

Use of Genetically Modified Glial Cells Overexpressing Laminin α 1-Chain Peptides in Neurite Outgrowth Studies

Gerald Webersinke,¹ Hans-Chr. Bauer,^{1,5} Claudia Danninger,¹ Istvan A. Krizbai,^{1,2} Johannes C. Schittny,³ Josef Thalhamer,⁴ and Hannelore Bauer¹

Received March 20, 2000; accepted March 17, 2000

SUMMARY

1. C6 glioma cells were transfected with two constructs carrying C-terminal laminin α 1-chain sequences of 117 and 114 bp length, respectively. These sequences are specifically known to code for peptides which have neurite-promoting activity.

2. The stable expression and secretion of the two peptides was detected by Northern and Western blot analysis.

3. Primary neuronal cultures derived from embryonic mouse forebrain were cocultured with these transfected cells and exhibited a substantial increase in neurite outgrowth and in survival time. Conditioned media from the transfected cells generated similar effects.

4. Organotypic cultures from embryonic mouse brain were used as a second system as being closer to the *in vivo* situation. Again, coculture of brain slices with transfected cells or treatment with laminin peptide-containing media increased neuronal outgrowth.

KEY WORDS: neurite outgrowth; laminin peptides; transfection, glia.

INTRODUCTION

Neurite outgrowth is part of neuronal differentiation and the key event for functional nervous communication throughout the body. Neurites adhere to particular surfaces and travel along pathways with guidance posts whose molecular composition is unknown. Laminin, a large glycoprotein of about 800 kDa and part of the extracellular matrix (ECM), is one of the well-studied adhesive matrix molecules which enable neurites to travel (Aumailley and Krieg, 1996; Sasaki *et al.*, 1988). Every laminin molecule consists of three different chains (α , β , γ) which assemble to structurally and functionally distinct laminin molecules. Genetically different isoforms of each

¹ Institute for Molecular Biology, Austrian Academy of Science, A-5020 Salzburg, Austria.

² Institute for Anatomy, University of Bern, CH-3000 Bern 9, Switzerland.

³ Institute for Biophysics, Hungarian Academy of Science, H-6701 Szeged, Hungary.

⁴ Institute for Biochemistry, University of Salzburg, A-5020, Salzburg, Austria.

⁵ To whom correspondence should be addressed at Institute for Molecular Biology, Austrian Academy of Science, Billrothstr. 11, A-5020 Salzburg, Austria. e-mail: hcbauer@oew.ac.at

chain are known. Laminin-1 (formerly known as EHS-laminin) possesses $\alpha 1$, $\beta 1$, and $\gamma 1$ chains and represents one of the best-studied isoforms. Like some other major ECM components such as fibronectin, thrombospondin, tenascin, and collagens, laminin is a multidomain molecule. It possesses individual domains which appear to have distinct biological activities. Laminin has been reported to influence cell shape, proliferation, motility, differentiation, and growth factor responsiveness as well as angiogenesis (Martin and Timpl, 1987; Goodman *et al.*, 1987; Grant *et al.*, 1992; McKerracher *et al.*, 1996; Malinda and Kleinman, 1996; Ryan *et al.*, 1996; Luckenbill-Edds, 1997).

Laminin also exerts striking effects on many types of neurons by promoting neurite outgrowth, migration, and regeneration (Lander, 1987; Letourneau *et al.*, 1988). In addition, it promotes early neural differentiation; for instance, embryonal carcinoma cells become neurons when grown on laminin substrates (Sweeney *et al.*, 1991).

Most experiments have been performed *in vitro* using laminin coats on glass or plastic culture dishes. These experiments have shown that neurites precisely follow laminin traces even when the width of the trace is in the range of some micrometers (Clark *et al.*, 1993). The phenotype of neurites varies depending on the laminin concentration: the higher the concentration of coated laminin, the longer and less branched will the elicited neurites grow (Buettner and Pittman, 1991).

The neuron-specific activities of the entire laminin molecule have been mapped to several domains of the laminin molecule as determined by elastase digestion. In this way, major neuron-specific activities were attributed to the E8 fragment, which starts at the amino acid position 1886 and comprises 223 amino acids of the α -helical region, and to a large globular heparin-binding domain (G-domain) at the end of the $\alpha 1$ chain (amino acid residues 2110–3060) (Edgar *et al.*, 1984). Besides promoting nonneuronal cell adhesion and spreading, the E8 fragment was found to stimulate neurite survival and outgrowth (Liesi *et al.*, 1989; Begovac *et al.*, 1991). In order to further elucidate this effect, several groups have synthesized peptides from the laminin $\alpha 1$ chain and have screened them for biological activities. A 19-mer peptide termed PA22-2 from just above the globular domain was found to promote cell adhesion, spreading, and migration of cells and neurite outgrowth. Even a smaller sequence within the 19-mer peptide, the pentapeptide IKVAV, was responsible for cell adhesion and neurite outgrowth (Tashiro *et al.*, 1989). A series of peptides from the globular domain were synthesized and tested for neurite outgrowth-promoting activity, several of which (termed GD 1–4) did indeed have a strong neurogenic effect (Skubitz *et al.*, 1991).

Although the ability of laminin to induce neuronal migration and survival may have considerable therapeutic potential as a means of regenerating and repairing damaged neuronal tissue or neuronal loss *in vivo*, no major effort has been made to investigate the availability of laminin-derived peptides in a gene therapy approach.

We have established stably transfected glial-cell subpopulations overexpressing two peptides of the laminin $\alpha 1$ chain (Fig. 1). The neurite-promoting activity of the transfected cells was tested in neural cultures as well as in an organotypic culture system. Cotransfection of the glial cells with a laminin construct and a *lacZ* reporter gene allowed us to study the adhesive capacity and migrational behavior of the

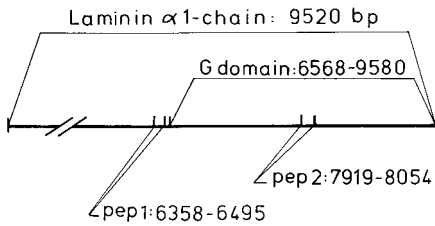


Fig. 1. Schematic diagram of pep1 and pep2 positions in the laminin α 1-chain sequence.

genetically modified glial cells in neural tissues. We demonstrate that the laminin peptides produced by glial cells markedly increase neurite outgrowth of primary neuronal cultures and of cultured neural explants and greatly enhance survival rates of neurons *in vitro*.

MATERIALS AND METHODS

Animals

NMRI inbred mice bred were used. For the *in vitro* experiments (neuronal cultures and brain slices; see below) pregnant mice of various stages were anaesthetized and killed; the embryos were removed and put into ice-cold phosphate-buffered saline. Stages of pregnancy were determined by plug checks in the morning after mating; noon was taken as 0.5 day of gestation.

Cell Cultures and Conditioned Media

Neurons were isolated as described by Bauer and Tontsch (1990). In short, forebrains from 14.5-day-old mouse embryos were carefully cleared from meninges, cut into small pieces, and digested for 5 min with 0.25% trypsin dissolved in glucose-enriched Dulbecco's medium at 37°C. After several rounds of gentle trituration, neurons were transferred into serum-free N2 culture medium (Bottenstein and Sato, 1979), plated onto poly-L-lysine-coated 35-mm plastic petri dishes, and cultured in a CO₂ incubator at 37°C. Culture medium was changed every second or third day. After about 1 week in culture, cells were differentiated and more than 95% of all cells proved to be neurons as determined by marker expression (neuron-specific enolase or neurofilaments, respectively) (data not shown). C6 glioma cells were kept in Ham's F12 medium enriched with 10% FCS. Conditioned media were collected from confluent cultures kept for 3 days in MEM without serum.

Organotypic CNS Cultures

Brain slices were basically prepared as described by Gähwiler (1981) and Stoppini *et al.* (1991). Slices were produced from forebrains of embryonic (E14–E17) and neonatal mice using either a McIlwain tissue chopper or surgical knives, depending on the size and stability of the tissue. Slices had a thickness of 200–400 μ m and

were cultured on Millicell CM (Millipore) or on nucleopore (Falcon) membranes which had been previously soaked with culture medium. The culture medium contained MEM supplemented with Hepes (50%), horse serum (12.5%), FCS (12.5%), and Hank's buffer (25%) at pH 7.2. Glucose was added to a final concentration of 6.5 mg/ml. Membranes were used uncoated except for certain experiments where either laminin was used as coating or transfected glial cells were seeded as feeder layer for the slice. During long-term cultivation (up to 2 months) the culture medium was gradually changed to a neuron-specific N2 medium enriched with 0.5–10% FCS, depending on whether or not glial cells were cocultured.

PCR and Cloning of the Laminin Constructs

Plasmids containing the full-length cDNA of the laminin $\alpha 1$ chain were a gift of Dr. Y. Yamada (NIH, Bethesda, MD). PCR amplifications were done using AmpliTaq polymerase (Perkin Elmer, USA). Amplification products were electrophoresed on 1.5% agarose gels and the DNA was extracted using a QIAEX II kit (Quiagen, USA). All PCR fragments were cloned into pBluescript KS II (pBSKII) vector (Stratagene) and sequenced using a Sequenase™ kit (USB). A 120-bp cDNA fragment containing the coding sequence of the original laminin signal peptide was generated by PCR with synthetic oligonucleotides 5'-GAGCGCAGGTA CCGGCGGCG-3' and 5'-GGCAGGGTCTAGACCTCTCTGC-3'. *KpnI* and *XbaI* restriction sites were introduced by primer modification. Pep1 and pep2 cDNA fragments were generated by PCR amplification using oligonucleotides 5'-GCA GAAATCTAGAGGAGATC-3' and 5'-GGTGTGGGATCCGTAGATTA AGTCTG-3' (pep1) and 5'-CGATCGATCTAGACAACCTG-3' and 5'-GCATG-GGTGGGATCCAAA AGTTAAGCGTC-3' (pep2), containing modifications to yield *XbaI* and *BamHI* restriction sites and artificial stop codons (Fig. 2).

A pBSK II vector was used as a basis for the laminin constructs. Restriction enzymes were obtained from Boehringer Mannheim (Germany). A part of the "long terminal repeat" (a 450-bp *NheI*–*KpnI* fragment) of the Moloney murine leukemia virus was chosen as a promoter (MoLTR). The cDNA fragment corresponding to the laminin signal peptide was cloned into the *KpnI* and a *XbaI* sites of pBSKII (see Fig. 2). The PCR-amplified fragments encoding pep1 and pep2 (positions 6366–6483 and 7926–8040) were digested with *XbaI* and *BamHI* and were cloned together with a *BamHI*–*EcoRI*-digested fragment containing the SV40 polyadenylation signal and a CAT (chloramphenicoltransferase) sequence downstream of the signal peptide into the *XbaI* and *EcoRI* sites of the polylinker. A hygromycin resistance gene under the control of the PGK (phosphoglycerate kinase) promoter was introduced into the *SalI* site of the constructs (see Fig. 2) in opposite transcriptional direction. All plasmid constructs were confirmed by restriction analysis and DNA sequencing. For transfection experiments, constructs were linearized by *HindIII* digestion.

Northern Blotting

Total RNA was isolated by phenol extraction using standard procedures and electrophoresed through a 1.2% agarose gel under denaturing conditions. RNA was

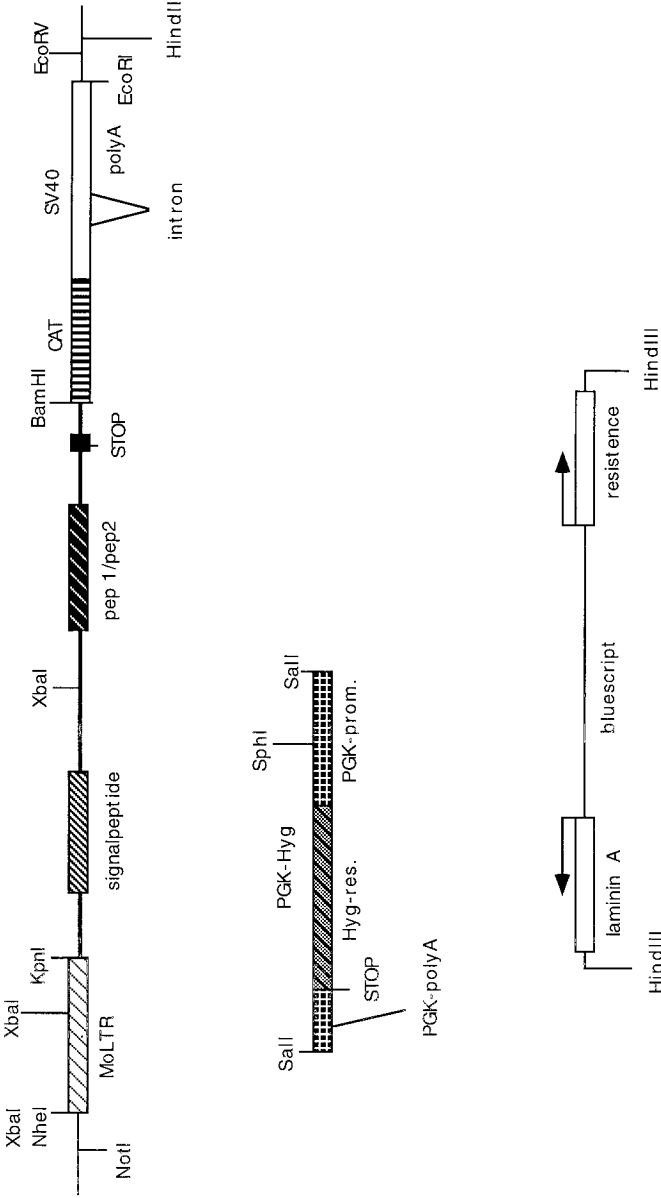


Fig. 2. Constructs of laminin peptides.

blotted onto Hybond membranes (Amersham, UK) overnight and probed with a ^{32}P -labeled *BamHI-EcoRI* fragment consisting of the CAT and the SV40 region of the laminin constructs.

Transfection of Glial Cells and Feeder Layers

C6 glioma cells and primary astrocytes were cultured in 250-ml plastic flasks (Falcon) to semiconfluence, removed with a rubber policeman, and washed three times with cold electroporation buffer containing 20 mM Hepes, 0.137 M NaCl, 5 mM KCl, 0.7 Na_2HPO_4 , 3 mM glucose, and 0.01% β -mercaptoethanol, pH 7.2. One ml of cell suspension (approx. 5×10^7 cells) containing 20 μg of linearized DNA (pep1, pep2) was electroporated at 1.5 kV (Biorad Gene Pulser™) at 4°C. Cells were plated on 90-mm plastic petri dishes and fed with medium containing 200 $\mu\text{g}/\text{ml}$ hygromycin. For cotransfection studies a *lacZ* reporter gene construct conferring neomycin resistance to the transfected cells was used. β -Galactosidase staining was done according to standard protocols. Remaining cells clones were isolated and tested for the transgene integration by Northern analysis. Fast-growing C6 cells carrying the laminin constructs were either kept in culture and their conditioned media harvested or were plated onto 35-mm plastic petri dishes and used as feeder layers for primary neuronal cultures. These feeder layers were first grown in Ham's F12 medium enriched with FCS, and after reaching confluence, isolated neocortical neurons were seeded upon and the medium was changed to N2 neuron-specific medium.

Quantification of Neurite Outgrowth and Neuron Survival

Primary neurons were grown in 35-mm plastic dishes either on feeder layers of transfected C6 glioma cells or in N2 medium enriched with 10–50% conditioned media derived from transfected C6 glioma cells. At various time intervals, neurite outgrowth was determined by counting neurites in five randomly chosen microscopic fields (about 2 mm^2). Means and standard errors are given. Three independent experiments were performed. For each experimental group three culture dishes were used. Survival of neurons was determined by counting cells which looked vital (firm adherence to the surface of the dish of both cell body and neurites) and by a colorimetric tetrazolium salt assay (MTT: 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide from Sigma; see Mosmann, 1983) following the protocol of Manthorpe *et al.* (1986). For the latter assay neurons were grown on 35-mm plastic petri dishes and on plastic 24-well plates (Primaria; Falcon). After 1, 2, and 3 weeks in culture either on feeder layers or treated with CMs of the transfected glioma cells (see Methods above) MTT sterile stock solution (1.5 mg/ml) was added at a ratio of 1:5. After incubation overnight, cultures were terminated by adding isopropanol–0.08 N HCl and after careful mixing the samples were read at 630 nm with a Hitachi spectrophotometer.

Antibodies and Western Blotting

For the detection of endogenous laminin in cell lysates, commercial antibodies were used (Sigma, USA). Two polyclonal antibodies against the laminin peptides

(pep1, pep2) were raised in rabbits using synthetic peptides as antigens. Western blots were performed using serum-free supernatants from C6 cells. The supernatants were collected under sterile conditions and 10–25 times concentrated using an Amicon concentration system. Protein concentration was determined according to Bradford (1976) using BSA as a standard. Equal amounts of protein samples (about 10 μ g/lane) were separated by SDS-PAGE (16%) according to Laemmli (1970) at 200 V for 1 hr and transferred to immobilon P membrane (Millipore). Following the transfer and blocking in 5% nonfat dry milk, membranes were incubated with the antibodies diluted to 1:2000 in TBS containing 0.1% Tween 20 (TBS-T) for 2 hr at room temperature. Membranes were then washed (2×15 min) in TBS-T and protein bands were visualized using the ECL Western blotting analysis kit (Amersham, UK).

RESULTS

Expression of Laminin Peptides in C6 Cells

Stably transfected glioma cells were selected by addition of hygromycin to the culture medium and subsequent cloning throughout several weeks. Four cell clones of each transfected population were randomly chosen and tested for the expression of laminin peptides by Northern blotting. A 32 P-labeled *Bam*HI–*Eco*RI fragment of the constructs was used as hybridization probe.

Pep2-transfected cells showed a generally weaker expression than pep1-transfected cells (Fig. 3). For further experiments only those clones showing strong expression of laminin peptides were used. Phase contrast pictures did not reveal any difference in the morphology of the transfected C6 cells versus nontransfected cells. Cell growth and proliferation rate were also unchanged in the transfected cells (data not shown). Expression of the peptides was detected in the supernatant of the cultured transfected cells using Western blot analysis and polyclonal antibodies directed against pep1 and pep2. Specificity of the antibodies was tested by staining

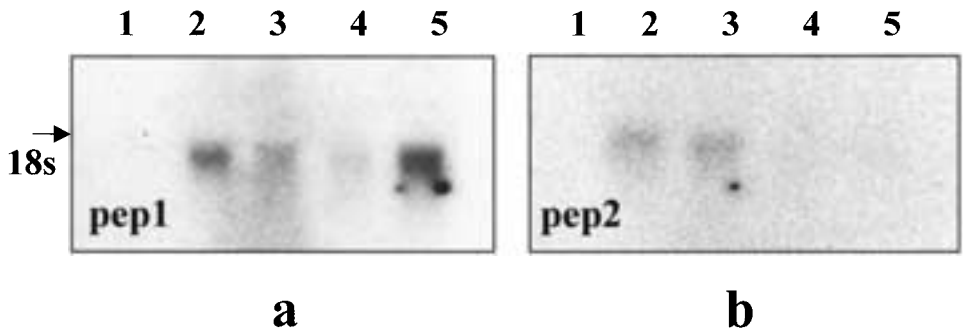


Fig. 3. Northern blot analysis of transfected C6 cells. (A) C6 cells transfected with pep1. Lane 1, untransfected C6 cells; lanes 2–5, four clones of transfected cells. (B) C6 cells transfected with pep2. Lane 1, untransfected C6 cells; lanes 2–5, four clones of transfected cells.

of pep2 supernatants with the pep1 antibody and vice versa. Both antibodies were shown to recognize specifically the proper antigen (Fig. 4). However, besides a specific reaction the antibodies exhibited also a weak unspecific reaction with untransfected cells, a problem which we could only partly overcome by increasing the NaCl concentration of the antibody reaction buffer to a maximum of 900 mM.

Cocultivation Studies Using Neuronal Cell Cultures

The effect of transfected cells on neurite outgrowth and neuronal survival was studied using both primary neuronal cultures and organotypic cultures derived from mouse forebrain. The neuronal cultures consisted to a very high extent of neurons as described earlier (Bauer and Tontsch, 1990). A few nonneuronal cells, probably of mesenchymal origin, were visible. Results from coculture experiments using C6pep1 and C6pep2 cells with primary neurons (freshly isolated neurons) are shown in Fig. 5. After 2 days of cocultivation, no differences in morphology and outgrowth could be observed between experimental groups and control cultures cocultured with untransfected C6 cells as feeder layers, although control cultures were less dense and exhibited significantly fewer neurites (Fig. 5a, d, h; Fig. 6A). Since transfected C6 glioma cells sometimes develop long extensions which could be mistaken for neurites, staining with antibodies against GFAP was performed routinely to avoid miscountings. It was virtually impossible to determine the exact cell numbers since cells had a strong tendency to form clusters, especially when grown on the pep1/pep2-transfected glioma cells. Therefore, another method for cell survival was employed using a colorimetry assay (MTT assay, see Materials and Methods section). After 1–2 weeks of cocultivation, control cultures showed a substantial decrease in cell density (fewer cell clusters and single cells) and the number of neurites was significantly reduced compared to the peptide-treated cultures, which also showed a significant decrease in neurite outgrowth (Fig. 5 and

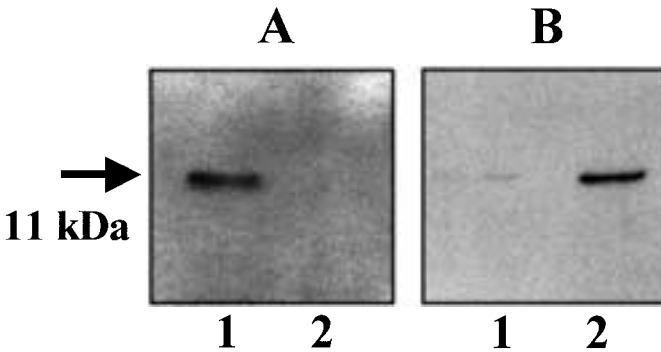


Fig. 4. Western blots demonstrating pep1 and pep2 in conditioned media from transfected C6 cells. Lane 1, conditioned medium from C6 cells transfected with pep1; lane 2, conditioned medium from C6 cells transfected with pep2. Immunoreaction was performed with peptide antibodies directed against (A) pep1 and (B) pep2.

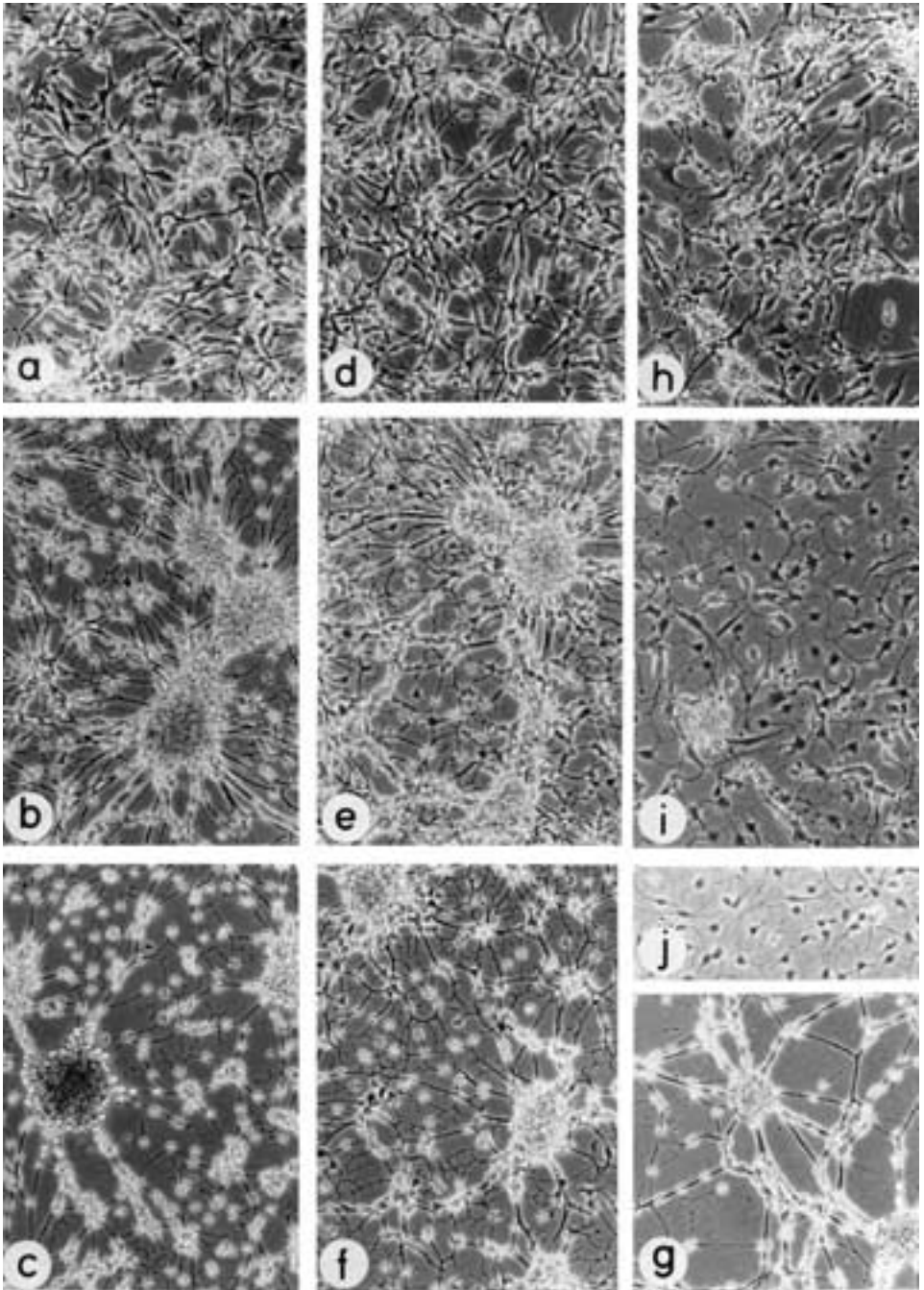


Fig. 5. Primary neuronal cultures grown on transfected C6 glioma cells. (a–c) Neurons grown on pep1-transfected cells for (a) 2, (b) 6, or (c) 13 days. (d–g) Neurons grown on pep2-transfected cells for (d) 2, (e) 6, (f) 13, and (g) 20 days. (h–j) Neurons grown on untransfected C6 cells for (h) 2, (i) 6, and (j) 13 days. (90 \times)

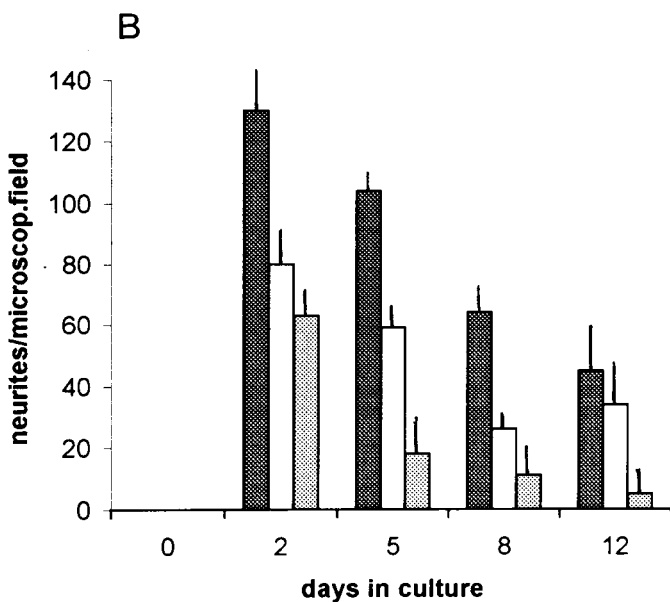
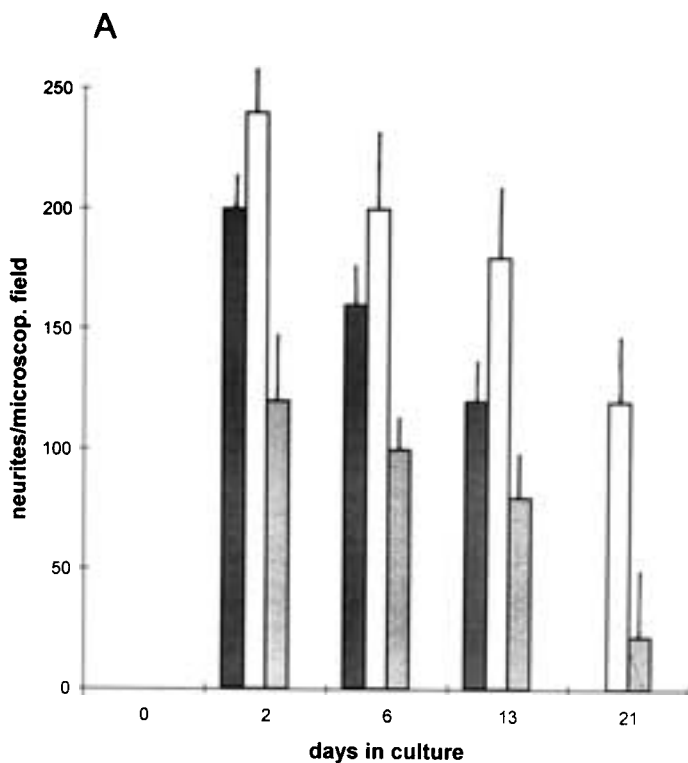


Fig. 6. Relations between neurite numbers and presence of laminin peptides. (A) Neurite numbers of primary neurons grown on feeder layers of C6 glioma cells transfected with laminin $\alpha 1$ -chain constructs. (B) Number of neurites of primary neurons grown in conditioned media derived from transfected C6 glioma cells. Black bars, pep 1; white bars, pep 2; shaded bars, C6 cells, controls. $n = 15$. Means and SE are given.

Fig. 6A). After 3 weeks of cocultivation only neurons grown on pep2-transfected C6 cells still exhibited many neurons interconnected with neurites (Fig. 5g). Neurons grown on pep2-transfected cells showed the strongest effect both on survival and on neurite outgrowth. Our results concerning cell survival have been confirmed by the MTT assay, demonstrating that pep1- and pep2-transfected C6 cells dramatically increase survival of cocultured neurons (Table I).

Experiments with Conditioned Media

Figure 7 shows the effects of various conditioned media (CM) on neurons in culture after 5, 8, and 12 days. At all three time intervals neuronal cultures treated with CM from C6pep1 and C6pep2 were viable and contained partly interconnected neurites. The total cell numbers were about equal in cultures treated with pep1 or pep2 CM (about 30–40 cells/mm²) during the observation period. Again, it was impossible to obtain exact numbers because of the tendency of the neurons to form clusters. In general, more cells survived and more neurites were observed in cultures treated with pep1 (6B). In control cultures treated with CM from untransfected C6 cells fewer cells survived (after 12 days about 10 cells/mm²) and the number of neurites was very low. The data obtained by the MTT assay revealed a similar tendency, showing maximal survival of neurons cultured with pep1 CM (Table I).

Studies with Organotypic Cultures

Organotypic cultures are useful model system to study neuronal functions or cell–cell interactions. Other than primary neuronal cultures, they are long-lasting: using the appropriate media, an organotypic brain culture derived from E13 or E16 mouse brain survived for up to 4 months in culture.

Figure 8a shows explants derived from E16 mouse brains cultured for 5 days on laminin-coated filters. As expected, long neurites were observed in these cultures. In order to study the effect of C6pep1/pep2-derived CMs on organotypic brain cultures, brain slices derived from mice of various developmental stages were cultured on filters and treated with the CMs. Cultures treated with CMs of pep1- or pep2-transfected C6 cells exhibited rapid sprouting; however, neurites were more abundant, but thinner and shorter than those in explants treated with laminin (not shown). After 3 weeks brain explants incubated with pep1 CM appeared thinned and spread out, but neurons were still branching (Fig. 8d), whereas in control

Table I. MTT Assay Testing Neuronal Survival^a

	C6 cells		Pep1–C6		Pep2–C6	
	+CM	Coculture	+CM	Coculture	+CM	Coculture
6 days	98 ± 13	183 ± 12	156 ± 6	207 ± 17	152 ± 4	257 ± 31
12 days	233 ± 51	439 ± 65	673 ± 48	663 ± 102	500 ± 58	699 ± 72
21 days	ND	230 ± 71	ND	478 ± 122	ND	490 ± 68

^a 100%: Neurons without glial CM or feeder layer. *n* = 6–12. Means and SE are given.

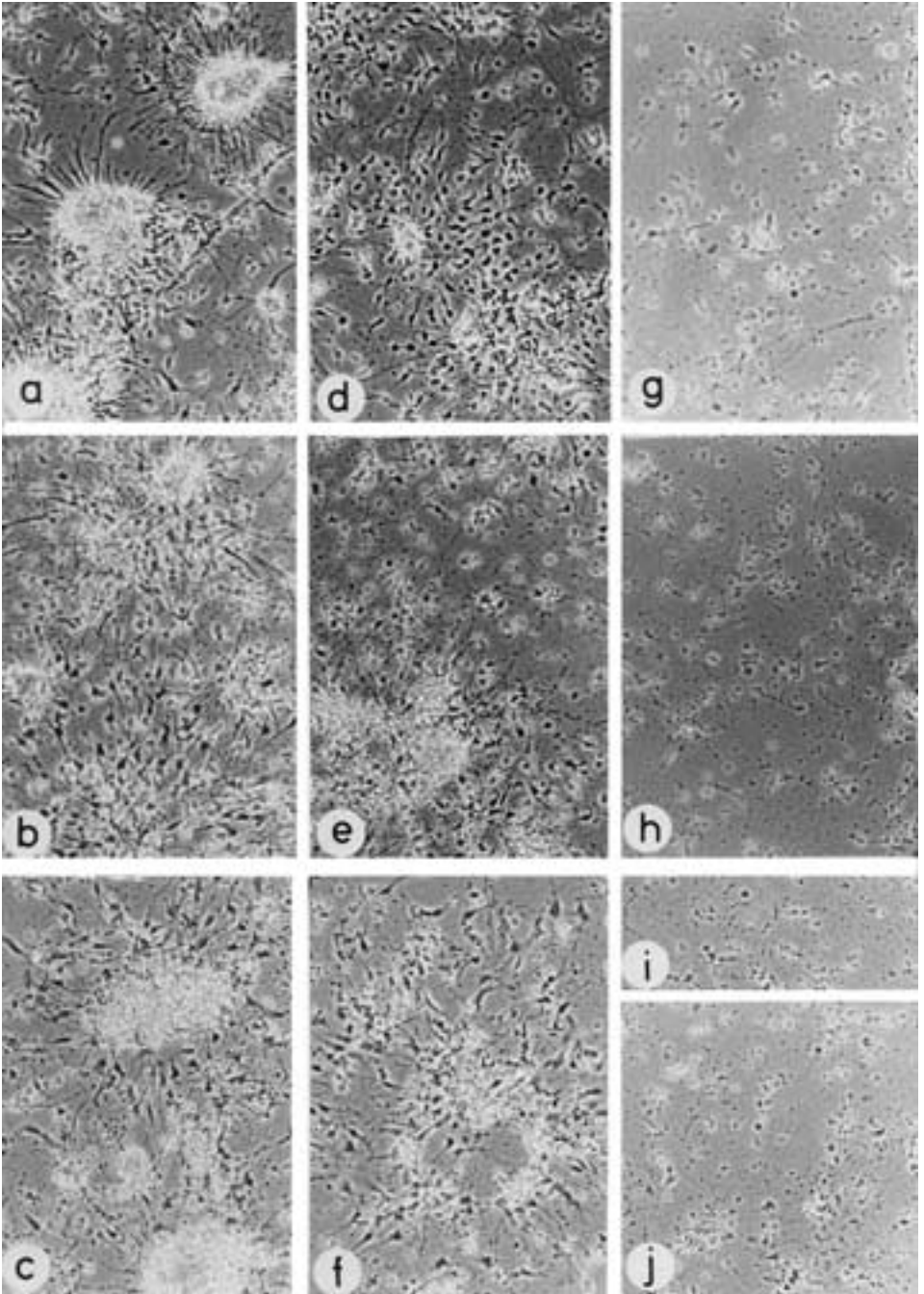


Fig. 7. Primary neuronal cultures grown in the presence of conditioned media (CM) derived from transfected C6 glioma cells for various lengths of time. (a–c) CM of pep1 glioma cells; (d–f) CM of pep2 glioma cells; (g–i) CM of nontransfected glioma cells; (j) no CM. (a, d, g) Neurons for 5 days in culture; (b, e, h) neurons for 8 days in culture; (c, f, i) neurons for 12 days in culture; (j) neurons for 8 days in culture. (90 \times)

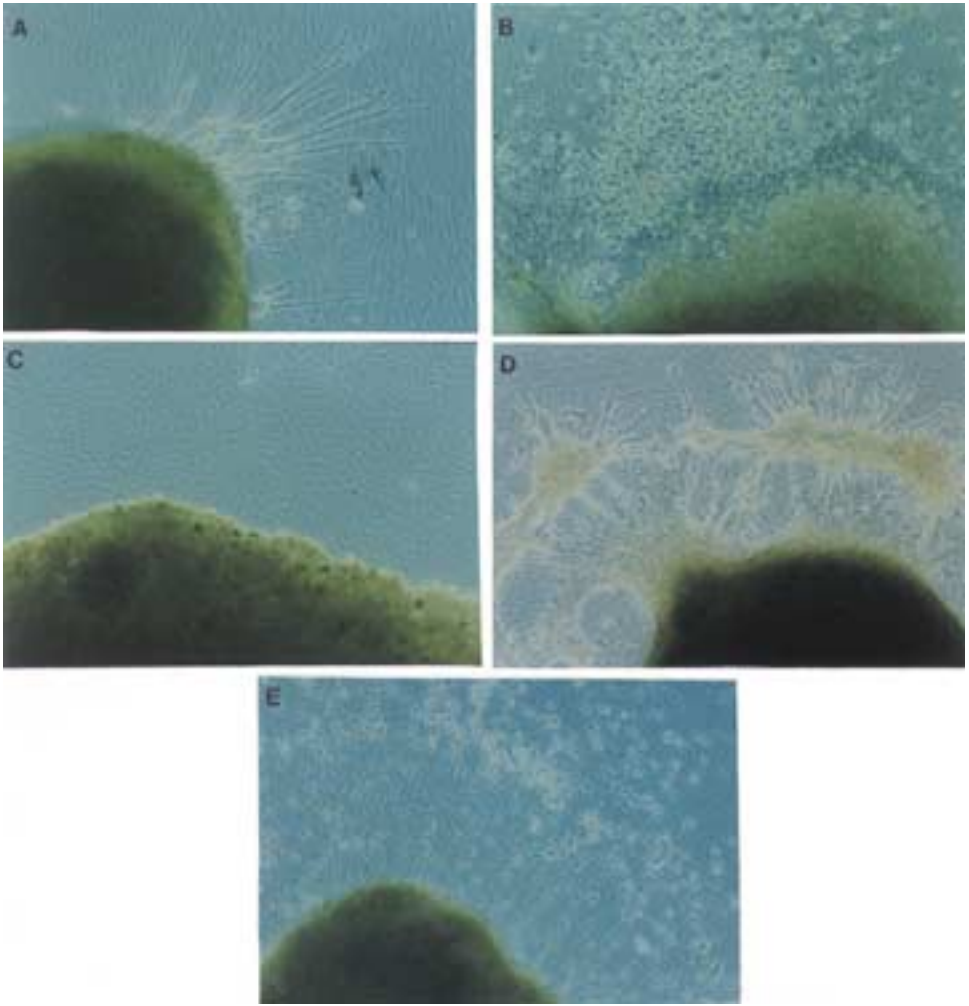


Fig. 8. Organotypic cultures derived from anterior embryonic (E16 resp. E13) mouse forebrain slices. (a) Five-day-old culture grown on laminin-coated filter. (b) Three-week-old culture cocultured with C6 glioma cells. (c) Three-week-old organotypic culture in medium enriched with 10% CM from non-transfected C6 cells. (d) Three-week-old organotypic culture in medium enriched with 10% CM from pep1-transfected C6 cells. (e) Five-week-old organotypic culture (derived from E13) in medium enriched with 10% CM from pep1-transfected C6 cells. (90 \times)

cultures hardly any neurites were left and numerous necrotic cells were visible (Fig. 8c). After 5–8 weeks in culture, pep1 CM-treated explants still showed neurites and only a few necrotic cells were visible (Fig. 8e). Pep2 CM had a similar effect. The density of the sprouting neurons was comparable to that of pep1 CM-treated cultures (data not shown). Slices of the same origin incubated with C6 CM showed no or very little neurite outgrowth (Fig. 8b).

DISCUSSION

In the present study the ability of recombinantly produced laminin peptides to promote neurite outgrowth and neuronal survival has been demonstrated. We studied the neurogenic effect of two peptides located in two structurally and functionally distinct regions of the laminin $\alpha 1$ chain. We generated two constructs containing the cDNA sequences known to encode small peptides corresponding to the amino acid regions from 2081 to 2108 (pep1) and 2615 to 2631 (pep2). Pep1 was encoded by a cDNA sequence located at the N-terminal end of the E8 domain of the laminin $\alpha 1$ chain. The pep1 fragment comprised a sequence previously described by Tashiro *et al.* (1989) coding for the PA-22 peptide, which was found to contain the pentapeptide IKVAV as its active core region. The IKVAV peptide was reported to promote hepatocyte attachment and neuronal process extension (Clement *et al.*, 1990; Sephel *et al.*, 1989; Tashiro *et al.*, 1989). Peptide 2 (pep2) was an expressed fragment of the globular domain (G-domain) of the laminin $\alpha 1$ chain encompassing the GD1 fragment previously described by Skubitz *et al.* (1991). This peptide was found to promote adhesion and spreading of fibrosarcoma cells and increased neurite outgrowth from dorsal root ganglion neurons (Sephel *et al.*, 1989).

To our knowledge, all studies demonstrating the neurogenic action of laminin have involved the addition of purified laminin protein or laminin peptides, particularly laminin $\alpha 1$ -chain fragments. Coating of culture dishes or addition to cell culture media has been the usual application form. Although all experimental evidence has clearly proven the neurogenic/neurotrophic effect of laminin, several difficulties arising with the use of purified peptides have been reported. For instance, experiments including E8 fragment showed that cell migration on E8 varied substantially among experiments. The explanation for this was suggested to be simple: E8 is considerably less stable than intact laminin, and preparations of it lose activity upon storage or when exposed to air (Tashiro *et al.*, 1989). On the other hand, it is impossible to know in a given experiment what fraction of a multidomain sequence is active. Thus, it is advantageous to work with the smallest entities possible if a restricted and distinct action of a laminin fragment is to be measured.

We first tested whether the transfected glial cells could promote neurite outgrowth of primary neuronal cells. As a result, cocultivation of neurons with transfected glial cells or addition of C6pep1/pep2-derived conditioned medium to primary neuronal cultures had a positive effect on neuronal survival and neurite outgrowth. The neurite-promoting effect of pep1 and pep2 was relatively slow (2–15 hr) compared to the entire laminin molecule adsorbed to the plastic dish. In addition, we found that not only was neurite outgrowth promoted, but so was neuronal survival, resulting in a survival period three times longer than untreated neuronal cultures.

Northern blotting revealed that the transfected cDNAs were transcribed in C6 cells, though at varying levels. For all experiments cell clones showing high levels of pep1/pep2 mRNA were chosen. Expression of laminin $\alpha 1$ chain in nontransfected C6 cells and primary astrocytes could not be detected by Northern blotting, but was visible by RT-PCR (data not shown). Addition of heparin to cocultures of neurons and C6pep1/2 cells did not reveal a significant change in the rate of neurite

outgrowth (data not shown), suggesting that heparin binding is not necessary for neurite outgrowth, but is probably involved in the adhesive function of laminin as described for other cells (Skubitz *et al.*, 1991).

In our studies we found that seeding of syngenic primary astrocytes overexpressing laminin peptides onto mouse brain slices resulted in an efficient and more rapid adhesion of the cells to the neural tissue (not shown). Since comparable results were obtained with C6 cells, this cell line was used for all our experiments because they grew much faster and gave comparable results to the transfected astrocytes.

Organotypic cultures represent a useful tool to mimic the *in vivo* situation and to maintain the "natural" cellular environment of the neuronal cells under study. In our experiments we successfully used slices from embryos of E13.5 to newborn mice at postnatal day 2. Generally, transplanted glial cells adhered better to embryonic slices, but the tissue was less stable and electron-optical studies revealed a more frequent appearance of necrotic cells (data not shown). Brain slices from older embryos or postnatal animals were more stable and necrotic events were only scarcely seen; however, transplanted cells did not adhere to the same extent as to slices from E13.5 embryos as evidenced by β -galactosidase staining.

Comparing the neurite outgrowth-promoting activity of pep1 and pep2 recombinant peptides, we found major differences in single-cell cultures when the peptide-secreting glial cells were used as feeder layers. Pep2-transfected cells elicited the most neurites and the longest lasting in culture. Neuronal survival was also best in cocultures of neurons with pep2-secreting glia cells, which may be explained by the presence of neurites which usually connect the cells or cell clusters, thus increasing survival rates.

As predicted by their amino acid sequence, pep1 and pep2 did not contain RGD sequences, indicating that no RGD-dependent integrin-receptor binding was involved in their actions on neuronal cells. Pep2, encompassing the GD1 peptide (Skubitz *et al.*, 1991), was reported to bind heparin with high affinity, suggesting that this peptide may interact with cells by way of their glycosaminoglycans or proteoglycans. In earlier years there was a simple picture concerning how the laminin α 1 chain acts on neuronal outgrowth and migration. In this respect, E8 was suggested to act via a single integrin receptor (α 6 β 1 integrin), accounting for all of laminin's neurogenic effect. However, during the last couple of years a more complicated picture has emerged. On one hand, integrin receptors have been found to be involved in laminin action, but on the other hand, various synthetic peptides from the different laminin chains, including the E8 domain, have been synthesized which were shown to promote cell adhesion and neuronal migration without using an integrin as a receptor (Graf *et al.*, 1987; Kleinman *et al.*, 1989; Liesi *et al.*, 1989; Tashiro *et al.*, 1989; Clement *et al.*, 1990; Hynes, 1992; McKerracher *et al.*, 1996; Powell and Kleinman, 1997).

Grafting of genetically modified neural or nonneural cells to the CNS as a source of neurotrophic factors holds promise for the treatment of neurodegenerative diseases. Interestingly, various cell lines such as PC12 cells, glial cells, C2C12 myoblasts, and 3T3 fibroblasts have been used for CNS transplantation studies (Blakemore and Franklin, 1991; Gage *et al.*, 1991). Cell lines offer some advantages over primary cells since they are essentially immortal and thus have a high potential

to survive following their implantation into the brain. On the other hand, this capacity of continuous cell division may also lead to intracerebral tumor formation (Shimohama *et al.*, 1989; Ushida *et al.*, 1989). A promising approach to overcome this problem has been reported by several authors using a polymer encapsulation of genetically modified cell lines secreting hCNTF or dopamine (Aebischer *et al.*, 1996; Jaeger *et al.*, 1996; Deglon *et al.*, 1996).

We have shown that genetically modified glial cells may be used as a source of secreted laminin peptides known to exert neurotrophic effects on CNS tissue. We have demonstrated that two distinct peptides of the laminin $\alpha 1$ chain can independently promote neuronal outgrowth. Survival and outgrowth of primary neurons as well as of neurons from CNS explants were shown to be substantially increased following cocultivation with glial cells overexpressing two peptides derived from the laminin $\alpha 1$ chain.

ACKNOWLEDGMENTS

We are grateful to R. Fuchs for her excellent technical assistance and to M. Bohrer for her help in preparing the manuscript. Part of this work was supported by the National Bank of Austria (Project number 6475), APART (H. Bauer), the Swiss National Science Foundation (grant number 31-45831.95), and the Hungarian Science Foundation (OTKA; I. A. Krizbai).

REFERENCES

- Aebischer, P., Pochon, N. A., Heyd, B., Deglon, N., Joseph, J. M., Zurn, A. D., Baetge, E. E., and Hammang, J. P. (1996). Gene therapy for amyotrophic lateral sclerosis (ALS) using polymer encapsulated xenogenic cell line engineered to secrete hCNTF. *Hum. Gene Ther.* **7**:851–860.
- Aumailley, M., and Krieg, T. (1996). Laminins: A family of diverse multifunctional molecules of basement membranes. *J. Invest. Dermatol.* **106**:209–214.
- Bauer, H. C., and Tontsch, U. (1990). Glial conditioned medium and attachment to ConA are essential for long term culture of cortical neurons. *Int. J. Dev. Neurosci.* **8**(2):151–158.
- Begovac, P. C., Hall, D. E., and Shur, B. D. (1991). Laminin fragment E8 mediates PC12 cell neurite outgrowth by binding to cell surface $\beta 1,4$ galactosyltransferase. *J. Cell Biol.* **113**(3):637–644.
- Blakemore, W. F., and Franklin, R. J. M. (1991). Transplantation of glial cells into the CNS. *TINS* **14**(8):323–327.
- Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **83**:6188–6192.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**:248–254.
- Buettner, H. M., and Pittman, R. N. (1991). Quantitative effects of laminin concentration on neurite outgrowth *in vitro*. *Dev. Biol.* **145**:266–276.
- Clark, P., Britland, S., and Connolly, P. (1993). Growth cone guidance and neuron morphology on micropatterned laminin surfaces. *J. Cell Sci.* **105**:203–212.
- Clement, B., Segui-Real, B., Savagner, P., Kleinman, H. K., and Yamada, Y. (1990). Hepatocyte attachment to laminin is mediated through multiple receptors. *J. Cell Biol.* **110**:185–192.
- Deglon, N., Heyd, B., Tan, S. A., Joseph, J. M., Zum, A. D., and Aebischer, P. (1996). Central nervous system delivery of recombinant ciliary neurotrophic factor by polymer encapsulated differentiated C2C12 myoblasts. *Hum. Gene Ther.* **7**:2135–2146.
- Edgar, D., Timpl, R., and Thoenen, H. (1984). The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO J.* **3**(7):1463–1468.

- Gage, F. H., Kawaja, M. D., and Fisher, L. J. (1991). Genetically modified cells: Applications for intracerebral grafting. *TINS* **14**(8):328–333.
- Gähwiler, B. H. (1981). Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Meth.* **4**:329–342.
- Goodman, S. L., Deutzmann, R., and von der Mark, K. (1987). Two distinct cell-binding domains in laminin can independently promote nonneuronal cell adhesion and spreading. *J. Cell Biol.* **105**:589–598.
- Graf, J., Ogle, C., Robey, F. A., Sasaki, M., Martin, G. R., Yamada, Y., and Kleinman, H. K. (1987). A pentapeptide from the laminin B1 chain mediates cell adhesion and binds the 67000 laminin receptor. *Biochemistry* **26**:6896–6900.
- Grant, D. S., Kinsella, J. L., Fridman, R., Auerbach, R., Piasecki, B. A., Yamada, Y., Zain, M., and Kleinman, H. K. (1992). Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) *in vitro* and induction of angiogenic behaviour *in vivo*. *J. Cell. Physiol.* **153**:614–625.
- Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69**:11–25.
- Jaeger, C. B., Greene, L. A., Tresco, P. A., Winn, S. R., and Aebischer, P. (1996). Polymer encapsulated dopaminergic cell lines as “alternative neural grafts.” *Prog. Brain Res.* **82**:41–46.
- Kleinman, H. K., Graf, J., Iwamoto, Y., Sasaki, M., Chasteen, C. S., Yamada, Y., Martin, G. R., and Robey, F. A. (1989). Identification of a second active site in laminin for promotion of cell adhesion and migration and inhibition of *in vivo* melanoma lung carcinoma. *Arch. Biochem. Biophys.* **272**:39–45.
- Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lander, A. (1987). Molecules that make axons grow. *Mol. Neurobiol.* **1**:213–245.
- Letoumeau, P., Madsen, A. M., Palm, S. M., and Furcht, L. T. (1988). Immunoreactivity for laminin in the developing ventral longitudinal pathway of the brain. *Dev. Biol.* **125**:135–144.
- Liesi, P., Närviäinen, A., Soos, J., Samola, H., and Snounou, G. (1989). Identification of a neurite outgrowth-promoting domain of laminin using synthetic peptides. *FEBS Lett.* **244**:141–148.
- Luckenbill-Edds, L. (1997). Laminin and the mechanism of neuronal outgrowth. *Brain Res. Brain Res. Rev.* **23**:1–27.
- Malinda, K. M., and Kleinman, H. K. (1996). The laminins. *Int. J. Biochem. Cell Biol.* **28**:957–959.
- Manthorpe, M., Fagnani, R., Skaper, S. D., and Varon, S. (1986). An automated colorimetric microassay for neurotrophic factors. *Dev. Brain Res.* **25**:191–198.
- Martin, G. R., and Timpl, R. (1987). Laminin and other basement membrane components. *Annu. Rev. Cell Biol.* **3**:57–85.
- McKerracher, L., Chamoux, M., and Arregui, C. O. (1996). Role of laminin and integrin interactions in growth cone guidance. *Mol. Neurobiol.* **12**:95–116.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**:55–63.
- Powell, S. K., and Kleinman, H. K. (1997). Neuronal laminins and their cellular receptors. *Int. J. Biochem. Cell Biol.* **29**:401–414.
- Ryan, M. C., Christiano, A. M., Engvall, E., Wewer, U. M., Miner, J. H., Sanes, J. R., and Burgeson, R. E. (1996). The functions of laminins: Lessons from *in vivo* studies. *Matrix Biol.* **15**:369–381.
- Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R., and Yamada, Y. (1988). Laminin, a multidomain protein. *J. Biol. Chem.* **263**(32):16536–16544.
- Sephel, G. C., Tashiro, K., Sasaki, M., Graetorex, D., Martin, G. R., Yamada, Y., and Kleinman, H. K. (1989). Laminin A chain synthetic peptide which supports neurite outgrowth. *Biochem. Biophys. Res. Commun.* **162**(2):821–829.
- Shimohama, S., Rosenberg, M. B., Fagan, A. M., Wolff, J. A., Short, M. P., Breakefield, X. O., Friedman, T., and Gage, F. H. (1989). Grafting genetically modified cells into the rat brain: Characteristics of *E. coli* β -galactosidase as a reporter gene. *Mol. Brain Res.* **5**:271–278.
- Skubitz, A. P. N., Letourneau, P. C., Wayer, E., and Furcht, L. T. (1991). Synthetic peptides from the carboxy-terminal globular domain of the A-chain of laminin: Their ability to promote cell adhesion and neurite outgrowth and interact with heparin and the β 1 integrin subunit. *J. Cell Biol.* **115**(4):1137–1148.
- Stoppini, I., Buchs, P., and Muller D. (1991). A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth.* **37**:173–182.
- Sweeney, T. M., Kibbey, M. C., Zain, M., et al. (1991). Basement membrane and the SIKVAV laminin-derived peptide promote tumor growth and metastasis. *Cancer Metastasis Rev.* **10**:245–254.
- Tashiro, K. I., Sephel, G. C., Weeks, B., Sasaki, M., Martin, G. R., Kleinman, H. K., and Yamada, Y. (1989). A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration, and neurite outgrowth. *J. Biol. Chem.* **264**(27):16174–16182.
- Ushida, K., Takamatsu, K., Kaneda, N., Toya, S., Tsukada, Y., Kurosawa, Y., Fujita, K., Nagatsu, T., and Kohsaka, S. (1989). Synthesis of L-2,3-dihydroxyphenylalanine by tyrosine hydroxylase cDNA-transfected C-6 cells: Application for intracerebral grafting. *J. Neurochem.* **53**:728–732.