smaller spike-like protrusions (Fig. 5B).

Fig. 2. Laminin is synthesized by PCC4uva cultures during neural differentiation. Laminin antibodies were used to immunoprecipitate [^{35}S]methionine-labeled (3 h) antigen from cell extracts, which was then applied to 5 % SDS–polyacrylamide gels. PCC4uva stem cell lysates show bands that correspond to laminin A and B chains, although the bands are of lower apparent molecular weight compared to EHS laminin chains (lane a). Similar bands are present in 48-h cultures (lane b). Lane c is a control in which a non-immune IgG was substituted for anti-laminin IgG. Cultures received equal amount of radioactivity and equal samples of cell lysates were loaded in each lane (see Materials and methods for details). The migration positions of Coomassie Blue-stained, purified mouse EHS tumor laminin A chains ($\sim 400\times10^3\ M_r$) and B chains ($\sim 210\times10^3\ M_r$) are indicated on the right; the migration position of 205, 116 and $97\times10^3\ M_r$ markers are indicated along the left margin.

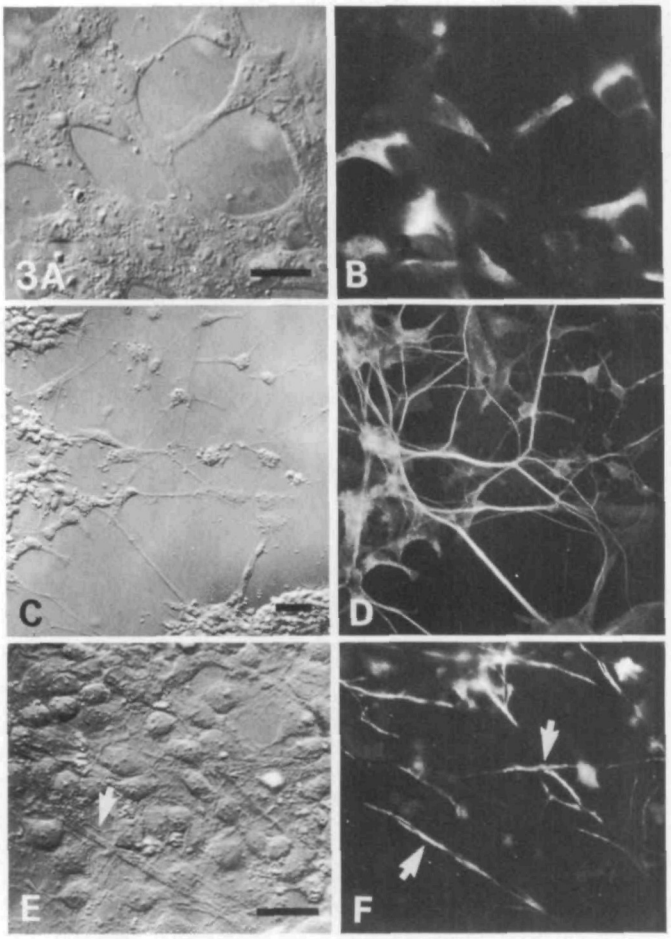

Fig. 3. Differentiating cells express a $67\times10^3\ M_r$ a laminin binding protein. Differential interference contrast optics and the corresponding immunolocalization of the $67\times10^3\ M_r$ laminin binding protein are shown. After 48 h of culture on laminin the formerly rounded, poorly attached stem cells have flattened dramatically (A). Immunofluorescence optics (B) shows immunoreactivity that, by careful focusing, appears to be associated with the basal cell surface of permeabilized cells. Neurons and most other cells in 7-day cultures (C) appear to express the $67\times10^3\ M_r$ laminin binding protein (D). Seven-day cultures immunolabeled without first making the cells permeable, show a considerably more restricted pattern of immunoreactivity (F). Note that the neural cell bodies were uniformly unstained, whereas discrete segments of neurites, primarily in regions in contact with the substratum (arrows), show marked immunostaining. Frequently, there was no immunoreactivity observed where neurites coursed over cell bodies (E). Bars, 25 μm.

Morphological differences between cells on laminin and all other substrata were first discernible by 15 h, and continued to be apparent throughout the culture period. Cell spreading on laminin was significantly greater ($P<0.01$) than on type I collagen, fibronectin and glass; 5 $\mu g\,ml^{-1}$ appeared to be the minimum concentration to permit significant spreading above background, but spreading was not dose-dependent. Type IV collagen substrata induced significant spreading compared to glass, but did not induce the separation between cell boundaries as observed for laminin (not shown). Spreading of cells grown on laminin in the absence of drug treatment was not significantly different from cell spreading on glass (not shown).

To assess the combined effects of cell attachment, spreading, survival and doubling rate, we measured culture confluence, which takes into account all these parameters. The degree of cellular confluence on laminin, after 15 h of drug treatment was dose-dependent and significantly greater than the degree of cellular confluence on all other substrata (Fig. 6).

Effects of laminin antibodies

Affinity-purified polyclonal antibodies to laminin blocked the laminin-induced increase in cellular confluence. Incubation of laminin substrata with anti-laminin antibody at 10 $\mu g\,ml^{-1}$, prior to subculture, abolished the effects of laminin on culture confluence (Fig. 7, circles). Addition of 25 $\mu g\,ml^{-1}$ laminin antibodies to cultures caused release of most cells. In contrast, non-immune rabbit IgG had no effect on culture confluence when incubated with laminin substrata prior to subculture, or when added to previously attached cells (Fig. 7, squares).

Effects of synthetic peptide sequences

Various laminin-derived and control synthetic peptides were tested for their ability to inhibit the effects of laminin substrata. Of the peptides tested (GRGDS, GRGDTP, GRGDN, PDGSR, PFGGS, YIGSR-NH_2, YIGSK-NH_2), only YIGSR-NH_2 caused a dose-dependent inhibition of

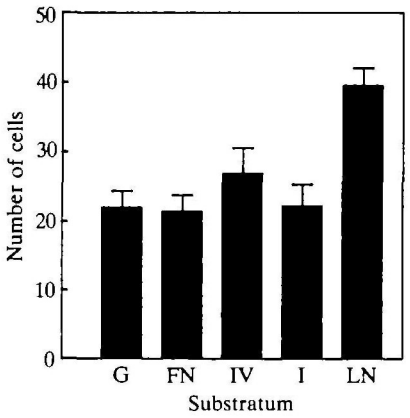

Fig. 4. Attachment of PCC4uva to various substrata. Glass coverslips, in 35 mm dishes, were treated with 1 ml (10 $\mu g\,ml^{-1}$) of various substrata, air dried overnight and then blocked with 2% bovine serum albumin (BSA) for 12 h as described in Materials and methods. One ml of medium containing 1.45×10^5 PCC4uva stem cells was plated into 35 mm tissue culture dishes ($n=25$) and allowed to attach for 2 h. The culture medium was aspirated and the cultures were fixed with 3% paraformaldehyde, stained with Toluidine Blue and the percentage confluence was measured as described in Materials and methods. Symbols: G, glass; FN, fibronectin; IV, type IV collagen; I, type I collagen; LN, laminin. Laminin promoted significantly more cell attachment than the other ECM components or BSA-coated glass. Fibronectin and collagen type I were not significantly different from BSA/glass. Collagen type IV promoted slightly more attachment than glass. Significance level of the statistical analysis was $P<0.001$.

the laminin-induced increase in cellular confluence (Fig. 8, squares) with partial inhibition at 10 $\mu g\,ml^{-1}$ of peptide and complete inhibition at 50 $\mu g\,ml^{-1}$. At a concentration of 250 $\mu g\,ml^{-1}$ or greater, cell detachment was observed. The peptide YIGSK-NH_2 in which lysine is substituted for arginine, did not significantly affect culture confluence or cell attachment on either laminin or glass (Fig. 8, circles). Addition of YIGSR-NH_2 appeared to perturb cell attachment to endogenous laminin. Differentiating PCC4uva stem cells cultured on glass (no exogenous laminin) were released from the substratum at concentrations similar to those that released cells on exogenous laminin, whereas the control peptide YIGSK-NH_2 had no effect on cell attachment (data not shown). Peptides PDGSR, PFGGS, GRGDS, GRGDN and GRGDTP applied at similar concentrations also showed no apparent decrease in culture confluence on laminin or glass substrata (not shown).

Influence of ECM components on neural differentiation

Laminin substrata promoted rapid and extensive neural differentiation when compared to all other substrata tested. Neurons were first observed by indirect immunofluorescence using TuJ1 after 24 h of drug treatment in laminin-coated dishes, and by 48 h were confluent and multi-layered. In contrast, neurons were not observed until 48 h after drug treatment in cultures plated on glass/BSA. In addition, 48 h glass/BSA cultures were always subconfluent for up to 25 days (Sweeney *et al.* unpublished). Most striking were the differences in cultures after 6 days, when clustering of neuronal cell bodies and neurite fasciculation were observed on laminin. In contrast, 6-day cultures initially seeded on glass appeared considerably less 'mature' (compare Fig. 9A,B with C,D).

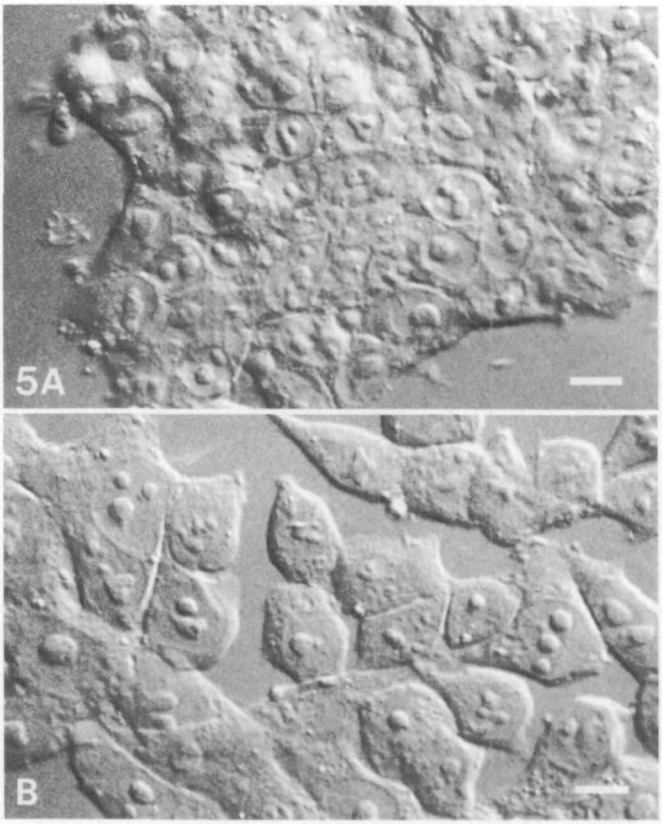

Fig. 5. Laminin substrata promote cell spreading. Differential interference contrast micrographs of 15-h cultures show a strikingly different morphology depending on whether they were cultured on laminin or glass (blocked with BSA). Cells initially seeded on BSA are poorly spread and tightly packed after 15 h (A). In contrast, cells initially seeded on a laminin substratum show a considerable degree of spreading behavior. No other substratum tested resulted in this marked spreading behavior. In addition, only cells initially seeded on laminin, separated and formed distinct cell margins. Bar, 10 μm.

Cultures seeded on all other substrata tested required an additional 2 days of growth to reach the same degree of differentiation as 6-day cultures seeded on laminin. Quantitation of the numbers of neurons in 6-day cultures was not possible because the cultures were confluent and multilayered. However, there appeared (subjectively) to be many more neurons in cultures on laminin than in subconfluent, 6-day glass/BSA cultures. Type IV collagen substrata yielded cultures with more neuronal cells compared to controls. However, the rate of cell body aggregation and neurite fasciculation was not accelerated. Type I collagen and FN substrata showed no apparent differences in the rate or extent of neural differentiation compared to glass/BSA.

Discussion

Laminin has dramatic effects on differentiating PCC4uva cultures

Determining what role laminin plays in early neural differentiation is hampered by the difficulty of perturbing or modulating endogenous laminin *in vivo*. Our previous work showed that PCC4uva embryonal carcinoma differentiates predominantly into neurons under defined short-

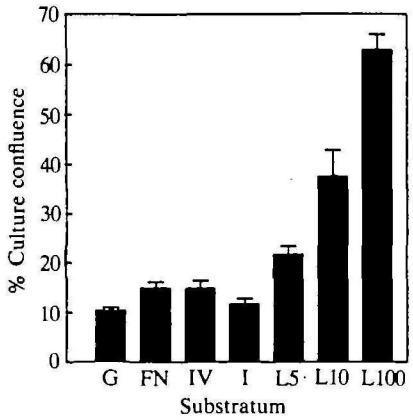

Fig. 6. Confluence of PCC4uva cells is influenced by growth on different substrata. These cultures were prepared like those in Fig. 4 except that in some cases increasing concentrations of laminin were used to coat coverslips. Confluence, the unit-area of a substratum covered by cells, is a parameter that encompasses cell attachment, doubling, spreading and survival. Laminin had a significant effect on cell confluence and showed a dose-dependent effect between 5 and 100 $\mu g\,ml^{-1}$. Symbols are the same as in Fig. 4 except L5=5 μg laminin ml^{-1}, L10=10 μg laminin ml^{-1}, L100=100 μg laminin ml^{-1}. Culture confluence was determined using an automated system, described in Materials and methods. Significance level of the statistical tests was $P<0.001$.

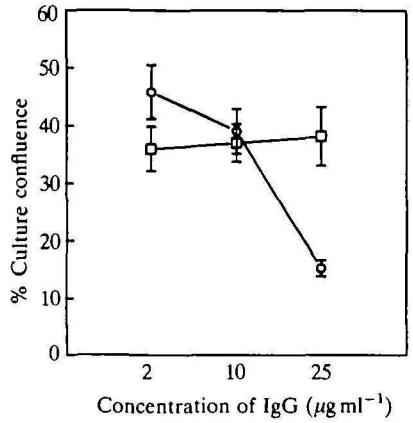

Fig. 7. Laminin antibodies perturb differentiating PCC4uva cultures. Coverslips, in 35 mm dishes, were coated with 10 $\mu g\,ml^{-1}$ of laminin then blocked with BSA. Dishes were seeded with 1 ml of medium containing 7.5×10^4 stem cells, allowed to attach for 24 h at which time the cultures were incubated with the indicated concentrations of affinity-purified rabbit anti-mouse laminin antibodies. A dose-dependent inhibition of confluence was observed with the laminin antibodies (circles). Non-immune rabbit IgG has no effect (squares). The culture confluence of fixed cells was automatically determined using digitized images of 25 randomly selected fields for each culture treatment. Significance level of the statistical tests was $P<0.001$.

term tissue culture conditions (Sweeney *et al.* unpublished). This culture system provides large numbers of differentiating neurons, which are easily manipulated and isolated for analysis. Using the defined culture conditions, this work shows that exogenous laminin strongly potentiates the neural differentiation of PCC4uva embryonal carcinoma.

Exogenous laminin substrata induced dose-independent

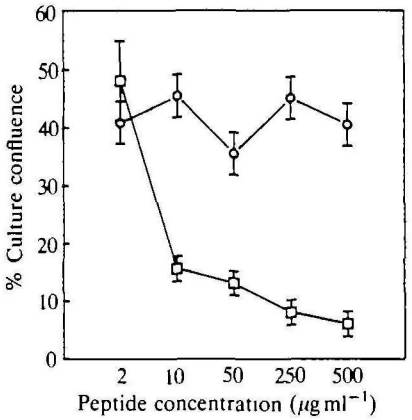

Fig. 8. Effect of YIGSR-NH_2 on cell confluences: laminin-treated substrata. Cultures were prepared as for Fig. 7, then incubated with various concentrations of YIGSR-NH_2 (squares) or YIGSK-NH_2 (circles) for 24 h. A dose-dependent decrease in confluence was consistently observed when laminin-treated cultures were incubated with YIGSR-NH_2. Each peptide was tested three times with similar results. The method by which confluence was assessed is described in Materials and methods. Significance level of the statistical tests was $P<0.001$.

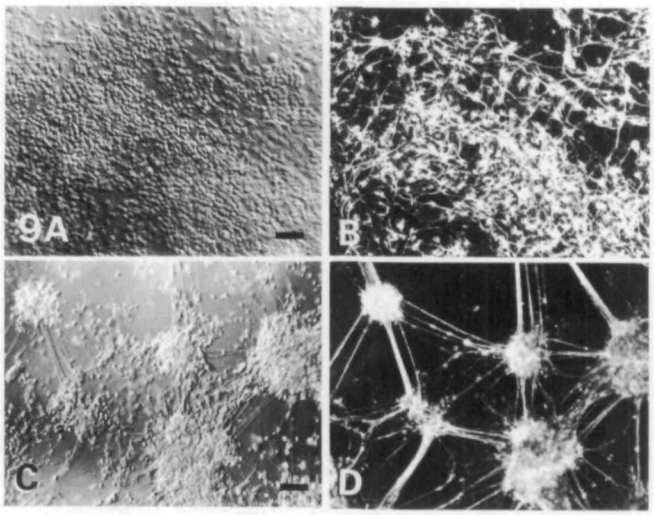

Fig. 9. Cultures seeded on laminin show accelerated neural differentiation. Differential interference contrast and immunofluorescence micrographs of cultures grown on BSA or laminin-coated substrata. Six days after PCC4uva stem cells were seeded on BSA-coated coverslips, the differentiated cultures contain an overlapping mat of cells when observed with DIC optics (A). Epifluorescence optics show TuJ1 immunolabeled neurons with relatively short unbundled neurites (B). In marked contrast, cultures derived from stem cells originally seeded on laminin substrata, display a more mature, tissue-like geometry. Clusters of neural cell bodies are interconnected by relatively long neuritic fascicles (C,D). Cultures that were originally seeded on glass substrata, such as the culture shown in A and B, do not reach this degree of maturity until an additional 2 days have passed. Bars, 50 μm.

(above a threshold of $5\,\mu g\,ml^{-1}$) spreading of differentiating neurepithelial-like PCC4uva cells. Laminin simultaneously induced a dose-dependent increase in culture confluence (confluence is a parameter that is the end result of multiple processes, including: plating efficiencies, cell spreading, cell survival and the doubling rate of differentiating cells). Most striking, laminin substrata induced more rapid and extensive neural differentiation of PCC4uva embryonal carcinoma cells compared to all other substrata tested. The acceleration of neural differentiation can be inhibited by addition of the synthetic peptide YIGSR-NH_2. In addition, YIGSR-NH_2 perturbs adhesion and neural differentiation of PCC4uva plated on glass/BSA. This strongly suggests that the YIGSR-NH_2 peptide is interfering with *endogenous* cell–laminin interactions.

Laminin may influence early mammalian stem cells

The effect of laminin on PCC4uva stem cell differentiation correlates with the fact that it is detected at the embryonic 2-cell stage (Cooper and MacQueen, 1989). Further, the independent and coordinated expression of each laminin subunit, suggests a functional role for this molecule (Cooper *et al.* 1981; Leivo, 1983; Grover and Adamson, 1985; Dziadek and Timple, 1985). It is known that during the very early stages of mouse development cell rearrangements appear to be necessary for differentiation of the inner cell mass (Ziomek and Johnson, 1980; Ducibella and Anderson, 1975; Lo and Gilula, 1979). Perhaps the coordinated expression of laminin chains partially mediates these cellular rearrangements.

Studies using various embryonal carcinoma cell lines provide evidence that laminin influences stem cell differentiation. Laminin synthesis is rapidly induced in F9 teratocarcinoma cultures after retinoic acid treatment (Strickland *et al.* 1980; Prehm *et al.* 1982; Carlin *et al.* 1983; Cooper *et al.* 1983; Grover and Adamson, 1985). Interestingly, the quantity of each laminin chain synthesized during F9 cell differentiation varies, similar to laminin subunit synthesis in early mouse embryos (Grover and Adamson, 1985). The commitment of cells from F9 aggregates and 1003 embryonal carcinoma cells to specific cell types is influenced by culture on laminin substrata. Again, this suggests that laminin exerts a direct influence on cellular differentiation (Grover *et al.* 1983; Darmon, 1982).

Why does exogenous laminin potentiate neural differentiation?

Assuming that laminin is required for neural differentiation, one possibility is that the lack of laminin on untreated substrata during the first few hours of cell culture may impose severe constraints on cell spreading behavior. In contrast, the availability of laminin in treated cultures during this crucial time may release the constraint. We have determined that stem cells synthesize laminin, thus stem cells subcultured on other substrata may have to await the accumulation of a critical amount of laminin before differentiation can progress rapidly.

Multiple laminin adhesion mechanisms have been reported in developing and mature animal cell systems (Buck and Horwitz, 1987; Martin and Timpl, 1987; Gehleson *et al.* 1988; Engvall *et al.* 1986; Tomaselli *et al.* 1987; Goodman *et al.* 1987; Kleinman *et al.* 1988; Eckstein and Shure, 1989), including a $67\times10^3\,M_r$ YIGSR mechanism (Graf *et al.* 1987*a*,*b*). Recent work using HT1080, a human fibrosarcoma cell line, suggests that the YIGSR site is not available for HT1080 cell binding in cross-shaped EHS–laminin. However, digestion of proteolytic laminin fragment E1-4 to fragment 1 rendered a site available for cell recognition. An accessibility problem was not observed in the PCC4uva system. Although we did not test proteolytic fragments, the YIGSR-NH_2 effect was dramatic and the $67\times10^3\,M_r$ binding protein was detected by immunofluorescence. Furthermore, all the data presently available regarding laminin subunit expression in mouse stem cell populations (see Fig. 2 above, and the Introduction) show that more B1 and B2 chains than A chains are produced. Presumably, this means that considerable laminin B1 chains are not in cross-shaped complexes, and that YIGSR sites are available for recognition.

Cell–laminin interactions

Synthesis of a coherent model of laminin–cell surface interactions during early stem cell differentiation is hampered by interpretation of studies that make use of widely differing cell lines for investigating multiple receptor systems. Perhaps the most troublesome problem is that most studies have relied on substratum adhesion assays as the only indicator of cell–laminin interactions. There is no evidence to suggest that cell–laminin interactions are restricted to adhesion. It is likely that contact with laminin can influence cell behavior by mechanisms other than providing mechanical adherence.

In view of recent work on laminin domains (Nurcombe *et al.* 1989; Panayotou *et al.* 1989), it is clear that there may be several mechanisms by which cells interact with laminin, independent of adhesive activity. We speculate that sequences such as YIGSR act in concert with other laminin binding sites in order to modulate cell surface–laminin interactions.

The laminin domains that encompass the YIGSR sequence appear to be highly conserved during evolution (Martin and Timple, 1987; Panayotou *et al.* 1989). In particular, the newly described s-laminin molecule contains the sequence YTGLR, which is found in the same relative position as YIGSR is in the B1 chain. Moreover, s-laminin may be an example of a laminin(s) that is not expressed in cross-shaped complexes.

Laminin and the commitment to a neural cell lineage

Our data indicate that extracellular laminin is an effective agent for influencing the fate of pluripotent stem cells. The first easily observed change in differentiating PCC4uva cultures is the onset of cell spreading. This drastic change in cell geometry seems to be a pivotal event in the life of these rounded, highly mitotic, pluripotent cells. Once the cells become larger and squamous, they have a markedly lower mitotic index (unpublished observations) and are presumably no longer pluripotent (Pierce, 1968). Treatment with retinoic acid induces the stem cells to leave the highly mitotic, poorly adherent state. Presumably, the cells are then subject to the influences of cell–ECM and cell–cell interactions. Changes in cell geometry in response to ECM signals are known to have profound effects on differential gene expression (Hay, 1984; Greenberg and Hay, 1986; Bissell and Barcellos-Hoff, 1987). It may be that these ECM 'signals' are partially responsible for commitment to a specific cell lineage.

In summary, laminin potentiates neural differentiation of retinoic acid and dbcAMP-treated PCC4uva embryonal carcinoma cells. We speculate that multiple interactions occur between the surface of PCC4uva stem cells (and

their immediate descendants) and laminin. We have shown that a 67×10^3 M_r receptor, which is reported to recognize YIGSR, is expressed on the surface of PCC4uva-derived cells during neural differentiation. The addition of laminin antibodies or YIGSR-NH_2 peptides results in a rounded cell shape. Since a spread morphology appears to be an obligatory step in neural differentiation of PCC4uva stem cells, the change to a rounded morphology interrupts subsequent neural differentiation. Thus specific alterations in cell–ECM interactions can have profound effects on the commitment of a pluripotent stem cell to a particular lineage.

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