The expression and distribution of laminin in the developing nervous system

DAVID EDGAR

Department of Human Anatomy and Cell Biology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England

Summary

The extracellular matrix glycoprotein laminin exerts profound effects on the survival and differentiation of neurons *in vitro*. Although principally confined to the basement membranes of the adult extracellular matrix, during development laminin immunoreactivity may be found both within the interstitial extracellular matrix and on the membranes of neural cells, in which location it may be expected to affect their development *in vivo*. To investigate the reasons for the occurrence of laminin outside basement membranes, the expression of genes coding for laminin subunits has been analysed in mouse sciatic nerves at different postnatal ages. The results show that the expression of genes coding for laminin subunits decreases from high levels at birth, very low steady state levels of the individual mRNAs being found in the adult. Thus, the predicted high rate of laminin synthesis during development might exceed the rate at which it may be incorporated into basement membranes. The effective change in distribution of laminin into the basement membrane is therefore likely to be simply a consequence of the down-regulation of gene expression, rather than being caused, for example, by the differential expression of laminin variants.

Key words: laminin, gene expression, basement membrane, nervous system, axonal growth.

Introduction

Neuronal survival and differentiation in both the developing peripheral and central nervous systems depend upon a variety of epigenetic factors. In addition to stimulating the rapid growth or regeneration of neurites by embryonic neurons (Collins, 1980), extracellular matrix molecules produced by a variety of cells can greatly potentiate the survival-promoting effects of neurotrophins such as the nerve growth factor (Edgar and Thoenen, 1982). The non-collagenous, extracellular matrix glycoprotein laminin is unique in that it evokes such effects on neurons both *in vitro* (Baron-Van Evercooren *et al.* 1982; Edgar *et al.* 1984) and *in vivo* (Kalcheim *et al.* 1987). Furthermore, laminin tissue culture substrates alone have been shown to enhance the expression of the enzyme, tyrosine hydroxylase, that regulates the synthesis of catecholaminergic neurotransmitters (Acheson *et al.* 1986), and such substrates also permit the survival of neuronal precursor cells, without the need for the presence of a neurotrophin (Ernsberger *et al.* 1989).

Analysis of the interactions of laminin variants with neural and other cells

Laminin isolated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma tumour (Timpl *et al.* 1979) has been cleaved by proteolysis to reveal that the site which evokes neurite outgrowth and neuronal survival is located towards the end of the long arm of this cruciform molecule (Edgar *et al.* 1984). Because tertiary protein structure and sites sensitive to proteolysis appear to be necessary for biological activity, then further attempts to increase the resolution of mapping of active site(s) have been largely unsuccessful (Edgar *et al.* 1988; Deutzmann *et al.* 1990). However, inhibition with domain-specific antibodies do show that the site responsible for neurite outgrowth, neuronal survival (Edgar *et al.* 1984) and induction of tyrosine hydroxylase (Acheson *et al.* 1986) is located at or near the large globular domain at the end of the long arm. Many cells are able to interact with this or a closely juxtaposed site (Aumailley *et al.* 1987; Dillner *et al.* 1988). However, the greater sensitivity to denaturation and/or proteolysis of the neurite-promoting activity of this region indicates that stimulation of neurite outgrowth requires laminin structures different from, or in addition to, those required for cell adhesion (Deutzmann *et al.* 1990).

Laminin was originally identified as being essentially only located in all basement membranes of the adult (Timpl *et al.* 1979). In this location, it is likely that long arm cell binding sites are available for interaction with their responsive cells: epitopes found on the long arm of laminin are accessible outside the basal lamina, indicating that this part of the molecule is exposed in the adult (Schittny *et al.* 1988; Abrahamson *et al.* 1989). It should be noted that a major potential cell binding site in the short arms of laminin is masked not only within the basement membrane (Schittny *et al.* 1988; Abrahamson *et al.* 1989), but also by the short arm globular domains (Nurcombe *et al.* 1989). Thus, in addition to sites overtly expressed by basement membrane-bound laminin, this matrix molecule also possesses sites which have the potential to interact with cells either after activation by proteolytic release, or alternatively, if the laminin were not confined to basement membranes.

Variants of laminin lack the epitopes recognised by blocking antibodies, owing to a different laminin subunit composition (Edgar *et al.* 1988). At least some of these variants can nevertheless stimulate neurite outgrowth

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(Edgar *et al.* 1988), indicating that antibody inhibition of EHS tumour laminin is likely to be by steric hindrance, the blocking antibodies recognising an epitope unique to EHS laminin which is close to (but not the actual) active site. Laminin variants may lack this epitope, but because some of them do promote neurite outgrowth they may be expected to share the active site which is likely to be non-antigenic (Edgar *et al.* 1988). One unfortunate practical consequence of this observation is that it is unlikely that antibodies blocking the activities of EHS tumour laminin will be of use in attempts to block the biologically important functions of other laminin variants *in vivo* (see below).

Immunohistochemical localisation of laminin in developing tissues

In contrast to its situation in the adult, it has been reported previously that laminin immunoreactivity in many different embryonic tissues appears as punctate deposits independent of basement membranes (Ekblom *et al.* 1980; Dziadek and Mitrangas, 1989). Such immunoreactivity is most noticeable in the interstitial extracellular matrix or mesenchyme, where this is a prominent feature of the tissue (Ekblom *et al.* 1980; Dziadek and Mitrangas, 1989). However in the nervous system, punctate deposits of laminin-immunoreactive material have been noted on the surface of neural cells, there being comparatively little interstitial extracellular matrix in the central nervous system (Liesi, 1985; Cohen *et al.* 1987; McLoon *et al.* 1988). Because this laminin appears transiently at the times when axonal growth is occurring, it is possible that laminin is one of the components within the immediate vicinity of the axonal growth cone which promotes axonal extension (see Bixby *et al.* 1987). This likelihood is strengthened by the observation that developing neurons also express receptors capable of inducing neurite outgrowth in response to intact mouse EHS tumour laminin at this time (Cohen *et al.* 1989).

Immunohistochemical analysis of laminin in the adult sciatic nerve has shown a differential distribution of laminin subunits (Sanes *et al.* 1990). Thus, while the laminin of the endoneurium was [$A^-B1^+B2^+M^+S^-$], as determined by subunit-specific antibody cross-reactivities, that of the perineurium was [$A^+B1^-B2^+M^-S^+$]. Significantly, preliminary experiments have indicated that the interactions of laminin variants with neural cells are influenced by the laminin subunit composition, so that while both A chain- and M chain-containing human laminins can support neurite outgrowth from sympathetic neurons, the rate of neurite growth is much faster on the A chain variant (unpublished observations; see also Hunter *et al.* (1989), for evidence that the S chain variant may actually inhibit neurite growth). In the light of these observations, it is clear that until the molecular composition, and consequently the biological activity, of the laminin responsible for any immunoreactivity seen *in vivo* is established, then it is not clear to what extent a response demonstrated by EHS tumour laminin *in vitro* can be used to confirm that an endogenous laminin variant is active and therefore likely to stimulate axonal growth: the variant(s) expressed in the developing nervous system may lack, or alternatively, have exposed different active sites. From the above discussion, it may be seen that this could either be due to their subunit structure, or because they are not in a basement membrane, or because they have been subjected to proteolytic degradation exposing novel active sites.

Developmental regulation of laminin gene expression

Not only the expression of laminin receptors and response (Cohen *et al.* 1989) are developmentally regulated: it is now clear that laminin gene expression is down-regulated in several tissues, as reflected by the decrease in steady-state levels of the mRNA coding for laminin subunits during development (Senior *et al.* 1988; Kücherer-Ehret *et al.* 1990*b*; Ekblom *et al.* 1990). Thus, in the sciatic nerve of the developing postnatal mouse it may be seen that mRNA levels decrease from birth to adulthood (Table 1). It is however, unknown what factors regulate laminin gene expression in this or other tissues, nor have the molecular mechanisms been established which determine the differential expression of laminin subunits (Cooper and Macqueen, 1983; Ohno *et al.* 1986; Kleinman *et al.* 1987; Liesi and Risteli, 1989; Hunter *et al.* 1989; Paulsson and Saladin, 1989; Ehrig *et al.* 1990; Ekblom *et al.* 1990). Significantly, there is currently little evidence for a developmentally regulated differential expression of the A or B1 and B2 subunits, which has been suggested to be responsible for alternative localisations of laminin either within, or extraneous to, basement membranes (see McLoon *et al.* 1988). Indeed, most tissues display very low levels of the laminin A chain, relative to the B chains, throughout development (Kücherer-Ehret *et al.* 1990*b*; Ekblom *et al.* 1990).

One consequence of high levels of laminin expression during development is likely to be that laminin accumulates at a rate faster than that with which it can be incorporated into basement membranes: it has been noted previously that laminin synthesis is not the rate-determining step in basement membrane production (Clark and Bunge, 1989). In the peripheral nervous system it been shown that the Schwann and other cells ensheathing the axons can synthesise and secrete laminin (Cornbrooks *et al.* 1983; McGarvey *et al.* 1984; Jaakkola *et al.* 1989). However, in the developing sciatic nerve it was demonstrated by immuno-electronmicroscopy that most of this laminin accumulates extracellularly where it is found in the interstitial extracellular matrix (mesenchyme), rather than being localised exclusively in the basement membranes as in the adult (Kücherer-Ehret *et al.* 1990*a*).

Although the laminin of the developing sciatic nerve is apparently associated mainly with interstitial collagen

Table 1. *Steady-state levels of laminin subunit mRNAs in developing mouse sciatic nerve*

Postnatal age (days)	Relative specific steady-state level		
	$mRNA_A$	$mRNA_{B1}$	$mRNA_{B2}$
1	<0.02	1.21±0.13	2.11±0.18
3	<0.02	1.41±0.15	2.49±0.52
8	<0.02	1.06±0.20	1.61±0.32
21	<0.02	0.32±0.05	1.11±0.03
60	<0.02	0.11±0.03	0.13±0.05

The values represent the specific steady state levels of mRNA at each time point, as determined by quantitative Northern blots. Means and standard errors of the means are shown from three independent assays, expressed relative to the specific mRNA steady state levels measured with RNA extracted from PYS2 cells (Kücherer-Ehret *et al.* 1990*b*). The limit of detectability using this assay was 0.02.

Table 2. *Developmental changes in amount and extractability of immunoreactive laminin in mouse sciatic nerve*

Postnatal age (days)	Amount of laminin per nerve (ng)	Percentage extractable	Percentage nonextractable
1	0.21	88	10
4	0.67	70	27
10	0.98	61	35
20	2.1	9	89
60	10.1	7	91

Immediately after dissection the nerves were placed in 5 volumes ice-cold Tris-buffered saline, pH 7.5, containing 2 mM PMSF. They were then homogenised using ground-glass homogenisers, and aliquots of the homogenate taken for determination of the total amount of laminin. The remaining homogenates were centrifuged (15 000 ***g*** for 15 min at 4°C), the supernatants removed and the pellets dissolved by boiling in 0.5 % SDS containing 2 mM 2-mercaptoethanol. The immunoreactive laminin was then estimated in all fractions by dot-immunoblots using purified mouse EHS tumour laminin as the standard (Kücherer-Ehret *et al.* 1990*b*). All samples including the laminin standard were treated before blotting by boiling in the presence of SDS and 2-mercaptoethanol. Means of two independent experiments are shown, the values differing by less than 8 %.

fibres (Kücherer-Ehret *et al.* 1990*a*), this association is extremely weak or even artifactual, possible arising from the tissue fixation procedures used in histochemistry: most of the laminin is freely extractable from unfixed tissue into physiological buffer solutions (Table 2). Similarly, it has been noted that the laminin and other basement membrane components are easily extractable from a variety of embryonic tissues, indicating that the phenomenon of soluble laminin is likely to be widespread throughout the tissues of the embryo (Dohrmann *et al.* 1986; Dziadek and Mitrangas, 1989).

Concluding discussion

One consequence of the change in levels of laminin gene expression during development is that this brings about an effective change in laminin distribution from the mesenchyme or interstitial extracellular matrix, to the basement membrane. Thus neural cells (and those of other tissues) have extensive access to laminin during development, although they have little or no contact with basement membranes in the adult. Furthermore, because the laminin is not sequestered into basement membranes during development, then other cell binding sites on the molecule might be able to interact with their receptors, such sites being masked within the basement membrane in the adult (Schittny *et al.* 1988; Abrahamson *et al.* 1989). Finally, laminin located outside basement membranes is more likely to be susceptible to proteolysis (see Dziadek and Mitrangas, 1989), giving rise to the possibility that cryptic cell binding sites within the molecule may be activated (Nurcombe *et al.* 1989).

Given the presence of laminin variants which are likely to have biologically active sites different from, or in addition to, EHS tumour laminin, then it is clear that laminin has a greater potential to influence the behaviour of neural and other cells during development than might be expected from its structures and distribution in the adult.

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