The Effects of Laminin on the Characteristics and Differentiation of Neuronal Cells from Epidermal Growth Factor-Responsive Neuroepithelial Cells

Dong-Sik Park, Jung-Sun Park, and Dong-Soo Yeon

Many extracellular matrix molecules are expressed in the embryonic nervous system and there is some evidence that they are important regulators of neural development. Of these molecules, laminin appears to be the most potent, affecting virtually all neurons of the peripheral and central nervous system. This study was undertaken to investigate the effects of laminin on the proliferation and differentiation of cultured neuroepithelial cells taken from fetal rat forebrains (embryonic day 17−19). The results are summarized as follows.

1) Neuroepithelial cells cultivated in epidermal growth factor containing serum-free medium subsequently differentiated into neurons, astrocytes, and oligodendrocytes. 2) Neuronal cells derived from neuroepithelial cells were immunoreactive for $\gamma$-aminobutyric acid (GABA) or substance P, but were not for serotonin and tyrosine hydroxylase. 3) In western blot analysis, the phosphorylated neurofilament content in neuronal cells was higher in culture on laminin than in culture on poly-L-lysine (PLL). 4) The proliferation rate of GABAergic neurons was higher in culture on laminin than in culture on PLL.

These results suggest that GABAergic and substance P-ergic neurons can be differentiated from neuroepithelial cells and that laminin promotes the differentiation of neuronal cells from neuroepithelial cells and the increased proliferation rate of GABAergic cells.

Key Words: Neuroepithelial cell, neuron, differentiation, epidermal growth factor, GABAergic neuron

The mammalian central nervous system (CNS) is comprised of neuronal and macroglial cells that are derived from neuroepithelium of the ventricular zone during early development (Jacobson, 1991; Davis and Temple, 1994). It is not totally clear how these neuroepithelial progenitor cells generate the diversity of cell types present in the CNS. Contrasting theories suggest that either there are multipotential progenitor cells that give rise to both neurons and macroglial cells or that there are separate progenitors which produce either the neuronal or the glial lineage. Recently, Von Vise et al. reported neuronal and glial cells could be reproduced and differentiated from a single neuroepithelial cell (Von Vise et al. 1994). Although little is known about the factors which regulate proliferation and differentiation of progenitor cells, evidence suggests that extracellular matrix components (Martin and Timple, 1987; Gordon-Weeks et al. 1992), cell adhesion molecules (Edelman, 1989; Masuda-Nakagawa and Wiedemann, 1992), and peptide growth factors may be involved in this process. These growth factors include epidermal growth factor (EGF) (Mytileneou
et al. 1992), tumor growth factor-α (Reynolds et al. 1992), basic fibroblast growth factor (Murphy et al. 1990), nerve growth factor (Lenoir and Honegger, 1983) and insulin-like growth factor (Cattaneo and McKay, 1990).

Laminin is a key component of extracellular matrix (ECM) and has diverse biological activities which include stimulating adhesion, migration, growth, and differentiation of various cell types such as embryonic kidney cells and endothelial cells (Adams and Watt, 1993; Juliano and Haskill, 1993). Laminin is expressed transiently in the embryonic nervous system (Letourneau et al. 1994) and only transiently induced in adult brain astrocytes by injury (Liesi et al. 1984; Zhang et al. 1994). There is considerable evidence indicating that laminin is an important regulator of neuronal development. Studies in neuronal cell culture revealed that laminin has many biological activities, including the capacity to enhance neuronal survival (Halfter et al. 1989), to induce specific enzymes in neuronal cells (Sephel et al. 1989; Nutcombe, 1992), and to guide growth cones (Krotoski et al. 1986).

The present study was undertaken to characterize the neuronal cells derived from neuroepithelial progenitor cells in EGF containing serum-free medium (SFM) and to investigate the effects of laminin on the proliferation and differentiation of neuroepithelial progenitor cells.

**MATERIALS AND METHODS**

**Primary neuroepithelial cell cultures**

Primary neuroepithelial cell cultures were prepared from fetal rat forebrains (Sprague-Dawley rats, gestation days 17–19) by a method similar to glial cell cultures (McCarthy and de Vellis, 1980; Reynolds and Weiss, 1992). Briefly, the forebrains were isolated under a dissecting microscope and the meninges were carefully removed. The tissues were triturated using 10 ml pipettes, and were incubated in Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (Gibco, Grand Island, NY, USA) containing 0.13% trypsin (Sigma, St. Louis, MO, USA) for 15 min in a magnetically-stirred flask at 37°C. The dissociated cells were filtered through Nitex (Tetko Elmsford, NY, USA) #210 followed by #130. The cell filtrates were centrifuged at 100 × g for 5 min. The resulting pellets were resuspended in HBSS and refiltered through Nitex #130 and repelleted. The pellets were resuspended again in serum-free medium (SFM) and filtered through Nitex #40. Cells were counted in a hemocytometer and were then plated at a density of 2.5 × 10⁶/ml on Linbro 6-well culture plates (ICN-Flow Labs; Horsham, PA, USA). Primary cultures were maintained in SFM at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 4–6 days until floating cell clusters were formed.

SFM consisted of an equal volume of Dulbecco’s modified Eagle medium (DMEM, Gibco) and Ham’s F-12 (Gibco) containing 50 nM hydrocortisone (Sigma), 100 nM putrescine (Sigma), 30 nM selenium (Sigma), 500 ng/ml prostaglandin F₂α (Sigma), 20 μg/ml transferrin (Sigma), 20 μg/ml insulin (Sigma), 20 ng/ml EGF (Sigma), and 5 mM HEPES (Sigma).

**Characterization of neuroepithelial cells and neuronal cells derived from neuroepithelial cells**

To characterize neuroepithelial cells, floating cell clusters were collected and replated (secondary culture) on poly-L-lysine (PLL, Mwt: 70,000-150,000, Sigma) coated chambered slides (Nunc, Naperville, IL, USA), and allowed to attach for 1 hr; immunocytochemistry was then performed using anti vimentin antibodies (Boehringer Mannheim, Indianapolis, IN, USA). To confirm neuroepithelial cells that could give rise to neuronal cells, astrocytes, and oligodendrocytes, then immunocytochemistry for neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), and galactocerebroside (GC) was performed after two weeks in secondary culture. Culture medium was exchanged every 4-5 days with fresh SFM.

To characterize the neuronal cells derived from neuroepithelial cells, immunocytochemistry for tyrosine hydroxylase (TH), γ-aminobutyric acid (GABA), substance P, serotonin, and glutamate was performed after 2 weeks in secondary culture.

**Effects of laminin on neuronal cell differentiation**

Neuroepithelial cells were replated on either PLL
or laminin-(Sigma) coated chambered slides and cultured for up to 2 weeks. For immunocytochemical visualization of phosphorylated neurofilament (pNF), we used monoclonal mouse antibodies specific for pNF isoforms of H and M peptides of neurofilament (SMI-31, Boehringer Mannheim). Phosphorylated neurofilament expression in neurons has been shown to increase with neuronal cell differentiation and maturation (Schilling et al. 1988).

For quantitative western blot analysis of pNF, cytoskeletal protein fractions were prepared from secondary cell cultures similar to the procedure described by Schilling et al. (1988). The protein concentration of samples was determined by the method of Lowry et al. (1951). Immunoblots were performed as described by Towbin et al. (1979) using 10% SDS-PAGE gels. Color development was performed with 1-chloro-4-naphthol. Densitometric analysis of the resulting bands was performed by Image-Pro Program.

Effects of laminin on GABAergic neuron proliferation

Proliferation rates of GABAergic neurons were accessed by measuring 5-bromo-2’-deoxyuridine (BrDU, Sigma) incorporated into the nucleus of the cells (Langan and Slater, 1992). Briefly, neuroepithelial cells were replated on either PLL or laminin-coated chambered slides and cultured for 2 weeks. These secondary cultures were pulse-labeled for 24 hours with BrDU and were processed for double immunocytochemistry against BrDU and GABA. Proliferation rates were calculated as follows:

\[
\text{Proliferation(\%) = } \frac{\text{the number of both GABA and BrDU positive cells}}{\text{the number of GABA positive cells}} \times 100
\]

The number of GABA-positive cells or both GABA and BrDU-positive cells were determined at a magnification of 100× using a fluorescence microscope. A count was performed on 4 to 5 microscopic fields per slide and the average number per microscopic field was calculated.

Immunocytochemistry

We used indirect double immunocytochemistry method. Briefly, cultured cells on chambered slides were fixed in 4% paraformaldehyde (Sigma) in 20 mM phosphate buffered saline (pH 7.4, PBS) for 30 min at 4°C and permeabilized in 0.1% triton X-100 (Sigma) for 20 min at 4°C. Non-specific binding sites were blocked with blocking solution consisting of 10% fetal bovine serum (Gibco) in 20 mM PBS (30 min, 37°C).

Primary antibodies and antisera were diluted with blocking solution to the following working concentration and incubated for 1 hr at 37°C: rabbit anti NSE (Sigma), anti GFAP (Sigma), and anti GC (Sigma); 1 : 100, mouse anti serotonin (Boehringer Mannheim); 1 : 10, mouse anti vimentin; 1 : 40, mouse anti BrDU (Boehringer Mannheim); 1 : 20, mouse anti TH, anti GABA; 1 : 500, mouse anti pNF (Boehringer Mannheim); 1 : 1000. The slides were washed extensively in 20 mM PBS.

For mouse primary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG secondary antibody (Boehringer Mannheim, diluted 1 : 500 in blocking solution) was incubated on slides for 30 min at 37°C; for rabbit polyclonal antisera, goat anti-rabbit IgG conjugated to rhodamine (Rhod) was used for secondary antibody (diluted 1 : 200). After washing extensively in 20 mM PBS, coverslips were mounted on slides with 50% glycerol in PBS with 0.05% propyl gallate(Sigma). All slides were examined with a fluorescent microscope equipped for epifluorescence (Olympus, Tokyo, Japan).

For double immunocytochemistry, mouse monoclonal antibody and rabbit polyclonal antisera were combined. For the staining of oligodendrocytes membrane surface antigens (GC), the permeabilization step with triton X-100 was omitted. When double immunocytochemistry involved BrDU and GABA, immunocytochemistry of GABA was processed to completion first (with primary and secondary antibodies) before cellular DNA was denatured with 2N HCl (10 min at 37°C).

Comparison by Student’s t-test was performed wherever appropriate.
The Effects of Laminin on Neuronal Cells

Fig. 1. Light microscopic appearances of EGF-responsive neuroepithelial cells from fetal rat forebrains. (A) Single neuroepithelial cell (arrowheads) after 2 days in primary culture (×200). (B) Dividing and proliferating neuroepithelial cells (arrowheads) after 4 days in primary culture (×200). (C) A cluster of cells (arrowhead) after 7 days in primary culture (×200). Floating cell clusters were replated on poly-L-lysine coated slides and allowed to attach for 1 hr; indirect immunocytochemistry was then performed using anti-vimentin antibodies. Virtually all cells expressed vimentin (D, ×400).

RESULTS

Characteristics of primary cultured neuroepithelial cells

After 1 to 2 days in primary culture, most of the plated cells had died (Fig. 1A). However, some round phase contrast-bright cells began to proliferate and to form small clusters (Fig. 1B). Between 4 and 6 days in culture, these proliferating clusters enlarged and floated free in suspension (Fig. 1C).

To characterize the EGF-responsive primary cultured cells, floating cell clusters were replated on PLL coated chambered slides and allowed to attach for 1 hr. After appropriate fixation, immunocytochemistry was performed using several different antibodies. As shown in Fig. 1D, virtually all cells expressed vimentin. However, at this time, almost no cells showed immunoreactivity for NSE, GFAP, or GC antibodies (data not shown). Furthermore, these clusters were negative for certain cell-specific markers such as fibronectin (fibroblast) or OX-42 (microglia/macrophage).

Replated clusters attached immediately to the PLL substrate. In the presence of EGF, clusters continued to proliferate and cells migrated from clusters (Data not shown, but see Fig. 1D). To determine the potentiality of EGF-responsive neuroepithelial cells, secondary cultures were maintained for up to 2 weeks and processed for immunocytochemistry. Within 3–5 days, some cells expressed GFAP. After 2 weeks in culture, many cells expressed GFAP and these cells had a flat, polygonal morphology (Fig. 2A). Some cells were immunoreactive
either GC (Fig. 2B) or NSE (Fig. 2C) after 2 weeks in culture. GC positive cells had a typical oligodendrocytic morphology. NSE positive cells were found at the center of clusters and had a round soma with fine, long processes. All NSE positive cells were positive for NF in double immunocytochemistry (Fig. 2D).

**Characterization of neuronal cells derived from neuroepithelial cells**

To determine what kind of neurotransmitters neuronal cells had, double immunocytochemistry for neurofilament and TH or serotonin, GABA and substance P were performed. Some neurofilament positive cells expressed either GABA or substance P (Fig. 3), but serotonin, glutamate, or TH positive cells could not be observed.

**Effects of laminin on neuronal cell maturation**

To investigate the effects of laminin on neuronal cell proliferation and maturation in these cultures, neuroepithelial cell clusters were plated on PLL or laminin-coated chambered slides and cultured for 2 weeks. When cultivated on laminin, the neurofilament-positive cells increased 4-fold on average and the cells had well-branched neurites (Fig. 4). Western blot analysis of pNF levels in secondary culture is shown in Fig. 5. After 2 weeks in secondary culture, pNF content in neuronal cells cultured on laminin was higher than cultured on
The Effects of Laminin on Neuronal Cells

**Fig. 3.** Characterization of neuronal cells differentiated from EGF-responsive neuroepithelial cells. After 2 weeks in secondary culture, cells were processed for indirect immunocytochemistry using several antibodies. Arrowheads indicate GABA-positive cell (A, ×400) or substance P-positive cell (B, ×400).

**Fig. 4.** Effects of laminin on neuronal cell differentiation from EGF-responsive neuroepithelial cells. Cell clusters were replated on poly-L-lysine (A) and on laminin (B). After 2 weeks in secondary culture, cells were processed for indirect immunocytochemistry using anti pNF antibodies. There are more abundant pNF expressed cells in culture on laminin (B, ×400) than in culture on poly-L-lysine (A, ×400).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>The number of neurofilament positive cells</th>
<th>pNF contents in neuronal cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly-L-lysine</td>
<td><strong>₈.₁ ± ₃.₆</strong></td>
<td><strong>₁.₂₅ ± ₀.₄₁</strong></td>
</tr>
<tr>
<td>laminin</td>
<td><strong>₃₂.₇ ± ₉.₅</strong>*</td>
<td><strong>₉.₇₆ ± ₂.₇₁</strong>*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE (n=4). Asterisks indicate values significantly different from the values of culture on poly-L-lysine group (P<0.01). Neurofilament content unit: calculated values of optical density unit and band width.

**Table 1.** The number of pNF positive cells and western blot analysis of pNF contents in neuronal cells

PLL (Table 1).

**Effects of laminin on proliferation of GABAergic neuron**

Neuroepithelial cells were cultivated on either laminin or PLL for 2 weeks. When cultivated on laminin, a substantial enhancement of neurite growth was observed as assessed by neurite length and the number of branching points per neurites (Fig. 6). The number of GABAergic neurons was 4-fold higher in culture on laminin than in culture on PLL.
Fig. 5. Western blot analysis of pNF H and M contents in neuronal cells differentiated from neuroepithelial cells. (A) Left lane: molecular weight marker. Mid lane: pNF H and M contents in neuronal cells cultured on poly-L-lysine. Right lane: pNF H and M contents in neuronal cells cultured on laminin. Note additional low molecular weight proteins recognized by anti pNF antibodies. (B) Densitometric analysis of the pNF H and M bands was performed as described in Materials and Methods.

Table 2. The number and proliferation rate of GABAergic neurons cultured on PLL or laminin coated chambered slides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>The number of GABA immunostained cells(A)</th>
<th>The number of both BrdU and GABA immunostained cells(B)</th>
<th>Proliferation rates (B/A x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly-L-lysine</td>
<td>6.3 ± 2.4</td>
<td>0.8 ± 0.4</td>
<td>12.7%</td>
</tr>
<tr>
<td>laminin</td>
<td>25.7 ± 7.8*</td>
<td>9.8 ± 3.2*</td>
<td>38.1%*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SE (n=4). Asterisks indicate values significantly different from the values of culture on poly-L-lysine group (P< 0.01). The number of cells was determined and the proliferation rate was calculated as described under Materials and Methods.

(Table 2).

To determine the effects of laminin on GABAergic neuron proliferation, secondary cultures were pulse-labeled for 24 hours and then double immunochemistry for GABA and BrDU was performed. Some cells were double-labeled with both GABA and BrDU in culture on laminin, but only a few cells had a colocalization of GABA and BrDU.
Fig. 6. Effects of laminin on GABAergic neuron proliferation. There are more GABAergic cells (arrowheads) in culture on laminin (B, ×200) than in culture on poly-L-lysine (A, ×200). The number of GABAergic neurons was determined as described under materials and methods.

Fig. 7. Double immunofluorescence microscopy of neurons reacted with anti GABA and BrDU antibodies. Cell clusters were replated on poly-L-lysine (A, B) and laminin (C, D). After 2 weeks in secondary culture, cells were processed for indirect immunocytochemistry using anti GABA (A, C) and anti BrDU antibodies (B, D). Arrowheads in A and B indicate identical cells which expressed GABA but did not express BrDU (×400). Arrowheads in C and D also indicate identical cells which expressed GABA but not BrDU (×400). Arrows in C and D indicate identical cells which expressed both GABA and BrDU.
in culture on PLL (Fig. 7, Table 2).

DISCUSSION

Most of the neuronal and glial cell types found in the mature CNS originate from precursor cells in the ventricular zone of the fetal brain (Davis and Temple, 1994). Recently, several studies have examined cell lineage in the vertebrate CNS to determine whether neuronal and glial cell types originate from a common precursor or from distinct progenitors. In the cortex, retina, and optic tectum, a single precursor cell can give rise to both neurons and glia as well as different types of neurons, even at the late stages of neurogenesis (Cameron and Rakic, 1991). These results imply that multipotential precursor cells persisted throughout CNS developmental stages.

However, another lineage analysis study using retroviral marking indicated that separate progenitors give rise to neurons and glia and these suggest that those progenitors are committed to a particular fate (Kornack and Rakic, 1995). Thus, the results from *in vivo* lineage analysis are not uniform.

The results presented here demonstrated that a single neuroepithelial cell from a fetal rat forebrain proliferated in the presence of EGF, and subsequently differentiated into neurons, astrocytes, and oligodendrocytes (Fig. 1, Fig. 2).

It is generally acknowledged that extrinsic factors such as cytokines, cell to cell contact and ECM must be important in generating the cellular diversity observed in the CNS (Cameron and Rakic, 1991). Johe *et al.* reported that platelet-derived growth factor supports neuronal differentiation and that ciliary neurotrophic factor act on stem cells to generate astrocytes and oligodendrocytes (Johe *et al.* 1996). The survival and development of neurons are influenced not only by soluble molecules such as cytokines, but also by neurotrophic factor or cell adhesion molecules (Barres *et al.* 1994).

Synaptic contact and neurotransmitter interactions between a motor neuron and its target muscle are known to support cell survival and differentiation of both neurons and muscle. For example, innervation regulates the structural integrity of muscle and growth factors synthesized in muscle regulate neuronal development and regeneration (Davis, 1983; Heinicke and Davis, 1985).

It is well known that astrocytes make a contact with neurons and regulate neuronal cell migration and differentiation (Schwartz *et al.* 1993). During the embryonic stage, radial glial cells participate in directing and facilitating neuronal migration. Laminin is expressed in radial glial cells and growing axones require a substrate to which they can adhere in order to extend (Sephel *et al.* 1989). Synthesis of laminin is developmentally regulated and may be involved in cell and tissue differentiation throughout embryogenesis (McLoon *et al.* 1988). The facts of the appearance of laminin at early embryonic stage and its interactions with neurons raise the possibility that their molecule has roles in nervous system development.

In this experiment, the migration of neuroepithelial cells was more prominent and fine process bearing cells were more abundant in culture on laminin than in culture on PLL. This means that laminin facilitates neuroepithelial cell migration as well as neuron cell migration.

In the nervous system, the major cytoskeletal intermediated filaments are composed of the type of NF polypeptides referred to as NF-C, NF-M, and NF-H. During neuronal cell differentiation and maturation, serine residues found in the consensus sequence Lys-Ser-Pro (KSP-motif), notably of NF-M and NF-H become highly phosphorylated (Shaw and Weber, 1982). Phosphorylate neurofilament content in neurons and NF-M/NF-H molar ratio can be used as a marker of neuronal differentiation and/or maturation (Schilling *et al.* 1988; Schilling and Pilgrim, 1990). The number of NF positive cells and pNF content in neuronal cells were increased in neuroepithelial cell culture on laminin compared to culture on PLL (Fig. 4, Table 1). These results are evidence that laminin influences the differentiation and maturation of neurons. It is well known that laminin controls the differentiation of several cell types including endothelial cells, myoblasts, macrophage, bone cells, and embryonic kidney mesenchymal cells (Klein *et al.* 1988; Sorokin *et al.* 1990; Brodky *et al.* 1993). However, in the nervous system, information regarding the proliferation and differentiation-promoting effects of laminin (as
distinct from neurite promoting effects) is scarce (Bryan et al. 1993). However, laminin proved to be essential for nerve growth factor induced-conversion of adrenal chromaffin cells into sympathetic-like neurons in vitro (Lein et al. 1992; Bryan et al. 1993). Moreover, laminin-stimulated levels of TH of chromaffin cells were associated with their conversion to adrenergic sympathetic cells (Otros et al. 1988; Leventhal and Feldman, 1996).

In these culture systems, neuronal cells derived from neuroepithelial cells contain either GABA or substance P as a neurotransmitter (Jacobson, 1991); these are two of the major neurotransmitters of the adult striatum in vivo. In contrast, these cultures did not contain cells that were immunoreactive for glutamate, serotonin, or TH. The reason for the restricted expression of phenotype is unknown, but it is possible (i) that EGF-responsive progenitor cells are limited to produce only cells containing GABA and substance P (ii) or that other phenotypes may appear at different times or under different culture conditions (Acheson et al. 1986; Frade et al. 1996).

The proliferation of GABAergic neurons increased when cultivated on laminin compared to when cultivated on PLL (Fig. 6, 7, Table 2). These results suggest that laminin stimulates proliferation of GABAergic neurons and does not affect the differentiation of GABAergic neurons from neuroepithelial cells. However, the possibility that the direct effects of laminin on the differentiation of GABAergic neurons from neuroepithelial cells cannot be excluded because astrocytes were expressed in neuroepithelial cell culture on PLL. It is well known that cultured astrocytes express laminin (Zhang et al. 1994).

In conclusion, our data show that GABAergic and substance P-ergic neurons can be differentiated from neuroepithelial cells and that laminin promotes the differentiation of neuronal cells from neuroepithelial cells and increases the proliferation rate of GABAergic neurons.

REFERENCES

Acheson A, Edgar D, Timpl R, Thoeuen H: Laminin increases both levels and activity of tyrosine hydro-


Kornack D, Rakic P: Radial and horizontal deployment


Langan TL, Slater MC: Astrocytes derived from long-term primary cultures recapitulated feature of astrogliosis as they re-enter the cell division cycle. Brain Res 577: 200-209, 1992


