Neurite Promoting Activity of Collagens on Embryonic Neurons: Decreased Effect at the Postnatal Stage

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HIROSE, H., KITAGUCHI, T. and TABIRA, T. *Neurite Promoting Activity of Collagens on Embryonic Neurons: Decreased Effect at the Postnatal Stage.* Tohoku J. Exp. Med., 1993, 170 (4), 207-218 — We studied the in vitro neurite outgrowth activity of fibronectin, laminin, heparan sulfate proteoglycan, type I collagen, type IV collagen, and type VIII collagen in cholinergic neuronal cell lines and primary cultured neurons. All these substances had high neurite promoting activity on primary cultured neurons from embryonic mouse brain. However, collagens had no such an effect on primary cultured neurons from postnatal brain. When neuronal cell lines were used, collagens and other extracellular matrix substances were equally and highly effective on cells originated from embryonic brain, but collagens were less effective on cells from postnatal brain. These findings suggest that postnatal neurons lose the neuritic responsiveness to collagens earlier than that of other ECM. ——— collagen; culture; extracellular matrix; neuron

The development of the nervous system involves generation of neuronal cells, cell migration, neurite outgrowth, and synapse formation within a complex and dynamic extracellular environment. It is now clear that interactions with the extracellular matrix (ECM) influence neuronal development, differentiation, and regeneration. In this context, several major constituents of the ECM, e.g., fibronectin (FN), laminin, and proteoglycans, have been extensively studied in the nervous system. FN, a cell-surface and serum-derived glycoprotein of molecular weight (MW) 440,000, can promote the adhesion, spreading, and migration of fibroblasts and certain other cells, including neuronal cells. FN enhances the capacity to support retinal neurite outgrowth (Akers et al. 1981), and local differences in FN concentration and availability in the matrix could be involved

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in the direction of neural crest cell migration by serving as a chemoattractant (Newgreen and Thiery 1980; Greenberg et al. 1981). Furthermore, FN promotes the adhesion of Schwann cells, and stimulates their proliferation and migration (Baron-Van Evercooren et al. 1982).

Laminin (LN), a high molecular weight glycoprotein, of MW 850,000 or greater, has been found in basement membrane throughout the body. Many studies have indicated an association between neuronal cells and LN. LN is a potent stimulator for neurite elongation of cultured neurons obtained from both central and peripheral neuronal tissues (Manthorpe et al. 1983; Rogers et al. 1983), and substrate-bound LN pathways guide the neurites of central and peripheral neurons (Hammarback et al. 1985, 1988). LN is concentrated in astrocytes, schwannoma cells, and Schwann cells in culture (Liesi et al. 1983; Palm and Furcht 1983; Chiu et al. 1991). It has also been shown that LN may be available on the cell surface and in the extracellular space as an adhesive ligand for growth cones during the formation of the ventral longitudinal pathway (Letourneau et al. 1988).

Proteoglycans are proteins that carry one or more glycosaminoglycan side chains; it has recently been reported that chondroitin sulfate proteoglycan and heparan sulfate proteoglycan (HSPG) are associated with the nervous system (Aquino et al. 1984; Ratner et al. 1985; Hantaz-Ambroise et al. 1987; Bertolotto et al. 1990; Carey et al. 1990; Herndon and Lander 1990; Sheppard et al. 1991).

Collagen (COL) is by far the most common constituent of the connective tissue and is ubiquitously distributed in the body. It plays an important role in cell proliferation, morphogenesis, and in the expression and maintenance of functional differentiation. However, there is a paucity of information regarding whether COL has any effect on neurons in the central nervous system, COL not generally being observed in the brain, except in the perivascular areas and meninges. Accordingly, we studied the effects of ECM constituents on the development of central cholinergic neuronal cell lines and primary cultured cerebral neurons, using an *in vitro* neurite outgrowth assay. We found that COL has a high neurite-promoting activity in cells from prenatal mouse brain, whereas it has little effect on the neurite outgrowth of postnatal neurons.

MATERIALS AND METHODS

ECM constituents

Of the ECM constituents used in the present study, type I collagen (COL-I), type IV collagen (COL-IV), and FN were kindly provided by Nitta Gelatin Inc. (Osaka). LN and HSPG, listed as "Extracellular Matrix, Human," were purchased from Collaborative Research, Inc. (Two Oak Park, Bedford, UK), and poly-L-lysine (PLL) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Type VIII collagen (COL-VIII) (Sawada et al. 1990) was a kind gift from Dr. Konomi at the Division of Mental Retardation and Birth Defect Research, National Institute of Neuroscience (Kodaira, Tokyo).

Preparation of culture dishes coated with ECM constituents

Two ml of 0.9% NaCl solution, containing 10 $\mu\text{g/ml}$ of PLL, was placed in a 35-mm plastic tissue culture dish (Falcon, Lincoln Park, NJ, USA). The dishes were incubated at room temperature for 2–4 hr, and then rinsed twice with 0.9% NaCl solution to remove free PLL. Next, the PLL-coated dishes (PLL-dishes) were coated again with each ECM constituents under the conditions described below according to the manufacturer's information for optimal doses.

Types I, IV, and VIII collagens. A PLL-dish containing 2 ml of 100 $\mu\text{g/ml}$ COL-I (porcine), COL-IV (bovine), or COL-VIII (bovine) solution was incubated at 4°C overnight. The COL-I-, COL-IV-, or COL-VIII-coated dishes (COL-I-, COL-IV-, or COL-VIII-dishes) were then washed three times with phosphate-buffered saline (pH 7.4), and finally washed with serum-free medium.

Fibronectin. A PLL-dish containing 2 ml of 3 $\mu\text{g/ml}$ FN (porcine) was incubated at 37°C overnight. The FN-coated dishes (FN-dishes) were washed with serum-free medium just before use.

Laminin. A PLL-dish containing 1 ml of 10 $\mu\text{g/ml}$ LN (murine) was incubated at room temperature for 1 hr. The LN-coated dishes (LN-dishes) were washed with serum-free medium just before use.

Heparan sulfate proteoglycan. A PLL-dish containing 1.25 ml of 10 $\mu\text{g/ml}$ HSPG (human) was incubated at room temperature for 2 hr. The HSPG-coated dishes (HSPG-dishes) were washed with serum-free medium just before use.

Cell lines

We used several cell lines that retained cholinergic neuronal characteristics for our experiments; the cell lines were SN6, SN49, SN50, and SN52 neuronal hybridomas, and all were kindly provided by Dr. B.H. Wainer at the University of Chicago, Chicago. The SN6 cell line was established by somatic cell fusion of N18TG2 neuroblastoma and septal neurons obtained from C57BL/6 mouse brain at embryonic day 14 (ED14) (Hammond et al. 1986). The SN49, SN50 and SN52 cell lines were established similarly from the brains of postnatal day 21 (PD21) C57BL/6 mice (Lee et al. 1990).

All cell lines were grown on 100-mm plastic tissue culture dishes (Falcon) in a medium consisting of 90% Dulbecco's modified Eagle medium (pH 7.2, Gibco, Grand Island, NY, USA), 10% heat-inactivated fetal bovine serum (Gibco), 100 $\mu\text{g/ml}$ streptomycin, and 100 units/ml penicillin (Gibco). Cells were dissociated for replating by vigorous trituration, and the growth medium was replaced twice a week. All cultures were maintained at 37°C in a humidified air atmosphere containing 5% CO_2 .

In the following experiments for assay of neurite-promoting activity, all tissue cultures were performed in serum-free conditions, as described below. The serum-free supplemented medium (Kamegai et al. 1990) was a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 (pH 7.4, Gibco) supplemented with 100 $\mu\text{g/ml}$ human transferrin, 25 $\mu\text{g/ml}$ bovine crystalline insulin, 7 ng/ml tocopherol, 7 ng/ml retinol, 100 μM putrescine, 10 μM L-carnitine (Boehringer Mannheim, Germany), 1 μM thioctic acid, 30 nM 3, 3', 5-triiodo-L-thyronine, 30 nM sodium selenate (Wako Pure Chemical Industries, Ltd., Osaka), 20 nM progesterone, 20 nM hydrocortisone-21-phosphate, 1 $\mu\text{l/ml}$ 1,000 $\times$ mineral mixture solution (Hutchings and Sato 1978), 100 $\mu\text{g/ml}$ streptomycin, and 100 units/ml penicillin. All supplements, except for L-carnitine, sodium selenate, and the mineral mixture, were purchased from Sigma Chemical Co.

Primary culture of neuronal cells

The primary culture of neuronal cells was prepared according to the method described (Hirose et al. 1993). Briefly, brains were obtained from BALB/cAJcl mice (Clea Japan, Tokyo) on ED14 or PD3. The cerebral hemispheres were dissected in Hanks' balanced salt

solution (pH 7.4, Gibco), minced, and digested with 0.03% trypsin (Sigma) and 100 units/ml deoxyribonuclease I (Sigma) in Hanks' balanced salt solution for 5 min at 37°C. After gentle trituration through a pasteur pipette, the reaction was stopped by adding trypsin inhibitor (Sigma). The dispersed cells were washed twice in Hanks' balanced salt solution, filtered through a nylon screen (pore size 63 μ m), suspended in the serum-free supplemented medium. Under these conditions, the amount of glial fibrillary acidic protein-positive cells was less than 4% at 120 hr after the initiation of culture.

The culture was initiated at a density of 1×10^5 cells per 35-mm culture dish for the study of neurite outgrowth of embryonic mouse neurons, and at a density of 5×10^5 cells per 35-mm culture dish for the study of neurons derived from postnatal mouse brains. For the maintenance of primary cultured neurons from postnatal mouse brain, we used the serum-free supplemented medium containing 1 mg/ml bovine serum albumin (bovine fraction V, Sigma).

Assay of neurite-promoting activity

Neurite-promoting activity was assessed as the proportion of neurite-bearing cells in the total number of cells in a culture. Neurites were defined as processes that exceeded one cell diameter in length. To confirm that these processes are neuronal origin, we performed immunostaining with monoclonal antibody to neurofilament polypeptide 200 kDa (NE14, Boehringer Mannheim, Germany). As shown in Fig. 1, most of the processes were stained

Fig. 1. Immunocytochemical staining of neurofilament in primary cultured neurons derived from prenatal mouse brain maintained 120 hr on PLL- (a), FN-(b), LN-(c), and COL-I-dishes (d). The cultures were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 1 hr at 4°C, and incubated with 0.2% Triton X-100 for 30 min at 37°C. Cultures were rinsed with PBS three times for 10 min each. For the following steps, the cultures were worked according to the "step by step procedure" outlined in the instruction manual included in the Immunoperoxidase Staining Kit (Biomedex Corp., Foster City, CA, USA). The first antibody was a monoclonal antibody to neurofilament polypeptide 200 kDa. Bar, 50 μ m.

with this antibody.

Cells of the SN6, SN49, SN50, and SN52 cell lines were washed twice with phosphate-buffered saline and then exposed to 0.025% trypsin (Difco, Detroit, MI, USA) and 0.02% EDTA (Wako) in phosphate buffered saline. After incubation at 37°C for 3–5 min, 10 ml of Dulbecco's modified Eagle medium containing 1% heat-inactivated fetal bovine serum was added. The cell suspensions were then centrifuged, pelleted, and washed three times in 10–20 ml of Hanks' balanced salt solution. Following the third wash, the cells were resuspended in the defined serum-free medium. The cell suspensions were then plated at 5×10^4 cells per 35-mm tissue culture dish that had been coated beforehand with PLL alone or with PLL and an ECM constituent, following which they were incubated as described above.

Phase-contrast photomicrographs were taken randomly at 12, 24, and 48 hr (and in addition, at 72 and 120 hr for some experiments) after the initiation of culture. Neurite-bearing cells in each culture were counted on three randomly-taken photographic prints at a final magnification of 160x; all experiments were performed in duplicate cultures. Total cell numbers counted were 100–400 for neuronal cell lines, 100–200 for primary culture cells from embryonic mouse brain, and 400–700 for primary culture cells from postnatal mouse brain. Statistical analysis was done by the two-tailed student *t*-test.

RESULTS

Effects of ECM constituents on neurite outgrowth of SN6, a cell line derived from prenatal (ED14) mouse brain

In uncoated dishes (control) and PLL-dishes, very few SN6 cells extended neurites, and those that did showed difficulty of maintenance over 48 hr in culture; the percentage of neurite-bearing cells was 6.2% at 24 hr for both control and PLL. The percentage of neurite-bearing cells in FN-dishes increased steadily, reaching 26.6% at 48 hr of culture. In LN-, HSPG-, COL-I-, COL-IV-, and COL-VIII-dishes, the percentages of neurite-bearing cells were highest at 24 hr (26.9%, 37.4%, 25.5%, 25.0%, and 30.6%, respectively), and they decreased slightly at 48 hr (Fig. 2). Thus, in all cultures the percentage of neurite-bearing cells was significantly higher than PLL.

Effects of ECM constituents on neurite outgrowth of SN49, SN50, and SN52, cell lines derived from postnatal (PD21) mouse brain

In SN49 cells, the percentages of neurite-bearing cells of control and PLL-dishes were 11.6% and 9.8%, respectively, at 12 hr. In all cultures with FN, LN, HSPG, COL-I, COL-IV, and COL-VIII, the maximal percentages of neurite-bearing cells were observed at 12 or 24 hr (31.3%, 58.3%, 47.3%, 25.0%, 30.3%, and 28.4%, respectively), and they were slightly reduced thereafter (Fig. 3). LN and HSPG showed highest neurite-promoting activity with SN49. On the other hand, the percentages of neurite-bearing cells in cultures with the three types of COL were also higher than those in the control and PLL-dishes, but were significantly lower than those in LN and HSPG dishes (Fig. 3). Thus, the three types of COL had less neurite-promoting activity than the other ECM constituents

Fig. 2. The promotion of neurite outgrowth of SN6 cells. A: SN6 cells maintained on normal culture dishes were dissociated with 0.025% trypsin and 0.02% EDTA in PBS and the cells were seeded on PLL-, LN-, COL-I-, or COL-IV-dishes in serum-free supplemented medium. Phase-contrast micrographs (160 $\times$) were taken 24 hr after seeding. Cells plated on PLL-dishes (a) extend very few neurites, whereas cells plated on two kinds of COL-coated (c and d) dishes exhibit their neurites as long as those exhibited with LN (b). Bar, 50 μ m. B: Data are expressed as means and S.D. of the percentage of neurite-bearing cells examined at 12, 24, and 48 hr of culture. Statistic analyses were done against PLL at 12 and 24 hr and Control at 48 hr. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

with SN49.

We also studied the neurite-promoting activity of ECM constituents with the SN50 and SN52 cell lines. The percentages of neurite-bearing cells in SN50 were generally lower than those in SN49. (The highest percentage was 23.6% at 12 hr with LN). In FN-, LN-, and HSPG-dishes, the percentages of neurite-bearing

Fig. 3. The promotion of neurite outgrowth of SN49 cells. A: A few cells plated on PLL-dishes (a) extend neurites. The neurite-bearing cells seeded on COL-I (c) and COL-IV (d) are fewer than those on LN (b). Methods: see legend to Fig. 2A. Bar, 50 μ m. B: Data are expressed as means and S.D. and statistic analysis were done all against PLL (see legend to Fig. 2B).

cells were 16.0–23.6% at 12 hr, which are significantly higher than those in control and PLL-dishes. In contrast, the percentages of neurite-bearing cells with COL-I and COL-IV were only 6.9 and 5.4%, respectively (Table 1). Thus, COL had less neurite-promoting activity than the other ECM constituents with SN50. As shown in Table 1, the percentage of neurite-bearing cells decreased at the later time. This was also observed in SN49. Since the number of neurons increased at the later time, the decrease is due to the cell proliferation particularly with FN, LN and HSPG.

The percentages of neurite-bearing cells in the SN52 cell line were even lower than those in SN50. (The highest percentage was 14.6% at 12 hr with FN.) In

TABLE 1. *Effects of ECM constituents on neurite outgrowth of SN50*

Culture time (hr)	Control	PLL	FN	LN	HSPG	COL-I	COL-IV
12	5.8	2.2	16.0	23.6	19.8	6.9	5.4
24	4.9	0.5	16.0	18.1	16.7	12.4	13.1
48	4.7	1.5	8.4	10.4	10.7	12.8	10.8

Data are expressed as % of neurite-bearing cells.

FN-, LN-, and HSPG-dishes, the percentages of neurite-bearing cells were about 4 times higher than those in control and PLL-dishes. In contrast, the percentages of neurite-bearing cells with COL-I and COL-IV were about two times higher than those with control and PLL (data not shown). Again, the COL had less neurite-promoting activity than the other ECM constituents with SN52 cell line.

These findings led us to speculate that COL had less effect on the neurite outgrowth of neurons from postnatal mouse brains than on neurite outgrowth in the neurons derived from prenatal brains. To confirm this, we performed further experiments with primary cultured neurons.

Effects of ECM constituents on neurite outgrowth of primary cultured neurons derived from prenatal (ED14) mouse brain

In PLL-dishes, very few primary cultured cells had neurites, the percentage being 9.9% at 12 hr. In all cultures with FN, LN, HSPG, COL-I, COL-IV, and COL-VIII, the percentages of neurite-bearing cells at 48 hr of culture were 46.9%, 67.4%, 60.1%, 54.0%, 43.8%, and 45.6%, respectively (Fig. 4). Further, the percentages increased steadily with time up to 120 hr. Thus, all ECM constituents showed significantly higher neurite-promoting activity than PLL.

Fig. 4. The effect of ECM on neurite outgrowth of primary cultured neurons derived from prenatal mouse brain. The neurons prepared according to Experimental Procedures were seeded on ECM-coated dishes in serum-free supplemented medium. Data are expressed as means and S.D. and statistic analyses were done against PLL (see legend to Fig. 2B).

Effects of ECM constituents on neurite outgrowth of primary cultured neurons derived from postnatal (PD3) mouse brain

In PLL-dishes, the percentage of neurite-bearing cells was 29.4% at 24 hr. This was higher than that of ED14 neurons (Fig. 4), because PD3 neurons had already had more neurites at the start of culture. In all cultures with FN, LN, HSPG, COL-I, and COL-IV, the maximal percentages of neurite-bearing cells were observed at either 12 or 24 hr (38.3%, 37.1%, 43.5%, 26.8%, and 28.4%, respectively), and they decreased significantly thereafter (Fig. 5).

In FN-, LN-, and HSPG-dishes, the percentage of neurite-bearing cells were significantly higher than that in PLL-dishes at 48 hr. In contrast, the percentages of neurite-bearing cells in COL-I- and COL-IV-dishes appeared to be lower and were closer to those in PLL-dishes. Thus, COL had less neurite-promoting activity with the primary cultured neurons derived from postnatal mouse brains than the primary cultured neurons derived from prenatal brain.

DISCUSSION

We examined the neurite-promoting activity of ECM constituents on cholinergic neuronal cell lines and primary cultured neurons. We found that PLL, which is commonly used as a substrate for neuronal cell culture, did not show neurite-promoting activity with any of the four cholinergic cell lines. However, all of the ECM constituents but PLL had specific neurite-promoting activity with these neuronal cells.

When SN6 was used, all the ECM constituents were equally effective. However, when SN49 was used, LN and HSPG had highest neurite promoting activity, but collagens were less effective. Since SN49 is more differentiated, the

Fig. 5. The effect of ECM on neurite outgrowth of primary cultured neurons derived from postnatal mouse brain. The neurons prepared according to MATERIALS AND METHODS were seeded on ECM-coated dishes in serum-free supplemented medium containing 1 mg/ml bovine serum albumin. Data are expressed as means and S.D. and statistic analyses were done against PLL (see legend to Fig. 2B).

response to LN and HSPG was expectedly high. Therefore, we judged that responses to collagens were rather decreased. It is most interesting to note, therefore, that the neurite-promoting activity of COL differed according to the stage of neuronal development. That is, COL-I, COL-IV, and COL-VIII had a significant neurite promoting activity with SN6, which is derived from prenatal mouse brain, while this activity was less in extent with SN49, 50, and 52, which are derived from postnatal brain. These differences were particularly true with primary cultured neurons. Since other ECM constituents affected both prenatal and postnatal neurons equally, it would appear that this difference is characteristic of COL.

Although COL is a major component of the ECM in the body, its role in the development of the central nervous system has not been known. It has generally been thought that COL has little association with the nervous system in vivo. Recently, however, it has been found that COL serves as a substrate for neuronal crest cells to migrate in vitro (Greenberg et al. 1981; Perris et al. 1991). Attachment, flattening, and neurite outgrowth of PC12 cells and embryonic chicken sensory cells from dorsal root ganglia has been shown to occur in dishes coated with COL (Vlodavsky et al. 1982; Turner et al. 1989), and COL has been shown to play a role in pathway finding and growth cone guidance (Carbonetto et al. 1983; Tomaselli et al. 1987). It has also recently been shown that COL-IV is located within the developing central nervous system of *Drosophila* (Mirre et al. 1992), and further, two glial cell lines, C6 and 9L, have been shown to produce COL-I in vitro (Ghahary et al. 1992). Our results suggest that COL may contribute to the neurite extension of central nervous system neurons in the developing brain. To see its significance in vivo, it is necessary to study whether COLs are expressed in the developing central nervous system of the mammals.

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