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Guidance of Neurite Outgrowth by Pathways of Substratum-Adsorbed Laminin

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Neurite outgrowth is guided by narrow pathways of bioactive laminin. These pathways are created by ultraviolet light irradiation of laminin-coated coverslips masked with electron microscope grids. Patterned outgrowth of neurites is independent of gross mechanical guidance and guidance caused by substrate limitation. Cells on unirradiated laminin are less readily displaced by shear forces than cells on irradiated laminin. This study suggests that ultraviolet light alters the adhesive properties of laminin and that differential cell-substratum adhesion may guide extending neurites on the purified naturally occurring substance, laminin.

Key words: laminin, neuron, guidance

INTRODUCTION

Growing axons extend toward synaptic targets along stereotyped pathways. Some of these pathways may be defined by substrate materials that axons extend on or through. Substrate pathways have been demonstrated in the central nervous system by observing the paths taken by growing axons of transplanted embryonic neurons [Katz and Lasek, 1981]. In the peripheral nervous system, distal stumps of severed nerves can act as substrate pathways for axons emerging from the proximal nerve stumps. The chemical or physical effectors of substrate guidance have not been identified. In vitro, paths of artificial substrate-bound materials can guide axon-like neuronal processes called neurites [Letourneau, 1977]. Neurite guidance by substrate pathways of natural, purified substances, though of great in vivo significance, has not been as extensively studied.

A guidance effect unique to an asymmetrically distributed substrate-bound substance is difficult to demonstrate. When serum is used in the culture medium, direct effects of any adsorbed purified material are unclear because serum can quickly alter the composition of the substrate [Rosenburg, 1960]. In addition, the methods used to create chemically anisotropic substrates [Smallheiser et al, 1984] often pro-

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duce light-microscopic substrate contours that can guide cells mechanically [Rovensky et al, 1971]. When substrate guidance does appear to be caused by chemical anisotropy, guidance often occurs only when pathways are bordered by substrates incapable of supporting neurite outgrowth [Collins and Garrett, 1980]. This type of guidance, caused by substrate limitation, is less specific than guidance occurring in the presence of an alternative substrate that supports neurite outgrowth. We have devised a method to test the ability of a substrate-bound naturally occurring molecule, laminin, to guide neurite outgrowth independent of serum components, mechanical guidance, and substrate-limitation effects.

Laminin, a basement membrane associated glycoprotein, was chosen as a good candidate for involvement in neurite guidance. Laminin appears early in embryonic development [Wartiovaara et al, 1980]. It is associated with skin, muscle [Wan et al, 1984], and peripheral nerve [Palm and Furcht, 1983] basement membranes that extending axons may encounter during nerve regeneration and embryonic development [Bignami et al, 1984; Sanes et al, 1978; Taylor and Roberts, 1983; McCloon, 1984]. Linear paths of laminin accessible to extending axons may be created by aligned extracellular spaces of cells attached to laminin-bearing basement membranes of the developing optic nerve and spinal cord [Singer et al, 1979; Krayanek and Goldberg, 1981]. In vitro, laminin adsorbed to tissue culture plastic promotes the initiation and growth of neurites from dissociated central nervous system and peripheral nervous system neurons [Rogers et al, 1983]. Together, these findings strongly suggest an in vivo guidance role for linear paths of laminin.

MATERIALS AND METHODS

Dorsal root ganglia were dissected from 8–9-day-old chicken embryos and dissociated as previously described [Rogers et al, 1983]. Cells were plated in serum-free Ham's F12 medium supplemented with 5 μg/ml insulin, 10 ng/ml β-nerve growth factor (a gift of Dr. Eric Shooter, Stanford University), 5 ng/ml sodium selenite, and 100 μg/ml human transferrin (Sigma Chemical Company). Approximately 20,000 cells at 13,000 cells/ml were placed in 35-mm plastic petri dishes (Falcon) containing ultraviolet light (UV)-patterned coverslips. Cells used in adhesion assays were plated in Ham's F12 containing 10% calf serum (GIBCO). Cultures were maintained at 37°C in a humidified atmosphere containing 95% room air and 5% CO_2.

Cells were plated on protein pathways made by first adsorbing purified proteins to nitric acid cleaned coverslips that had been extensively rinsed with distilled water and heat sterilized. Laminin from the mouse Engelbreth-Holm-Swarm (EHS) tumor and human plasma fibronectin were isolated as previously described [Rogers et al, 1983]. One hundred microliters of either protein at 100 μg/ml in 0.05 M carbonate buffer (pH 9.6) containing 0.2 mg/ml sodium azide was applied to one side of the coverslips. In some experiments, 1 mg/ml fatty acid free bovine serum albumin (Sigma Chemical Company) was used to coat coverslips. After 1 hr at room temperature, these coverslips were rinsed with distilled water and air dried. Substrate pathways were made by placing electron microscope grids directly on the coated air dried coverslips before irradiating these coverslips with ultraviolet light. Coverslips were irradiated for 1 hr with a bactericidal lamp (Westinghouse Sterilamp 782L-30) at a distance of 20 cm, and then the electron microscope grids were removed, uncovering invisible grid patterns of unirradiated protein.

Affinity-purified rabbit anti-laminin and anti-fibronectin were used to detect substrate patterns. Preparation of these antibodies was described earlier [Rogers et al, 1983]. Cells on UV-patterned substrates were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 20 min at room temperature. The coverslips were then incubated with 1 mg/ml sodium borohydride in PBS for 15 min and incubated with rabbit anti-laminin or anti-fibronectin followed by a rhodamine-labeled goat anti-rabbit secondary antibody (Cappel) as described previously [Letourneau, 1981]. In experiments where patterned neurite outgrowth was blocked, anti-laminin antibody at 23 μg/ml in PBS was applied to UV-patterned laminin substrates 1 hr prior to plating the cells. In addition, stock anti-laminin (230 μg/ml in PBS) was added to the culture medium to a final concentration of 23 μg/ml.

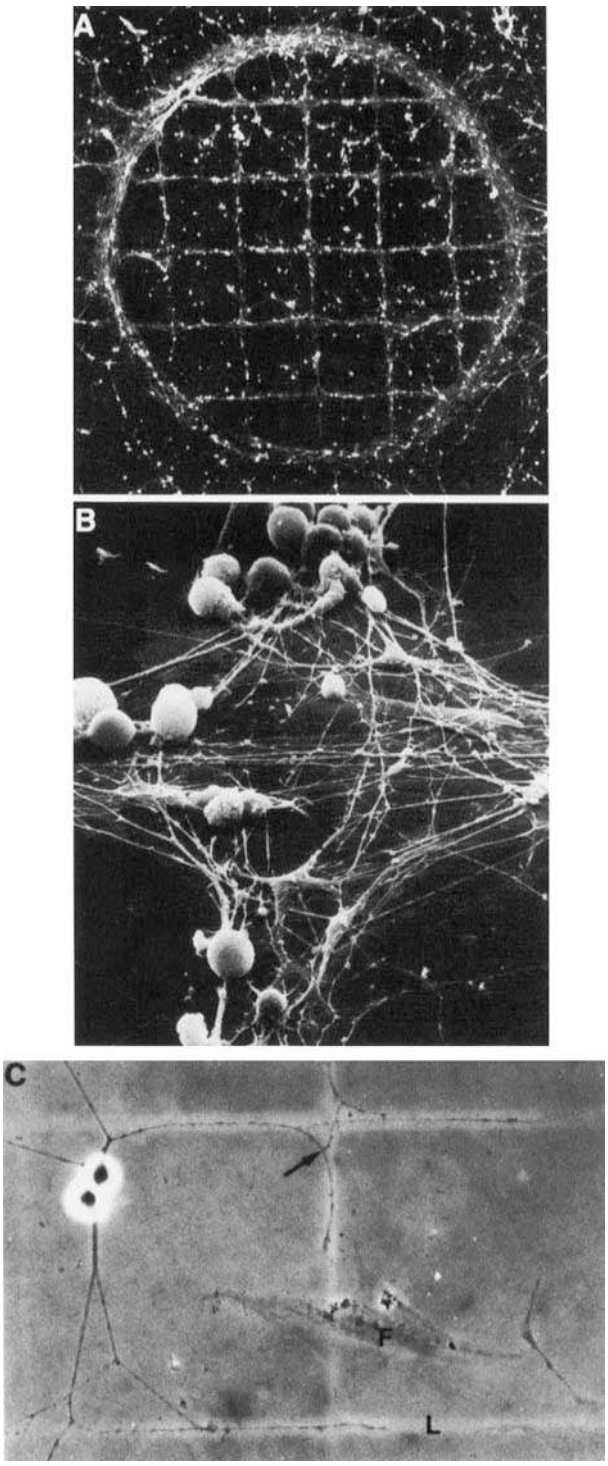
^{3}H-laminin [Jentoft and Dearborn, 1979] was used to determine the loss of adsorbed unirradiated versus adsorbed UV-irradiated laminin from coverslips during cell culture. Coverslips were coated with ^{3}H-laminin as described above for laminin. Duplicate irradiated or unirradiated coverslips were evaluated for ^{3}H-laminin bound by rinsing them in serum-free medium and then boiling them for 10 min in glass scintillation vials containing 1 ml of 1% sodium dodecyl sulfate followed by liquid scintillation counting (Beckman LS 6800).

The resistance to liquid shearing force of cells attached to UV-irradiated versus unirradiated laminin substrates was used as a measure of cell-substrate adhesion. Halves of laminin-treated coverslips were UV-irradiated to create sharp borders between adsorbed unirradiated laminin and adsorbed UV-irradiated laminin. Dissociated dorsal root ganglion cells were cultured on the coverslips for 3 hr. Some coverslips were then subject to low shear by processing them for immunofluorescence as described above. Other coverslips were subjected to high shear by placing them on a rotatory shaker for 1 min at 200 rpm followed by fixation and processing for immunofluorescence as described above. Cells were counted in microscope fields at $\times$400. These fields were immediately adjacent to each other, one field on adsorbed laminin and the other on UV-irradiated laminin. Ten paired fields per coverslip were counted, two coverslips per shear condition; each coverslip was subjected to a shear condition separately.

Scanning electron microscopy was performed on cultures fixed with 2% glutaraldehyde for 1 hr and alcohol dehydrated through 15%, 25%, 50%, 75%, 90%, and 100% ethanol. Cultures were critical-point dried (Sorvall apparatus) and sputter-coated with Au-Pd.

RESULTS

Grid patterns of unirradiated laminin have a dramatic effect on neurite outgrowth from dissociated dorsal root ganglion (DRG) neurons (Fig. 1A). The spatial relationship between extending neurites and unirradiated laminin can be identified precisely using immunofluorescence because only the unirradiated laminin binds anti-laminin antibody. Using this method, a guidance effect on individual neurites is clearly demonstrated (Fig. 1C). The transient presence of electron microscope grids on air-dried laminin-coated coverslips has no effect on neurite outgrowth. Even at high cell densities, outgrowth appears to be guided through interaction with unirradiated laminin paths because extensive neurite-neurite fasciculation is not seen and


A
B
C
F
L

the paths continue to be clearly demonstrated by the pattern of neurite outgrowth (Fig. 1B).

UV-irradiated laminin is not toxic to cells. Neurites are initiated and extend farther than 1,000 μm on UV-irradiated laminin (Fig. 1A). Neurites initiated on UV-irradiated laminin are capable of following the grid patterns of unirradiated laminin. Therefore, a selective toxicity of UV-irradiated laminin to a subpopulation of neurons capable of following unirradiated laminin paths is not responsible for the guidance effect.

Guidance is not elicited by all UV-irradiated proteins or glycoproteins. Substrata containing UV-light-patterned plasma fibronectin or bovine serum albumin prepared in the same way as UV-light-patterned laminin does not elicit guided neurite outgrowth, even though the fibronectin pattern can be clearly identified using anti-fibronectin antibodies. Guidance on laminin can be blocked by adsorbing additional laminin after the grid patterns of unirradiated laminin have been made. High levels of anti-laminin antibodies blocks most cell attachment and neurite outgrowth on laminin patterns if the patterns are incubated with anti-laminin before the cells are plated and antibody is present in the culture medium. By these criteria, guidance by paths of unirradiated laminin is mediated through specific neuron-laminin interactions.

UV-irradiation and subsequent culture of cells on the laminin-coated coverslips does not create microscopic substrate contours that could guide extending neurites mechanically. Scanning electron microscopy shows no visible ridge at the border of the unirradiated laminin (Fig. 1B). A greater loss of adsorbed laminin after UV-irradiation could create mechanical guides and cause a greater loss of cells from UV-irradiated laminin. Either of these possibilities might result in the patterning of cells observed in Figure 1. However, there is no statistically significant difference in the amount of ^{3}H-laminin retained on surfaces containing UV-irradiated versus unirradiated adsorbed ^{3}H-laminin. When 100 μg/ml of ^{3}H-laminin was used to coat glass coverslips, 194,000 $\pm$ 11,000 dpm (mean $\pm$ standard error, n = 2) were initially bound; after 1 day in culture with dissociated DRG cells, 137,000 $\pm$ 10,000 dpm (n = 3) of ^{3}H-laminin remained bound while 121,000 $\pm$ 9,000 dpm (n = 3) of UV-irradiated ^{3}H-laminin remained bound. Finally, growth cones (i.e., tips of extending neurites) and neurites do not accumulate at the borders of unirradiated laminin pathways as one might expect if growth cones were guided by a mechanical border at this site (Fig. 1B).

Greater adhesion of growth cones and filopodia to the unirradiated laminin paths may be responsible for guidance of neurite outgrowth. In experiments where extending neurites have the opportunity to extend on more than one type of substrate, the preferred substrate has been found to be the most adhesive [Letourneau, 1975]. We

Fig. 1. A) Scanning electron microscopy of dissociated dorsal root ganglion cells on an ultraviolet light (UV)-patterned laminin substrate after 2 days of culture in serum-free medium. Neurite ougrowth is guided by laminin protected from ultraviolet light, resulting in a pattern of dense neurite outgrowth resembling an electron microscope grid. The ultraviolet-light-exposed laminin bordering the grid pattern supports neurite outgrowth (diameter of grid pattern = 3 mm). B) High magnification of center of grid pattern in A. Evidence of a mechanical barrier at the border between laminin and UV-irradiated laminin can not be found. Neurites do not accumulate at the border and they do not tend to fasciculate. C) Simultaneous phase contrast and epi-flourescent microscopy identifies the spatial relationship between neurites and the unirradiated laminin paths. These 7-μm-wide paths (L) are precisely followed by extending neurites that often branch near intersections of paths (arrow). Many fibroblastic cells (F) do not seem to be aligned by the laminin paths.

found a substantially greater adhesion of DRG cells to unirradiated versus UV-irradiated laminin as measured by the resistance of DRG cells to displacement from the coverslip by shear forces produced on a rotatory shaker (Table 1).

DISCUSSION

Our findings are consistent with the hypothesis that guidance of neurite outgrowth is based on differential cell-substratum adhesion. Unirradiated laminin is more adhesive for dissociated DRG cells in culture medium containing serum than UV-irradiated laminin. However, the relationship between overall adhesion of a mixed population of DRG cell bodies and adhesion of the growth cone, where guidance occurs, is not obvious. We are now employing an adhesion assay using the calcium chelating agent EGTA that allows direct observation of growth cone attachment. Detecting differential adhesion that is important in guiding neurite outgrowth may be difficult for several reasons. Guidance may depend on turnover rates of adhesions or the localization and shape of adhesions on the growth cone. Time-lapse interference reflection microscopy could help identify the temporal and spatial features of cell-substrate contacts but contacts having similar size and shape may not have similar adhesive properties.

The photochemistry of proteins is not well documented, and chemical studies will have to be done to determine the critical molecular effect of UV-irradiation on adsorbed laminin that results in the guidance phenomena illustrated in Figure 1. Preliminary results indicate the adhesivity of adsorbed fibronectin is not lowered by UV-irradiation. This may explain the inability of UV-patterned fibronectin to guide neurite outgrowth.

The inability of UV-irradiated fibronectin or albumin to elicit oriented outgrowth, and the blockage of guided outgrowth by adsorbing additional laminin to the UV-patterned substrata indicate specificity for laminin in guiding neurite outgrowth under our experimental conditions. It is not possible to determine from this study if

TABLE I. Resistance to Shear of Cells on Unirradiated Laminin Versus Cells on UV-irradiated Laminin*

Treatment	Substratum	Average No. cells/field[a]	Ratio A/B[b]
Low shear	Laminin	57.30 ± 2.73	
	UV-Laminin	37.40 ± 2.11	1.54 ± .04
High shear	Laminin	40.55 ± 3.49	
	UV-Laminin	6.80 ± 1.09	5.98 ± .23

*Halves of laminin-coated coverslips were UV-irradiated to create sharp borders between adsorbed unirradiated laminin and adsorbed UV-irradiated laminin. Dissociated dorsal root ganglion cells were cultured on these coverslips in medium containing serum for 3 hours, and then some coverslips were subject to low shear by processing them for immunofluorescence. Some coverslips were subject to high shear by placing them on a rotatory shaker at 200 rpm for 1 minute. Cells remaining on coverslips were counted in microscope fields at ×400. Fields were immediately adjacent to each other, one field on unirradiated laminin and the other on UV-irradiated laminin (see Materials and Methods for more details).

[a]Mean ± standard error, n = 20.

[b]A is the average number of cells per field on laminin, and B is the average number of cells per field on UV-irradiated laminin, mean ± standard error, n = 2. Difference between A/B ratios of low shear versus high shear is significant by unpaired Student's t-test, $P < .005$, n = 2.

laminin is interacting directly with the cell surface or through interaction with molecules produced by the cells in culture or present in serum-free culture medium.

Recently, we have found that neurites of dissociated spinal cord cells from 6-day chick embryos are also guided by UV-patterned laminin. Other preliminary results indicate that dissociated chick heart fibroblasts do not align or accumulate on laminin pathways. In addition, some fibroblastic cells from DRGs do not align on laminin pathways although a subpopulation of these cells may align (Fig. 1C). Cell-type-specific guidance was not found in a study using artificial substrates [Harris, 1973]. More work is needed to determine the basis of the cell-type-specific effects of laminin pathways and whether there are effects on non-nerve cell migration or only on cell alignment.

The use of UV-light-patterned substrates has identified a chemically specific and possibly cell-specific guidance effect of a naturally occurring molecule of potential significance for development and regeneration of the nervous system. The ease in constructing laminin pathways with UV will facilitate study of the cellular and molecular mechanisms of chemically based cell guidance.

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REFERENCES

Bignami A, Chi NH, Dahl D (1984): Laminin in rat sciatic nerve undergoing wallerian degeneration. J Neuropathol Exp Neurol 43:94–103.

Collins F, Garrett JE, Jr (1980): Elongating nerve fibers are guided by a pathway of material released from embryonic nonneuronal cells. Proc Natl Acad Sci USA 77:6226–6228.

Harris AK (1973): Behavior of cultured cells on substrata of variable adhesiveness. Exp Cell Res 77:285–297.

Jentoft N, Dearborn DG (1979): Labeling of proteins by reductive methylation using sodium cyanoborhydride. J Biol Chem 254:4359–4365.

Katz, MJ, Lasek RJ (1981): Substrate pathways demonstrated by transplanted mauthner axons. J Comp Neurol 195:627–641.

Krayanek S, Goldberg S (1981): Oriented extracellular channels and axonal guidance in the embryonic chick retina. Dev Biol 84:41–50.

Letourneau PC (1975): Cell-to-substratum adhesion and guidance of axonal elongation. Dev Biol 44:92–101.

Letourneau PC (1977): Regulation of neuronal morphogenesis by cell-substratum adhesion. In Cowan WM, Ferrendelli JA (eds): "Society for Neuroscience Symposium." Bethesda: Society for Neuroscience, pp 67–81.

Letourneau PC (1981): Immunocytochemical evidence for colocalization in neurite growth cones of actin and myosin and their relationship to cell-substratum adhesions. Dev Biol 85:113–122.

McCloon SC (1984): Distribution of laminin in the developing visual system of the chick. Soc Neurosci Abstr 10 (in press).

Palm SL, Furcht LT (1983): Production of laminin and fibronectin by schwannowa cells: cell-protein interactions in vitro and protein localization in peripheral nerve in vivo. J Cell Biol 96:1218–1226.

Rogers SL, Letourneau PC, Palm SL, McCarthy J, Furcht LT (1983): Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev Biol 98:212–220.

Rosenberg MD (1960): Microexudates from cells grown in tissue culture. Biophys J 1:137–159.

Rovensky YA, Slavnaja IL, Vasiliev JM (1971): Behavior of fibroblast-like cells on grooved surfaces. Exp Cell Res 65:193–201.

Sanes JR, Marshall LM, McMahan UJ (1978): Reinnervation of muscle fiber basal lamina after removal of myofibers. J Cell Biol 78:176–198.

Singer M, Nordlander RH, Egar M (1979): Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: the blueprint hypothesis of neuronal pathway patterning. J Comp Neurol 185:1–22.

Smalheiser NR, Crain SM, Reid LM (1984): Laminin as a substrate for retinal axons in vitro. Dev Brain Res 12:136–140.

Taylor JSH, Roberts A (1983): The early development of the primary sensory neurones in an amphibian embryo. J Embryol Exp Morphol 75:49–66.

Wan Y, Wu T, Chung A, Damjanov I (1984): Monoclonal antibodies to laminin reveal the heterogeneity of basement membranes in the developing and adult mouse tissues. J Cell Biol 98:971–979.

Wartiovaara J, Leivo I, Vaheri A (1980): Matrix glycoproteins in early mouse development and in differentiation of teratocarcinoma cells. In Subtelny S, Wessells NK (eds): "The Cell Surface: Mediator of Developmental Processes." New York: Academic Press, pp. 305–324.