An inhibitor of neurite outgrowth produced by astrocytes

Linda C. Smith-Thomas1, Juin Fok-Seang1, James Stevens1, Jian-Sheng Du1, Elizabeth Muir1, Andreas Faissner3, Herbert M. Geller4, John H. Rogers1 and James W. Fawcett1,2,*

1Physiological Laboratory, University of Cambridge, Downing St, Cambridge CB2 3EG, UK
2MRC Cambridge Centre for Brain Repair, Cambridge, UK
3Neurobiology Department, University of Heidelberg, Germany
4Department of Pharmacology, University of Medicine and Dentistry of New Jersey, USA

*Author for correspondence at address 1

SUMMARY

We have produced a number of astrocytic cell lines, some of which promote abundant neurite outgrowth, some of which are poor promoters of neurite outgrowth. The critical difference between these lines lies in the extracellular matrix, cell lines that are good promoters of axon growth producing a matrix that promotes axon growth, cell lines that are poor promoters of axon growth producing a non-permissive matrix. We were unable to find any consistent correlations between promotion of axon growth and production of proteases, protease inhibitors, N-cadherin, growth cone collapsing activity, and several extracellular matrix molecules. In the present study we have compared the least permissive of our cell lines, Neu7, with the most permissive, A7. Medium conditioned by the cell lines has the same properties as the matrix, since dorsal root ganglia (DRGs) grown in conditioned medium from the Neu7 line grow axons poorly, while DRGs grown in medium conditioned by A7 or primary astrocytes grow many long axons. Since matrix produced by all the cell lines contains large amounts of laminin, we looked to see whether the cells were producing laminin-blocking activity. Medium from the Neu7 line blocked laminin, while that from the A7 and primary astrocytes did not. However, when the conditioned media were heat-treated to remove neurite-promoting activity, they all had laminin-blocking activity: the blocking activity is heat stable. The neurite-promoting properties of the conditioned media therefore probably reflect a balance between promoting molecules and blockers. The laminin-blocking activity could be reduced by treatment of the heat-treated conditioned media with trypsin, keratanase, chondroitinase ABC, but not chondroitinase AC or heparitinase. Fractionation of the conditioned medium on an ion-exchange column revealed that the laminin-blocking activity was found in the sulphate-labelled fractions, which are predominantly proteoglycan. Whole Neu7 extracellular matrix was treated with enzymes, and its neurite-promoting activity could be increased by chondroitinase ABC and to a lesser extent by keratinase, but not by heparitinase. We conclude that the critical difference between matrix produced by astrocytic cell lines that promote axon growth and those that do not lies in the level of production of a dermatan/keratan sulphate proteoglycan.

Key words: astrocyte, extracellular matrix, proteoglycan, laminin, axon regeneration

INTRODUCTION

Most axons in the adult vertebrate central nervous system (CNS) fail to regenerate following injury. This failure is partly due to the CNS environment being inhibitory to axonal growth. Within this environment, both astrocytes and oligodendrocytes have inhibitory or non-permissive properties (Schwab et al., 1993; Fawcett et al., 1989; McKeon et al., 1991).

In order to investigate the inhibitory properties of astrocytes on axon growth, we have developed a number of clonal astrocytic cell lines that vary in the degree to which they promote axon growth (Fok-Seang et al., 1994). From these we chose to characterise four cell lines, which cover the spectrum from non-permissive to permissive. The aim of this exercise was to identify molecular properties of the cells that corresponded to their axonal growth-promoting characteristics. We studied most of the molecules and activities that have been implicated in the promotion of axon growth by astrocytes: N-cadherin, proteases, protease inhibitors, growth cone collapsing activity, laminin, fibronectin, tenascin, and CS56 antibody staining for chondroitin sulphate proteoglycans. However, we were unable to find consistent differences across the four cell lines that correlated with their axon growth-promoting activities. The main correlation we saw was an anatomical one; the cells that were poor neurite outgrowth promoters could be seen in aggregate cultures to be producing large amounts of matrix, and also to be forming more compact aggregates with little extracellular space. When the cells were lysed from the culture surface a layer of extracellular matrix remained, and these matrices were then tested for their ability to promote axon growth. We found that matrix from cell lines that were poor promoters of axon growth was itself a poor outgrowth promoter, while the matrix...
from the permissive lines was a good promoter of growth. Taking this result together with the lack of correlation between ability to promote axon outgrowth and other specific molecules, we concluded that the properties of the extracellular matrix were the main determinant of the ability of the different cells to promote axon outgrowth.

Since the matrix produced by all the cells contains large amounts of the neurite outgrowth-promoting molecule laminin, we considered that the inhibitory cell lines were probably producing an inhibitory molecule that was able to block the outgrowth-promoting effects of laminin and probably other growth-promoting molecules as well.

In the experiments reported in this paper, we have concentrated on the most inhibitory and the most permissive of the cell lines described in the previous paper, Neu7 and A7. We demonstrate that Neu7 cells secrete an inhibitor of neurite outgrowth, which is also produced in smaller amounts by primary cultures of cortical astrocytes. We make a preliminary identification of this inhibitor as a dermatan/keratan sulphate proteoglycan.

**MATERIALS AND METHODS**

**Tissue culture**

Dorsal root ganglia (DRGs) were obtained from P1 or P2 rats. Production and characterisation of A7 cells has been described previously (Geller and Dubois-Dalcq, 1988). The production and characterisation of the Neu7, T34.2 and 27A1 cell lines is described by Fok-Seang et al. (1994).

In order to assay neurite outgrowth, whole DRGs were placed on the tissue culture surface of 35 mm Petri dishes, then covered with a thin layer of medium, so that the tissue was held down by surface tension. On the following day, the medium was topped up to a total of 2 ml.

**Production and use of conditioned medium**

A7, Neu7, T34.2 or 27A1 cells were maintained in DMEM with 10% foetal calf serum (FCS). Cortical astrocytes were grown from cerebral cortex taken on the day of birth, dissociated in trypsin, and cultured in DMEM +10% FCS. Oligodendrocytes and microglia were removed by shaking (McCarthy and de Vellis, 1980). To produce conditioned medium, the serum-containing medium was removed and then the cells were incubated in ITS+ serum-free medium (DMEM with ITS+ (insulin transferrin and selenium with linoleic acid/albumen; Collaborative Research) and antibiotics) for 2 to 3 days. In some experiments, conditioned medium (CM) was heated to 90°C for either 20 or 60 minutes. Some conditioned medium was concentrated using a Diaflo ultrafilter with a membrane with a 3000 molecular mass cut off.

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**Growth of neurites on extracellular matrix**

Astrocyte cell lines were cultured on plastic in DMEM + 10% FCS for 3 to 5 days. The cells were then washed twice with Hanks’ balanced salts solution (HBSS) and treated with distilled water twice for 20 minutes each, usually with 0.2% Triton X-100, followed by vigorous washing off of cellular debris with a Pasteur pipette. The matrix preparations were then washed twice with HBSS and DRGs were cultured in a thin film of serum-free medium with NGF and AraC for 48 hours.

**Enzyme treatment of conditioned medium**

Neu7 or primary astrocyte conditioned medium was heated to 90°C for 20 or 60 minutes and then treated with either chondroitinase ABC at 0.2 unit/ml, heparitinase at 1 unit/ml, trypsin at 100 µg/ml, chondroitinase AC at 0.1 unit/ml or keratanase at 1.25 units/ml (all from Sigma) at 37°C either for 3 hours or overnight. Trypsin activity was stopped by adding an equal amount of soybean inhibitor or by heating to 90°C for 20 minutes, and GAG lyase treatment was either stopped by heating to 90°C, or treated CM was used with the enzymes still active. The control comprised heated Neu7 CM mixed with a similar volume of PBS to that in which the enzymes were added; 1 ml of the enzyme-treated CM was then preincubated with 1 ml of laminin solution in a plastic dish overnight at 37°C. The laminin solution was then removed and DRGs were cultured in ITS+ with NGF and AraC for 48 hours.

**Enzyme treatment of extracellular matrix**

Extracellular matrix preparations were made in 35 mm dishes from Neu7 cells as described above. Chondroitinase ABC, heparitinase, and keratanase in PBS at the concentrations above were put on the matrix for 4 hours at 37°C, then the preparation was washed with HBSS, and DRGs plated onto it in serum-free medium with NGF and AraC.

**Ion exchange chromatography of Neu7 conditioned medium**

Neu7 CM was labelled with 35SO4 by growing Neu7 cells in medium containing radioactive sulphate (50 µCi/ml) for 2 days; 5 ml of radioactively labelled medium and 20 ml of unlabelled Neu7 CM were collected. The labelled and unlabelled CM were then heated up to 90°C for 25 minutes and 1 ml of radioactively labelled heated Neu7 CM was applied to a 10 ml Sephadex G25 column pre-equilibrated with 50 mM Tris-HCl, pH 7.5, + 0.5% Triton X-100 (buffer A), to remove unincorporated radioactivity. The radioactive fractions from the Sephadex G25 column, which came through the column unretracted and therefore contained radiolabelled macromolecules, were collected and 150 µl of this was mixed with 15 µl of unlabelled heated Neu7 CM that had been dialysed against two changes of buffer overnight at 4°C. This mixture was then applied at room temperature to a DE52 anion exchange column, which had been preequilibrated in the same buffer. The column was first washed with buffer A (fractions 1 to 32, Fig. 9 were eluted in this), then buffer A + 8 M urea (fractions 33 to 49 were eluted in this) followed by a salt gradient (0-0.5 M NaCl) in the same buffer (fractions 50-123 were eluted in this). Fractions 124-130 were eluted in 1 M NaCl wash. 100 µl of each 1 ml fraction was counted in a scintillation counter, and assayed for protein content. Fractions were then pooled, dialyzed against 5 mM ammonium bicarbonate, freeze-dried and reconstituted in DMEM.

The ability of the fractions to block the neurite-promoting activity
of laminin was assayed by mixing 35 µl of fraction with 950 µl DMEM, and adding this to 1 ml of 1 mg/ml laminin in a 35 mm dish overnight at 37°C, then removing the mixture, and plating DRGs in serum-free medium with NGF and AraC in the dish for 48 hours.

Quantification of results
The number of axons emanating from each DRG was counted and the length of the 5 longest axons was recorded using a calibrated linear graticule for each DRG. The average length of the 5 axons was taken as the length of axon outgrowth for each DRG. This figure was used rather than one obtained by choosing axons at random because the axons fasciculate, and therefore the shorter axons cannot reliably be distinguished. DRGs with less than 5 axons were not counted. An estimate of the total axon length for each DRG was then calculated by multiplying the number of axons by the average axon length. This gives a figure slightly larger than the actual total, but the halo of axons surrounding a DRG are all very similar in length, and this figure produces the most reliable estimate. Some results were plotted directly as axon length, some were calculated as a percentage of control cultures.

RESULTS

Neurite outgrowth on extracellular matrix
Extracellular matrix was prepared by water lysis of monolayer cultures. As described by Fok-Seang et al. (1994), total axon outgrowth was much less on Neu7 matrix than on A7 matrix, indicating that the matrix retained the permissive or non-permissive properties of the cells. Addition of detergent (Triton X-100) to the water, in order to ensure removal of any remaining membrane (as was done routinely) made no difference, neither did treatment of the matrix for 4 hours with 2 M NaCl in order to remove molecules such as basic fibroblast growth factor, which might have bound to the glycosaminoglycan (GAG) chains (see Fig. 7) (Lillien et al., 1990). Immunostaining of the matrix remaining after lysis of the cells revealed that, although nothing could be seen under phase contrast, there was a matrix footprint left behind by each lysed cell, and that this remaining matrix contained considerable amounts of laminin (Fig. 1A and B). This result indicates that Neu7 cells secrete the neurite-promoting molecule laminin into the matrix, and suggests that they also produce an extracellular matrix molecule that is an inhibitor of the neurite outgrowth-promoting properties of laminin.

The effects of conditioned medium on neurite outgrowth
DRGs were grown in medium conditioned by either the A7 or Neu 7 cell line or by primary astrocyte cultures. Serum-free medium was conditioned for 2-3 days, mixed in equal amounts with fresh NGF-containing medium, and the DRGs were plated on polylysine-coated dishes in this medium. DRGs grown in Neu7 conditioned medium had a much smaller total axon length than those grown in A7 conditioned medium, having fewer, shorter, axons. However, we saw no difference in growth cone morphology in the two different conditioned media. These results are summarised in Fig. 2. Some DRGs did not attach or had less than 5 axons and these DRGs were not counted; many more DRGs did not attach or had less than 5 axons in Neu7 compared to A7 CM. Despite using serum-free medium and AraC, Schwann cells and fibroblasts migrated out of the DRGs. Axons were rarely ahead of the Schwann cell halo when DRGs were grown in Neu7 CM, but were always well ahead when DRGs were cultured in A7 CM (Fig. 3A and B). The conditioned media could be stored at −70°C for several
weeks and at 4°C for at least 2 weeks, and still gave the same results.

In order to see whether the behaviour of the conditioned medium was affected by heat, A7 or Neu7 CM were heated to 90°C for 20 minutes and DRGs were grown in the heated CM mixed 50:50 with fresh medium containing NGF and AraC, on polylysine-coated plastic. There was no neurite outgrowth from any of the DRGs. We assume that this was because all the neurite-promoting molecules such as laminin were destroyed by heat, or heating had made an inhibitory degradation product.

**Effects of conditioned media on the neurite-promoting activity of laminin**

Since matrix produced by the Neu7 cell line contained large amounts of laminin, we assumed that there must also be molecules present that were able to block its neurite-promoting effects. In order to assay for this, we mixed conditioned media with laminin, and allowed the mixture to attach to the surface of polylysine-coated dishes, into which were subsequently placed DRGs and fresh serum-free medium with NGF and AraC.

When DRGs were grown in such dishes preincubated with laminin mixed with A7 or cortical astrocyte CM, the amount of axon growth was similar to that of DRGs grown on dishes coated with laminin alone. However, when DRGs were grown on laminin preincubated with Neu7 CM, the total axon length was significantly less (Fig. 4). The same overall result was observed if laminin was applied to the dish first, then conditioned media were added over the laminin overnight. These results indicate that Neu7 CM contains an inhibitor of laminin neurite-promoting activity.

When A7 CM or cortical astrocyte CM were heated to 90°C for 20 minutes, all were able to inhibit laminin neurite-promoting activity.
promoting activity; presumably the neurite outgrowth-promoting activity of the CM, much of it due to laminin, had been destroyed, leaving behind only the inhibitory activity. Heated laminin by itself had no blocking activity. Neu7 CM is inhibitory even when not heated but A7 and cortical astrocyte CM are not. However, when A7 CM and cortical astrocyte CM are heated to 90°C for 20 minutes they inhibit LN neurite-promoting activity. (ITS, n=20; ITSh, n=8; A7, n=18; A7h, n=15; N7, n=8; N7h, n=7; ca, n=8; cah, n=8.)

The inhibitory activity in Neu7 CM could be diluted out. A considerable reduction in inhibitory activity was observed at a 1:5 dilution and most of the activity was diluted out by a 1:50 dilution.

**Inhibitory conditioned medium can block the neurite-promoting effect of A7 conditioned medium**

Our results indicate that the neurite-promoting activity of astrocyte conditioned medium depends on a balance between promotors such as laminin (Lander et al., 1985) and inhibitors. Neu7 CM probably contains an excess of inhibitor over promotors, while A7 CM probably contains an excess of neurite-promoting activity. In this series of experiments, therefore, we see whether Neu7 conditioned medium can cancel out the neurite-promoting activity in A7 conditioned medium.

In the first part of this experiment, A7 CM was mixed with either A7 CM heated, Neu7 CM, Neu7 CM heated, cortical astrocyte CM or cortical astrocyte CM heated. This mixture was then left for 4 hours at room temperature. DRGs were then grown in a 50:50 mixture of the conditioned medium mix and serum-free medium containing NGF and AraC. The results of this experiment are summarised in Fig. 5. Numbers of DRGs are low in heated CM experiments as many became detached, or had fewer than 5 axons, and were therefore not counted. All of the media inhibited the growth of DRGs in A7 medium relative to A7 medium alone (control). Thus, even when not heated, Neu7 and to a lesser extent cortical astrocyte CM are able to block the neurite-promoting activity in A7 CM. When A7, Neu7 or cortical astro CM are heated, there is a larger reduction of the permissive effect of A7 CM on neurite outgrowth, with heated Neu7 CM being the most inhibitory, then cortical astrocyte CM and then A7 CM.

The experiment was repeated, with the variation that the test CM and the A7 CM were mixed, left on polylysine-coated plastic overnight at 37°C, then removed and replaced with fresh serum-free medium with NGF and AraC, in which DRGs were grown. The results, shown in Fig. 6, are very similar to those obtained when the conditioned media were left in place for the duration of the experiment.

These results indicate that the permissive and inhibitory cell lines and normal cortical astrocytes all produce an inhibitor of A7-promoted neurite outgrowth but the amount of the inhibitor produced varies according to how permissive or inhibitory the cells are to neurite outgrowth.

**Treatment of Neu7 extracellular matrix with enzymes**

Treatment of Neu7 matrix with chondroitinase ABC significantly increased the neurite-promoting activity of Neu7 matrix with total axon outgrowth being increased by a factor of 4 over control. Keratanase also increased outgrowth, but to a lesser extent, while heparitinase was without effect (Fig. 7). This suggests that the neurite-promoting molecules in Neu7 extracellular matrix are blocked by chains containing chondroitin sulphate or dermatan sulphate and keratan sulphate.

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**Fig. 4.** The effects of conditioned media on the neurite-promoting activity of laminin. Laminin and CM were mixed together and allowed to condition the surface, then DRGs were grown on the surface in fresh medium. ITS, unconditioned serum-free medium; ca, cortical astrocyte conditioned medium; h after the name indicates that the medium was heat-treated. Neu7 CM is inhibitory even when not heated but A7 and cortical astrocyte CM are not. However, when A7 CM and cortical astrocyte CM are heated to 90°C for 20 minutes they inhibit LN neurite-promoting activity. (ITS, n=20; ITSh, n=8; A7, n=18; A7h, n=15; N7, n=8; N7h, n=7; ca, n=8; cah, n=8.)

**Fig. 5.** The neurite-promoting activity of A7 CM can be blocked by other CMs. The DRGs were grown in the presence of the mixed CMs on a polylysine-coated surface. All of the media inhibited the growth of DRGs in A7 medium relative to A7 medium alone (control). When A7, Neu7 or ca CM are heated, there is a larger reduction of the permissive effect of A7 CM on neurite outgrowth. Heated Neu7 CM is the most inhibitory. (A7, n=15; A7/A7h, n=11; A7/N7, n=15; A7/N7h, n=11; A7/ca, n=7; A7/cah, n=8.)
Treatment of conditioned medium with enzymes

Treatment of heated Neu7 CM with either chondroitinase ABC (0.2 units/ml), chondroitinase AC (0.1 unit/ml) or keratanase (1.25 units/ml) reduced the blocking effect of Neu7 CM on the axon growth-promoting effect of laminin. Keratanase removed almost all the blocking activity, chondroitinase ABC removed about one third of the blocking activity, and chondroitinase AC had a small but not statistically significant effect (Student’s t-test). Incubation of heated Neu7 CM with heparitinase had no effect whereas trypsin removed about half the activity. The effect of chondroitinase AC was not significant.

Neu7 CM. The proteolytic enzyme trypsin removed about half the blocking effect in Neu7 CM. These results are summarised in Fig. 8. This spectrum of activity would indicate that the inhibitory activity lies in a keratan/dermatan sulphate proteoglycan, possibly with some chondroitin sulphate. The sensitivity to trypsin suggests either that the GAG chains have to be held in a particular conformation in order to inhibit, or that there is some direct activity in the protein components. Essentially identical results were obtained with heat-treated conditioned medium from cortical astrocytes, indicating that the proteoglycan secreted by the Neu7 line is not a peculiarity of this line alone, but is secreted by astrocytes in general.

Incubation of pure chondroitin sulphate GAGs (Sigma) with laminin overnight, at concentrations of 10 µg/ml, 20 µg/ml or 40 µg/ml actually increased the growth of axons from DRGs considerably, as has been reported previously (Lafont et al., 1992). We found that the axon outgrowth (length × number of axons) from the DRGs was increased by a factor of 1.8 to 1.9 in cultures treated with chondroitin sulphate GAGs at all three concentrations.

Ion exchange chromatography of Neu7 conditioned medium

Ion exchange chromatography was performed on heated Neu7 conditioned medium, some of which had been labelled with 35S, and in the presence of 8 M urea so as to allow clean separation of the proteoglycan fraction. Almost all the sulphate-containing material secreted by cells is likely to be proteoglycan. Most of the protein eluted in a single peak at 0 to 0.1 M NaCl. Then one broad peak of radioactivity eluted at 0.25 to 0.5 M NaCl. The protein peak was well separated from the peak of radioactivity. Fractions were pooled as follows.

When the fractions were tested for their ability to inhibit laminin neurite-promoting activity, fractions 1, 2, and 9 were permissive but fractions 3 to 8 all exhibited some degree of inhibition. All of these except fraction 3 lay within the peak of radioactivity. These results are summarised in Fig. 9. Similar results were obtained in two other fractionations. This result demonstrates that the most inhibitory fractions lie within the proteoglycan peak.

**DISCUSSION**

We have compared the extracellular matrix made by the astrocyte cell line Neu7 on which axons grow poorly, with that made by the highly permissive line A7. As reported elsewhere (Fok-Seang et al., 1994), and summarised in the introduction, extracellular matrix preparations from the two cell lines retain the neurite-promoting or non neurite-promoting nature of the cells. We find that conditioned medium has the same inhibitory or permissive activity as the cells from which it was derived, indicating that at least some of the molecules responsible for governing axon growth on the extracellular matrix are also soluble and secreted. Since the extracellular matrix from both cell lines contains large amounts of the neurite-promoting molecule laminin, we looked to see whether the Neu7 line was producing a laminin-blocking activity, and indeed we found that conditioned medium from the Neu7 line will mask the neurite-promoting activity of laminin.

We have three lines of evidence suggesting that the inhibitory molecule secreted by Neu7 cells is a keratan sulphate/dermatan sulphate proteoglycan. (1) The inhibitory activity is heat stable. (2) The inhibitory activity of conditioned medium is reduced after chondroitinase ABC but not AC digestion and removed by keratanase digestion; whole extracellular matrix is also rendered more neurite promoting by chondroitinase and keratanase treatment. (3) The inhibitory activity fractionates with the sulphated proteoglycan fraction after ion-exchange chromatography.
The blocking activity of Neu7 conditioned medium is also reduced by trypsin digestion, indicating some activity in the protein core, or a need for the sugar chains to be held in a particular conformation by the protein core. Pure chondroitin sulphate has no inhibitory activity.

It is possible that more than one inhibitory proteoglycan is being produced. Keratanase treatment of Neu7 conditioned medium renders it completely non-inhibitory, while chondroitinase only has a partial effect. However, enzyme treatment of whole matrix shows chondroitinase having a greater effect than keratanase. This may indicate that there is a mixture of inhibitory molecules, one of which is predominant in conditioned medium, another predominant in whole matrix.

The inhibitory activity is not just made by the Neu7 cell line, but also by the permissive A7 line, and by primary cultures of cortical astrocytes. We have demonstrated this by heat treating the conditioned medium from the cells, so as to inactivate laminin and other neurite outgrowth-promoting molecules. Heated conditioned medium from all three cell types does not promote axon growth, and also has the ability to mask the neurite-promoting activity of laminin. The laminin-blocking effect in Neu7 CM and in primary astrocyte CM is sensitive to keratanase and chondroitinase, and is probably therefore due to the same proteoglycan in the two types of cell. It is probable that the difference between the various cells lies in the quantity of the inhibitory proteoglycan produced, Neu7 producing the most, A7 the least, with cortical astrocytes producing intermediate amounts. In the Neu7 line there is more inhibitory activity than neurite-promoting activity, while in A7 there is insufficient activity to mask the neurite-promoting molecules in the extracellular matrix.

A number of studies have demonstrated that proteoglycans can influence axon growth either positively or negatively. There is also evidence that various chondroitin, dermatan and keratan sulphate proteoglycans are inhibitory to neurite outgrowth in vitro, and some are positioned anatomically so as to exclude growing axons from areas of the developing nervous system, thus guiding them into appropriate pathways (Cole and McCabe, 1991; Fichard et al., 1991; Grumet et al., 1993; Oohira et al., 1988, 1991; Snow and Letourneau, 1992; Snow et al., 1990a,b; Hoffman et al., 1988; Rauch et al., 1991; Brittis et al., 1992; Oakley and Tosney, 1991). Identification and characterisation of the large number of different proteoglycans, many of them developmentally regulated (Herndon and Lander, 1990; Ruoslahti, 1988; Margolis and Margolis, 1993), is still at a relatively early stage.

There are a number of reports suggesting that astrocytes can make inhibitory proteoglycans. Fractionated astrocyte conditioned medium has been found to contain a number of proteoglycans of heparan sulphate, chondroitin sulphate, and dermatan sulphate classes, the heparan sulphate proteoglycan being neurite-promoting, the others inhibitory (Ard and Bunge, 1988; Johnson-Green et al., 1991, 1992; Dow et al., 1993). Bovolenta et al. (1993b) have extracted heparan/chondroitin sulphate proteoglycan from injured brain, while astrocytes from injured adult brain have been shown to have on their surface CSPG, together with tenascin (McKeon et al., 1991; Pindzola et al., 1993). Groves et al. (1993) report CSPG and HSPG on the surface of an immortalised astrocyte line on which axons grow poorly. The inhibitory proteoglycan or proteoglycans made by our astrocyte cell lines may be the same as some of those identified by Dow et al. (1993). However, it differs from that reported by Bovolenta et al. (1993a,b) in being resistant to heparinase. Muir et al. (1989) report a proteoglycan produced by Schwannoma cells, which has a biological activity very similar to that produced by our astrocyte line, but again, this activity was sensitive to heparinase, while our activity is not. A number of other CNS proteoglycans have been identified, none of which appear to be the same as is produced by our astrocytes, either because of different biological activity, being produced by different cell types or having different GAG lyase sensitivity. These molecules include astrochondrin (Streit et al., 1993), NG2 (Stallcup and Beasley, 1987), 1D1, 3F8 (Grumet et al., 1993; Rauch et al., 1991), the various CSPGs extracted by Oohira et al. (1991), Cat-301 (Zaremba et al., 1990), and 473 (Faisson, 1988).

While there is general agreement that some proteoglycans can inhibit axon outgrowth, there appear to be several mechanisms by which they exert their inhibitory effect. In the majority of reports, enzymatic removal of the GAG chains inactivates the molecules, as in our experiments, indicating that the main activity resides in the chains. However, there are also examples where the core protein left behind after enzymatic digestion of the GAG chains has the same activity as the whole molecule (Oohira et al., 1991; Grumet et al., 1993). In our experiments, as in those of Muir et al. (1989), both digestion of the GAG chains and proteolytic enzymes reduce the activity of the molecule, which might indicate that the GAG chains need to be held in a particular conformation, or anchored by the protein core. The activity of inhibitory proteoglycans may be through blocking other permissive matrix molecules such as laminin, as Muir et al. (1989) and the results in this paper have demonstrated, and also through effects on adhesion molecule interactions (Grumet et al., 1993).

Astrocytes cultured as monolayers are excellent substrates for axonal growth from many types of neurone. This is because they have appropriate adhesion molecules on their surface, and produce an extracellular matrix in which neurite-promoting activity is greater than the inhibitory activity due to the inhibitory proteoglycans. Why, then, are astrocytes in the CNS environment of a three-dimensional tissue?

Part of the explanation may lie with the effects of the geometric arrangement of the cells on local amounts of protease, or availability of adhesion molecules. However, it may be that the inhibitory extracellular matrix proteoglycans of which we have made a preliminary identification are important. In this case, it may be that inhibitory molecules accumulate in high concentration in the small amount of extracellular space in such tissues, or that axons become more susceptible to inhibitory influences when they are having to penetrate the complex environment of a three-dimensional tissue.

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