Effects of Inhibition of Proteoglycan Synthesis on the Differentiation of Cultured Rat Schwann Cells

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Abstract. Schwann cells synthesize two heparan sulfate proteoglycans, one that is a component of the Schwann cell basement membrane and a smaller one that is an integral component of the Schwann cell plasma membrane. To determine the functions of these molecules, Schwann cell–nerve cell cultures were grown in medium containing a specific inhibitor of proteoglycan biosynthesis, 4-methylumbelliferyl-β-D-xyloside. Treatment with 1 mM β-D-xyloside caused a 90% reduction in the accumulation of $^{35}SO_4$-labeled proteoglycans in the cell layer of the cultures. Gel filtration analysis revealed that both the basement membrane and plasma membrane proteoglycans were affected. Inhibition of proteoglycan biosynthesis was accompanied by an inhibition of laminin deposition into extracellular matrix as determined by immunostaining of cultures and by immunoblotting of cell-associated proteins. This occurred even though there was no decrease in the amount of laminin detected in the medium of β-D-xyloside–treated cultures. Deposition of collagen type IV was similarly affected. In addition, there was no myelin produced in β-D-xyloside treated cultures. However, when β-xyloside–treated cultures were supplied with exogenous basement membrane, Schwann cells produced numerous myelin segments. These results indicate that Schwann cell proteoglycans play an essential role in basement membrane assembly, and that the integral plasma membrane proteoglycan is not required for the basement membrane to exert its effects on Schwann cell differentiation.

THE extracellular matrix (ECM)[1] plays an essential role in the development of many tissues, affecting such cellular functions as adhesion, migration, cell shape, proliferation, and gene expression (reviewed by Hay [14]). The ability to purify several of the major molecular constituents of the ECM has made it possible to study their properties in detail, and is beginning to provide an understanding of ECM action at the molecular level.

An example of a cell whose development is dependent upon ECM contact is the Schwann cell. The principal function of Schwann cells is to provide myelin and unmyelinated ensheathment for peripheral nerve fibers. In addition, Schwann cells synthesize a portion of the peripheral nerve connective tissue matrix, including the basement membrane that surrounds each Schwann cell axon unit (4–8, 11, 28, 29). Experiments performed with primary tissue cultures of Schwann cells and nerve cells have demonstrated that contact with this basement membrane by Schwann cells is essential for their ensheathment and myelination of axons (6, 10, 30).

These findings have led to investigations into the biochemical composition of the Schwann cell ECM, with the goal of understanding the mechanism of its effect on Schwann cell differentiation. These experiments have established that Schwann cells synthesize several collagen types (5), including collagen type IV (8), the basement membrane glycoprotein laminin (11), and two heparan sulfate proteoglycans (HS-PGs) (29). Biochemical studies of these HS-PGs have revealed that one is a component of the Schwann cell basement membrane, and the other is an integral component of the Schwann cell plasma membrane (9, 29).

It is of interest to determine the role these HS-PGs play in Schwann cell development. Investigations into the functions of PGs have been hampered by the difficulty of purifying these molecules in an intact, native state. For this reason most studies assessing PG function have relied upon correlating the absence or presence of these molecules with alterations in particular cellular functions. This has been done by correlating changes in PG metabolism with cellular function (2, 41), or by removing PGs, either with enzymes (17) or by using drugs which specifically inhibit PG biosynthesis (26, 34, 38).

We have used 4-methylumbelliferyl-β-D-xyloside as a specific inhibitor of PG biosynthesis to investigate the functions of the Schwann cell HS-PGs. In this paper we present evidence that inhibition of PG biosynthesis in rat Schwann cell–nerve cell primary cultures prevents basement membrane deposition as well as myelin formation by Schwann cells. The inhibition of myelin formation by Schwann cells can be reversed, however, by supplying these cultures with

1. *Abbreviations used in this paper*: ECM, extracellular matrix; HS, heparan sulfate; PG, proteoglycan.

exogenous basement membrane. These results indicate that HS-PGs are required for assembly of the Schwann cell basement membrane. Furthermore, Schwann cells are capable of myelin formation in response to exogenous basement membrane contact even under conditions in which they lack their cell surface HS-PG.

Materials and Methods

Cell Culture

Primary dissociated cultures containing only Schwann cells and sensory nerve cells were prepared by previously published methods (4, 7, 10, 30). Briefly, dorsal root ganglia were dissected from rat embryos, dissociated with trypsin, and seeded onto 2-cm diam Aclar mini-dishes coated with collagen type I (from rat tail tendons). For the first 5–7 d the cultures were fed an antimitotic medium containing 10^{-5} M 5-fluorodeoxyuridine and 10% FCS. The cultures were then maintained in the serum-free medium N2. Schwann cell basement membrane production was initiated by switching the cultures to N2 supplemented with ascorbic acid (50 μg/ml) and BSA (1 mg/ml) (supplemented N2) (Carey, D. J., and M. S. Todd, manuscript submitted for publication). For cultures grown with exogenous basement membrane, dorsal root ganglion explants previously grown for 1 wk oh type I collagen in antimitotic medium were transferred to basement membrane-coated dishes; dissociated cultures grown in N2 medium for 3–4 wk were embedded in basement membrane gel as described earlier (10).

Basal media components were from Gibco Laboratories (Grand Island, NY). Sera were obtained from KC Biologicals (Lenexa, KS); other medium additives were from Sigma Chemical Co. (St. Louis, MO). Solubilized matrix proteins from the Englebreth-Holm swarm sarcoma used to prepare reconstituted basement membrane (20) were generously provided by Dr. Hynda Kleinman, National Institutes of Health.

PG Analysis

PGs were labeled by incubating cultures in Ham's F12 medium supplemented with transferrin (100 μg/ml), insulin (5 μg/ml), and 200 μCi/ml $^{35}SO_4$ (carrier-free, from ICN Radiochemicals, Irvine, CA) as described previously (9, 29). After incubation, culture media were harvested and mixed with protease inhibitors (10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cells and matrix were solubilized in guanidine buffer (4 M guanidine-HCl, 50 mM sodium acetate, 10 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). Unincorporated $^{35}SO_4$ was removed by chromatography on a Sephadex G-50 column eluted with guanidine buffer. Molecules that eluted in the void volume fractions were pooled and an aliquot was counted to determine the total $^{35}SO_4$ incorporation. The pooled fractions were applied to a 1 × 100-cm column of Sepharose CI-4B and eluted with guanidine buffer at a flow rate of 6 ml/h. Alkaline hydrolysis was carried out in 0.05 M NaOH, 1 M $NaBH_4$ at 45° for 16 h. Nitrous acid hydrolysis was in 0.25 M $NaNO_2$, 1.9 M acetic acid at room temperature for 2 h.

Immunofluorescence

Indirect immunofluorescence was carried out on whole-mount preparations of primary cultures as described previously (8, 11). The cultures were rinsed with Leibovitz L-15 medium containing 10% heat-inactivated horse serum and incubated for 1 h at 4°C with rabbit antiserum specific for laminin or collagen type IV (diluted 1:20 in L15-horse serum). The cultures were rinsed and then incubated for 1 h at 4°C with affinity-purified goat anti-rabbit IgG (Sigma Chemical Co.). After rinsing, the cultures were fixed for 1 h at room temperature with 3% paraformaldehyde, mounted on glass slides, and viewed with a Nikon Diaphot inverted microscope (Nikon, Inc., Garden City, NY) equipped for epifluorescence.

Immunoblotting

Aliquots of conditioned culture medium or cells (extracted with 0.0625 M Tris-HCl, 2% SDS, pH 6.8) were subjected to electrophoresis in SDS-polyacrylamide slab gels prepared according to the method of Laemmli (21). After running, the gels were soaked for 30 min in transfer buffer (12.5 mM Tris base, 96 mM glycine) and electrophoretically transferred to nitrocellulose sheets (BioRad Laboratories, Richmond, CA) at a constant voltage of 70 V for 40 min. After transfer the nitrocellulose sheets were soaked for 1 h in blocking solution (BLOTTO, 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5% Carnation nonfat dry milk [16]). The sheets were then incubated overnight at 4°C in first antibody solution (diluted 1:100 or 1:250 in BLOTTO). After rinsing with BLOTTO, the sheets were incubated for 2 hr in a solution containing affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad Laboratories, diluted 1:3,000 in BLOTTO). After rinsing, the bound antibodies were visualized by their peroxidase reaction products with 4-chloro-1-naphthol as substrate.

Antisera

Antisera to laminin and collagen type IV were produced in rabbits by multiple subcutaneous injections of the purified proteins (100–200 μg/injection; obtained from Bethesda Research Laboratories, Gaithersburg, MD) in complete (first injection) or incomplete Freund's adjuvant. Antibody specificity and titer were tested by staining of proteins transferred to nitrocellulose as described above.

Myelin Formation

Myelin formation was assessed by examining the living cultures in a Nikon Diaphot inverted microscope using bright-field optics. As reported previously, structures that can be identified as myelin by immunostaining and histologic staining can be clearly visualized using this method (10).

Results

Effects of 4-Methylumbelliferyl-β-D-Xyloside on Schwann Cell PG Synthesis

To examine the effects of the PG biosynthesis inhibitor on Schwann cells we used primary dissociated cultures of rat Schwann cells and nerve cells which were grown initially (for 4 wk) in N2 medium. Cultures maintained in N2 medium produce a dense network of nerve fibers which become populated with Schwann cells that proliferate in response to nerve fiber contact (30, 35, 36). In this medium, however, Schwann cells do not produce EMC and do not myelinate nerve fibers (7, 29). ECM production and myelination were induced in these cultures by switching them to N2 medium supplemented with ascorbic acid (50 μg/ml) and BSA (1 mg/ml) (supplemented N2) (Carey, D. J., and M. S. Todd, manuscript submitted for publication). This medium switch results in the deposition into ECM of laminin, collagen type IV, and the basement membrane HS-PG (29; Carey, D. J., and M. S. Todd, manuscript submitted); the plasma membrane HS-PG is present in approximately the same levels in both N2 medium and supplemented N2 (29).

To test the effect of the PG synthesis inhibitor on Schwann cell HS-PG synthesis, the drug was added to the culture medium at the time of switching to supplemented N2, i.e., at the time of initiation of basement membrane formation. Sibling cultures switched to supplemented N2 but without inhibitor served as controls. After 1 wk the cultures were labeled with $^{35}SO_4$ for 18 h and the medium and cell-associated $^{35}SO_4$-labeled macromolecules were quantitated and analyzed by gel filtration chromatography.

As shown in Table I, incorporation of $^{35}SO_4$ into cell-associated macromolecules was reduced to 24% of control by 1 mM β-xyloside, whereas incorporation into culture medium macromolecules was increased nearly threefold. These results are consistent with the mode of action of the drug, which is to compete with xylose residues (attached to serines on the core proteins) for glycosyltransferases that add the next sugar of the newly initiated glycosaminoglycan

Table I. Effects of β-Xyloside on $^{35}SO_4$ Incorporation

Compartment*	$^{35}SO_4$ incorporated		Ratio
	Without xyloside	With xyloside	
	cpm/culture		
Culture medium	25,450	68,030	2.7
Cell layer	60,000	14,340	0.24

Groups of duplicate cultures were switched to supplemented N2 medium without or with 1 mM β-xyloside and 1 wk later were labeled for 18 h with $^{35}SO_4$ in medium without or with the drug; incorporated radioactivity was measured as described in Materials and Methods; numbers represent the average of duplicate measurements; this experiment was repeated twice with identical results.

* The culture media were removed and mixed with protease inhibitors; after washing, the cell layers were solubilized in 4 M guanidine-HCl, 0.1% Triton X-100, 50 mM sodium acetate, pH 5, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride.

chains. This results in the synthesis of core proteins lacking glycosaminoglycan and also free glycosaminoglycan bound to the drug (26, 37). The latter are unable to associate with the matrix and accumulate in the culture medium (37).

These conclusions were confirmed by the results of gel filtration analysis performed on Sepharose CL-4B columns under dissociative conditions. The cell-associated material from control cultures was resolved into two major peaks, eluting at K_{av}s of ~0.2 and 0.4 (Fig. 1 *a*). We have shown previously that these represent the basement membrane and plasma membrane HS-PGs produced by Schwann cells (29). These peaks were dramatically reduced in the cell-associated material obtained from cultures treated with 1 mM β-xyloside (Fig. 1 *c*). Quantitation of the amounts of radioactivity

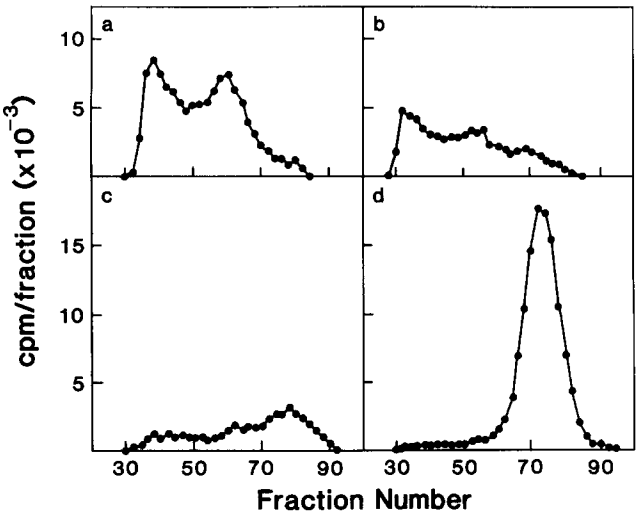

Figure 1. Gel filtration chromatography of radiolabeled macromolecules from control and β-xyloside-treated cultures. Schwann cell-nerve cell cultures were labeled with $^{35}SO_4$ and the cell-associated and culture medium macromolecules were subjected to gel filtration chromatography on Sepharose CL-4B columns as described in Materials and Methods. (*a*) Cell-associated proteins from control cultures; (*b*) culture medium from control cultures; (*c*) cell-associated proteins from cultures treated with 1 mM β-xyloside; (*d*) culture medium from cultures treated with 1 mM β-xyloside. In each case material from an equivalent number of cells was applied to the columns. Recoveries were >85%.

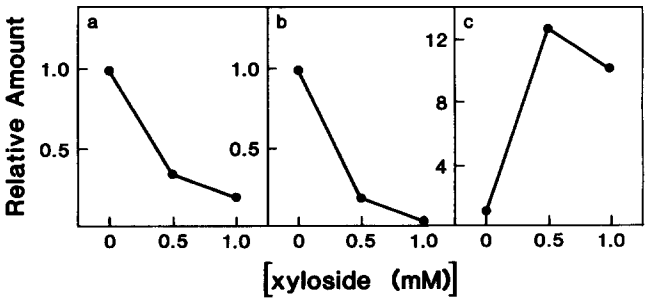

Figure 2. Concentration dependence of β-xyloside effects on PG and glycosaminoglycan accumulation. Schwann cell-nerve cell cultures that had been grown for 1 wk in medium containing the indicated concentrations of β-xyloside were labeled with $^{35}SO_4$ and the cell-associated and culture medium macromolecules were analyzed by gel filtration chromatography as in Fig. 1. All values are expressed relative to control values (without β-xyloside). (*a*) Cell-associated PGs; (*b*) culture medium PGs; (*c*) culture medium glycosaminoglycans.

recovered from the column indicated that these HS-PGs were present at ~10% of their levels in control cultures. The cell-associated material from the β-xyloside-treated cultures contained an additional peak that eluted at a K_{av} = 0.7 that was not present in the control cultures. The elution position of this peak was not altered by treatment with mild base. Digestion of this material with nitrous acid and chondroitin ABCase indicated it contained a mixture of heparan sulfate and chondroitin sulfate chains (not shown). Identical results were obtained in cultures treated with β-xyloside for 2 wk (not shown).

Gel filtration on Sepharose CL-4B of the medium from control cultures resulted in the elution of radiolabeled macromolecules in a broad region with several poorly resolved peaks (Fig. 1 *b*). Additional characterization of these macromolecules indicated they were PGs (data not shown). In medium from cultures treated with 1 mM β-xyloside, these peaks were absent and were replaced by a large sharp peak eluting at K_{av} = 0.7. (Fig. 1 *d*). This peak was insensitive to base hydrolysis but was degraded by nitrous acid, indicating it contained free HS chains (data not shown).

Fig. 2 summarizes the effects of β-xyloside tested at two concentrations on the accumulation of cell-associated and medium PG and medium HS. β-Xyloside inhibited HS-PG accumulation in the cell layer by 70% at 0.5 mM and 90% at 1 mM.

β-Xyloside Inhibits Deposition of Laminin into the Schwann Cell ECM

To assess the effect of inhibition of PG synthesis on the production of basement membrane by Schwann cells, we used indirect immunofluorescence microscopy to compare the deposition of the glycoprotein laminin into ECM of control and β-xyloside-treated cultures. Laminin is a major structural component of basement membranes (39), including the basement membrane synthesized by cultured Schwann cells (11). As in the experiments described above, the drug was added to Schwann cell-nerve cell cultures at the time of switching from N2 medium to supplemented N2.

In control cultures significant accumulation of laminin on the surfaces of the Schwann cells was evident by 4 d after the

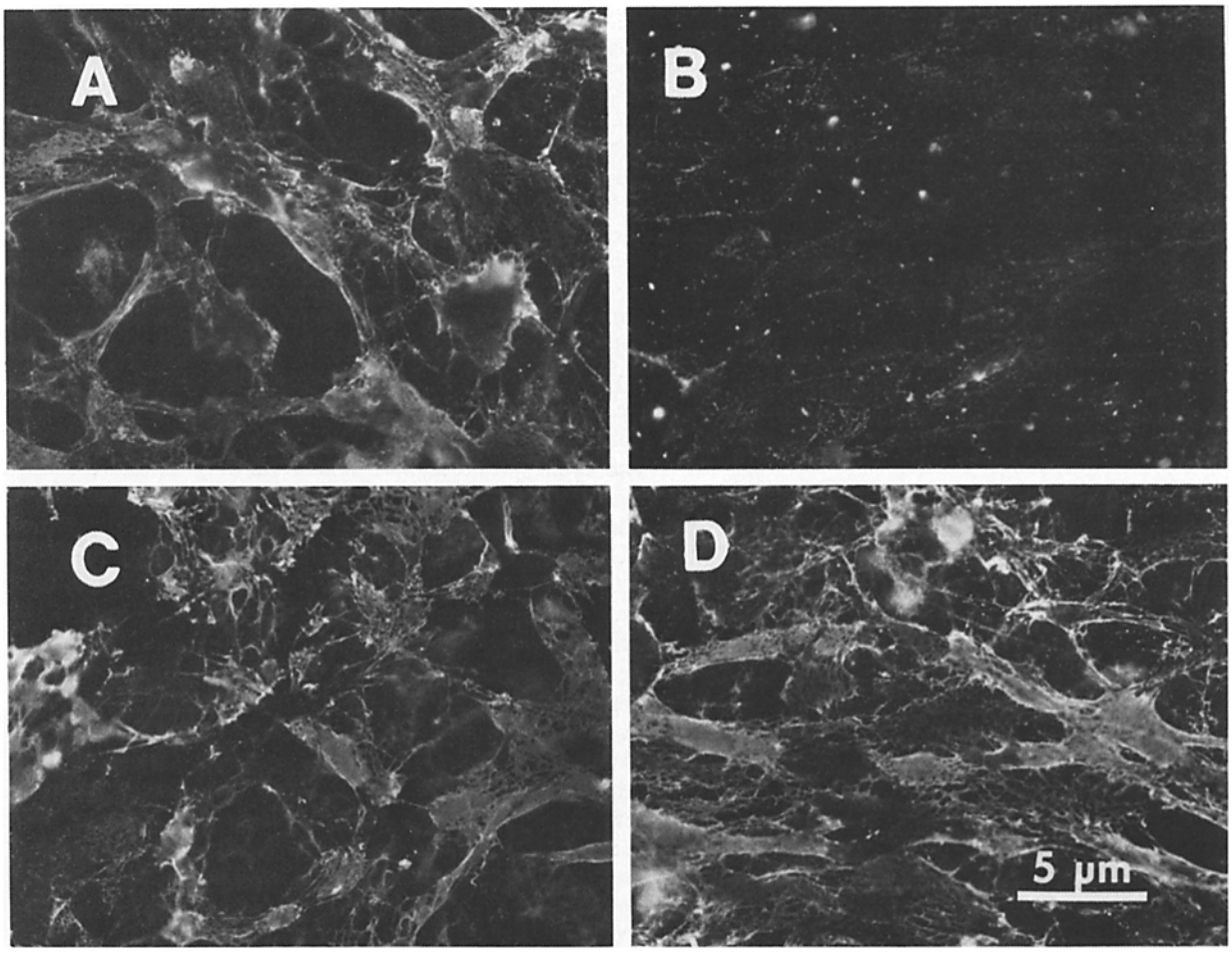

Figure 3. Inhibition of laminin deposition is specific to β-D-xyloside. Schwann cell–nerve cell cultures were switched to supplemented N2 medium containing no additions (*A*) or 1 mM concentrations of 4-methylumbelliferyl-β-D-xyloside (*B*), α-methyl-D-xyloside (*C*), or 4-methylumbelliferyl-β-D-glucoside (*D*). After 4 d the cultures were processed for indirect immunofluorescence with anti-laminin antiserum. The fields shown were photographed and printed under identical conditions.

medium switch (Fig. 3 *A*). By 8 d the cells were covered by a continuous layer of laminin (not shown). In contrast, in cultures given 1 mM β-xyloside, there was almost no laminin detectable on the surfaces of the Schwann cells at 4 d (Fig. 3 *B*); by 8 d some laminin deposition had occurred, but it was sparse and patchy (not shown) in comparison to the control cultures. After 2 wk of β-xyloside treatment, laminin deposition was increased, but still was sparse in comparison to control cultures (not shown).

To demonstrate that this reduced laminin deposition was caused by inhibition of PG biosynthesis, we examined the effects of compounds that were chemically similar to β-xyloside but that do not inhibit PG biosynthesis. As shown in Fig. 3, addition of 1 mM methyl-α-D-xyloside (Fig. 3 *C*) or 1 mM 4-methylumbelliferyl-β-D-glucoside (Fig. 3 *D*) to supplemented N2 did not inhibit the deposition of laminin into the Schwann cell ECM.

The reduced laminin deposition demonstrated by immunofluorescence could have been caused either by an inability of secreted laminin to assemble into the basement membrane or by an inhibition of laminin synthesis and/or secretion as a result of β-xyloside treatment. To determine which of these possibilities was occurring, we measured the amounts of laminin present in the medium and cell layers of cultures 4 d after switching to supplemented N2 with or without 1 mM β-xyloside. This was done by electrophoresing aliquots of medium or cell extracts in SDS-slab gels, electrophoretically transferring the proteins to nitrocellulose, and detecting bound laminin with antibodies.

Consistent with the immunofluorescence data presented above, the immunostained blots of cell-associated proteins showed a marked inhibition by β-xyloside of laminin deposition (Fig. 4). In contrast, the blots of medium proteins showed no apparent differences between the control and β-xyloside–treated cultures. Blots of extracts of cultures treated with β-xyloside for 2 wk showed some laminin accumulation, but much less than was observed in control cultures (not shown). These results indicate that the failure of laminin deposition into ECM is not due to an inhibition of laminin synthesis or secretion.

Treatment with β-xyloside caused abnormal accumulation of free glycosaminoglycans in the culture medium (see Fig. 1). To eliminate the possibility that this caused the observed inhibition of matrix assembly, we grew cultures in supplemented N2 containing 50 ng/ml each of heparin and chondroitin sulfate. This is more than 20 times the calculated concentration of glycosaminoglycan in the medium of β-xyloside–treated cultures. Addition of these molecules had no

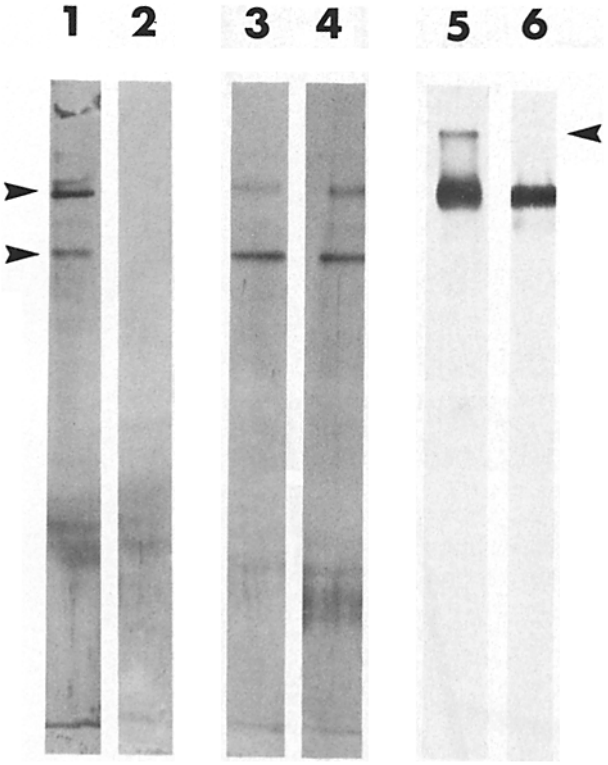

Figure 4. β-Xyloside inhibits laminin deposition but not laminin secretion. Schwann cell–nerve cell cultures were switched to supplemented N2 without (lanes *1* and *3*) or with (lanes *2* and *4*) 1 mM β-xyloside. After 4 d the cell-associated proteins (lanes *1* and *2*) and conditioned culture media (lanes *3* and *4*) were subjected to electrophoresis in 6% polyacrylamide gels, transferred to nitrocellulose, and stained with anti-laminin antiserum as described in Materials and Methods. Lane *5* shows staining of authentic mouse laminin; lane *6* shows staining of rat kidney homogenate, revealing the specificity of the antiserum. The *upper left arrow* indicates the position of migration of the small (M_r = 200,000) subunit of laminin. Under these conditions of transfer and staining the large (M_r = 400,000) subunit of laminin (*right arrow*, lane *5*) is poorly detected. The *lower left arrow* indicates an immunostained band of M_r 150,000 present in the Schwann cell samples that may be a degradation product of laminin.

observable effects on deposition of laminin into the ECM (data not shown).

β-Xyloside Inhibits Accumulation of Collagen Type IV in the Schwann Cell Basement Membrane

The other major structural component of the Schwann cell basement membranes is collagen type IV (8). We conducted experiments to determine whether its assembly into basement membrane was also blocked by β-xyloside. Control cultures examined 1 wk after switching to supplemented N2 exhibited bright, linear extracellular staining with anti-collagen type IV antibodies (Fig. 5). Staining of β-xyloside–treated cultures was significantly reduced, being absent in some areas and punctate or fibrous in other areas. These results indicate that β-xyloside treatment also blocks collagen type IV deposition into basement membrane. Other experiments indicated that secretion of collagen type IV was not inhibited by β-xyloside treatment (not shown).

Effects of β-Xyloside on Schwann Cell Spreading and Myelin Formation

Previous studies have shown that formation of myelin segments around axons by Schwann cells is dependent upon contact with a suitable ECM (6, 10, 30). In culture this is usually the basement membrane synthesized by the Schwann cells (5). Not surprisingly, in β-xyloside–treated cultures there was a complete inhibition of myelin formation 2 wk after switching to supplemented N2, when there were numerous myelin segments present in the control cultures (not shown).

To determine whether β-xyloside treatment had an effect on Schwann cell myelin formation separate from its effects on basement membrane assembly, we grew Schwann cell–nerve cell cultures in N2 medium containing both β-xyloside and exogenous basement membrane (20). We have shown previously that this exogenous basement membrane, which contains laminin, collagen type IV, and basement membrane PG in the proportions 35:1:1, plus small amounts of entactin (20), can substitute for the Schwann cell basement membrane in stimulating myelin formation (10). To ensure that the Schwann cells were depleted of the cell surface HS-PG, the cultures were grown for 2 d in medium containing 1 mM β-xyloside before addition of exogenous basement membrane. Pulse-chase radiolabeling experiments had shown that in cultures grown in N2 medium this HS-PG is degraded with a $t_{½}$ of ~9 h (not shown). Thus, after 48 h <5% of the original HS-PG pool should remain.

Previous studies have indicated that the first visible effect of basement membrane contact is the longitudinal spreading of Schwann cells onto axonal surfaces (7, 10). Light microscopic observation of the cultures revealed that β-xyloside treatment did not inhibit Schwann cell spreading (Fig. 6 *c*). We attempted to quantitate the matrix-induced cell spreading by measuring the diameters of Schwann cell nuclei. The spreading of Schwann cells causes a narrowing of their nuclear diameters perpendicular to the neurite axis (7). The nuclear diameters of control cultures without basement membrane were 21.7 ± 2.6 μm. In cultures with basement membrane the diameters were 10.5 ± 1.9 and 10.9 ± 1.9 μm, without and with β-xyloside. Thus, the response of the Schwann cells to basement membrane contact in β-xyloside–treated cultures was not significantly different from that of Schwann cells in control cultures.

To assess myelin formation, cultures were inspected by bright-field microscopy 2 wk after basement membrane addition. In the presence of exogenous basement membrane, β-xyloside–treated cultures formed numerous myelin segments that were indistinguishable in the light microscope from those present in control cultures (Fig. 6 *d*). Average counts of numbers of myelin segments per field did not differ significantly between control and β-xyloside–treated cultures (not shown).

Discussion

The data presented here have demonstrated that Schwann cell PG biosynthesis can be inhibited 90% by treatment with 1 mM 4-methylumbelliferyl-β-D-xyloside. In our experiments the basement membrane and plasma membrane HS-PGs produced by Schwann cells (9, 29) were equally affected

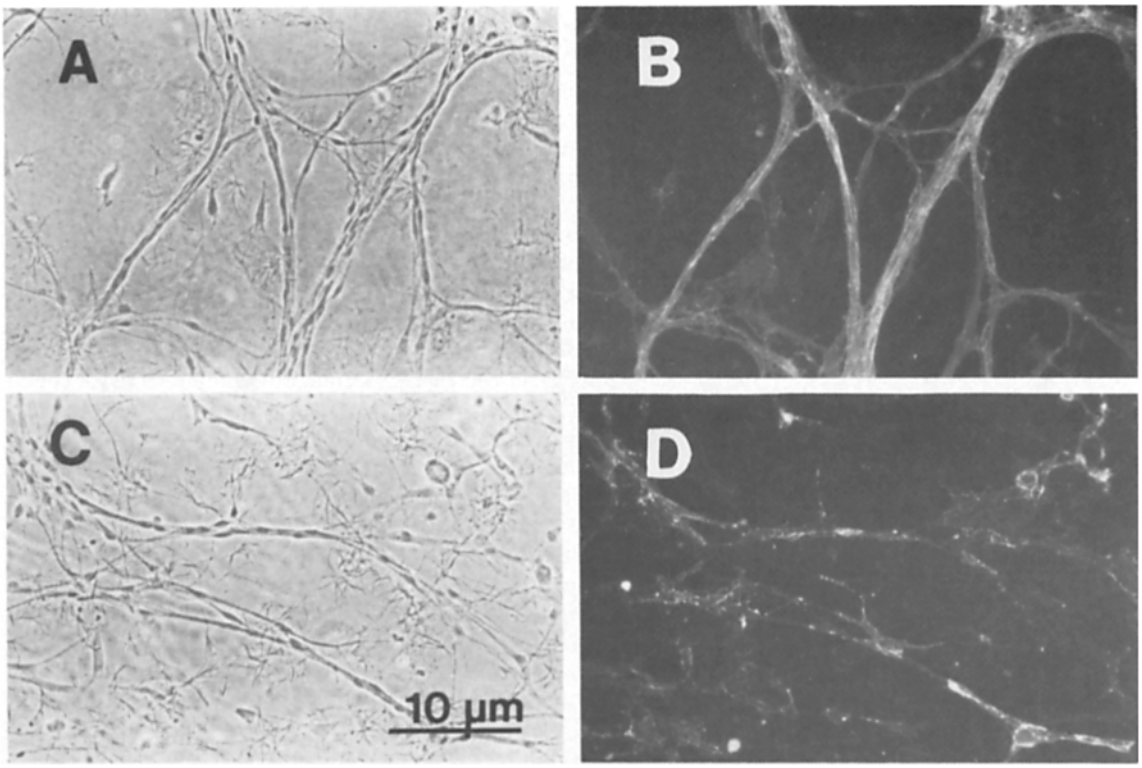

Figure 5. β-Xyloside inhibits collagen type IV deposition. Schwann cell–nerve cell cultures were switched to supplemented N2 without (*A* and *B*) or with (*C* and *D*) 1 mM β-xyloside. After 7 d the cultures were processed for indirect immunofluorescence with anti–collagen type IV antiserum. The fields shown in the figure were photographed with phase contrast (*A* and *C*) and fluorescence (*B* and *D*) optics. The fluorescence images shown in *B* and *D* were photographed and printed under identical conditions.

by this inhibitor. Inhibition of HS-PG biosynthesis resulted in an inability of Schwann cells to assemble the basement membrane they would normally produce in culture (5), which, in turn, resulted in an inhibition of myelin formation by the Schwann cells. That the effect on myelin formation was due to the lack of basement membrane was demonstrated by the observation that cultures given exogenous basement membrane were able to produce myelin in the presence of the inhibitor. The ability to form myelin in the presence of the drug also indicates that cell viability was not seriously affected by the long exposure time (2 wk) needed to observe myelin formation in culture.

Our experimental protocol for studying the effects of the PG biosynthesis inhibitor involved first growing the cultures in the serum-free medium N2. In this medium the nerve cells produce neurites and the Schwann cells proliferate vigorously in response to neurite contact (30, 35, 36). However, under these conditions no ECM material is deposited by the Schwann cells. As a consequence, Schwann cell terminal differentiation, which entails spreading along the neurites and ensheathment and myelination of the axons, does not occur (6, 7, 30). Previous studies have established that these processes do not occur in the absence of basement membrane contact (10). Production of basement membrane by the Schwann cells and subsequent terminal differentiation was initiated by growing the cultures in N2 medium supplemented with ascorbic acid and bovine serum albumin (Carey, D. J., and M. S. Todd, manuscript submitted for publication). By simultaneously providing the inhibitor and supplemented medium to the cultures, we were able to examine the effects of the inhibitor on Schwann cell terminal differentiation without the complications of possible effects on such things as neurite outgrowth or Schwann cell proliferation. This was especially important in light of the recent report demonstrating that the neurite mitogen that stimulates Schwann cell proliferation may be a PG (34). In addition, we were able to investigate effects of inhibition on basement membrane assembly in the absence of preformed extracellular matrix. Because of the presumed cooperative nature of basement membrane assembly (43), the presence of basement membrane on the Schwann cell surface could have complicated the analysis and interpretation of the experiments.

The results presented here provide strong evidence that HS-PGs play an essential role in basement membrane assembly. Current models of basement membrane structure postulate a framework consisting of a molecular lattice formed by collagen type IV molecules onto which other basement membrane molecules attach through specific noncovalent interactions (24, 40, 43). Collagen type IV molecules are capable of spontaneous self-assembly into such a network via noncovalent associations at their amino and carboxyl terminal ends (1, 12, 13). Basement membrane HS-PGs have been shown to bind in vitro to specific sites on both laminin and collagen type IV molecules (24). This raises the possibility that one function of basement membrane HS-PGs is to provide cross-links between collagen and laminin that stabilize the molecular network formed by these two molecules. In the absence of HS-PG, e.g., in β-xyloside–treated cultures, the lack of these cross-links would prevent the formation of stable basement membrane structures. Alternatively, the bind-

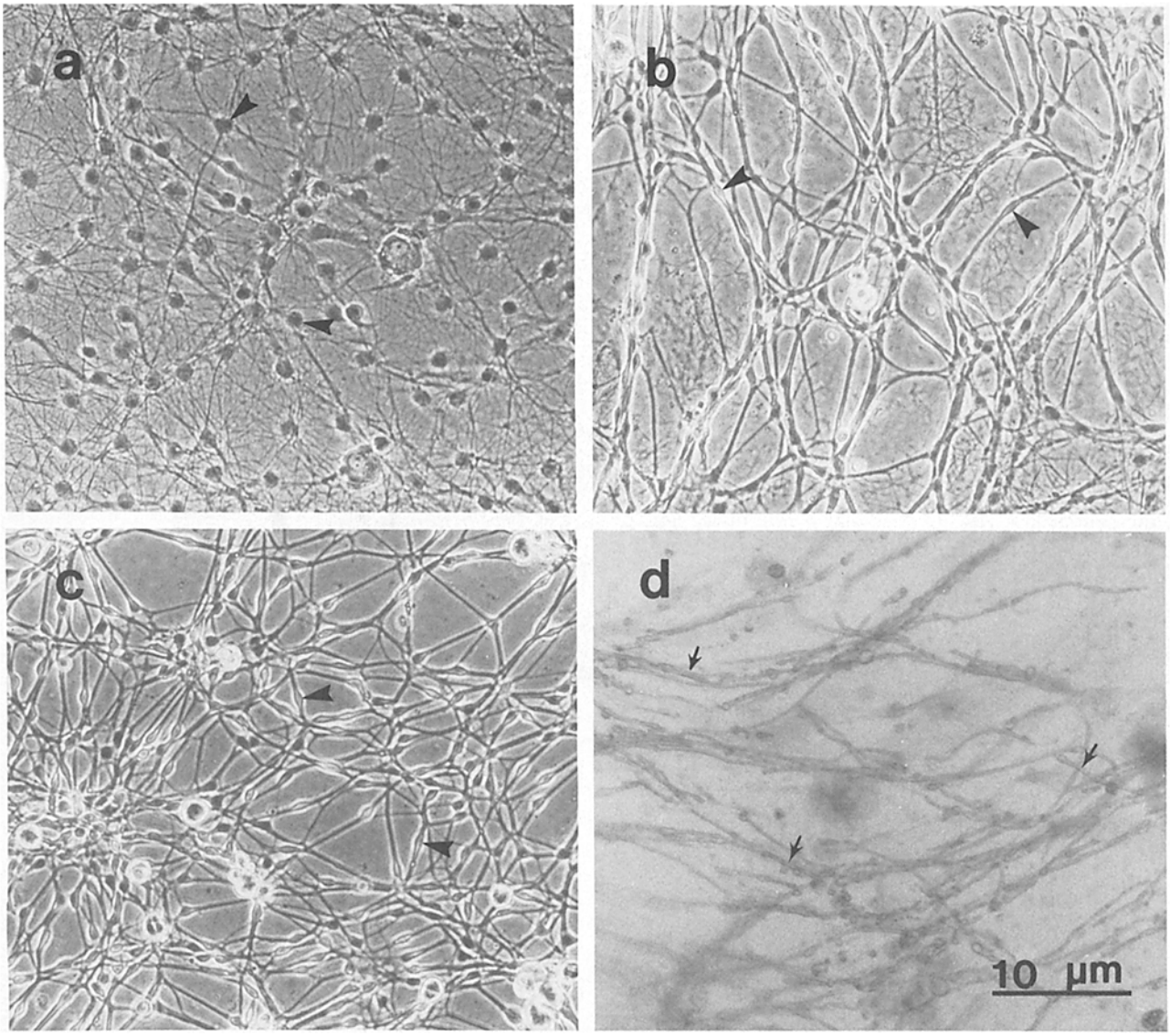

Figure 6. Effects of β-xyloside and exogenous basement membrane on Schwann cells. Schwann cell–nerve cell cultures were grown for 4 wk in N2 medium. Cultures were then given N2 medium alone (*a*), N2 medium plus exogenous basement membrane (*b*), or N2 medium plus 1 mM β-xyloside and exogenous basement membrane (*c* and *d*). The latter cultures were given β-xyloside 2 d before basement membrane addition (see text). Living cultures were photographed after 4 d with phase-contrast optics (*a–c*) or after 2 wk with bright-field optics (*d*). Schwann cell nuclei are indicated by *arrowheads*. Note the change in Schwann cell morphology elicited by the exogenous basement membrane both with and without β-xyloside present. The fibrillar noncellular material visible in *b* is in the collagen type I gel coating the culture dish and does not affect Schwann cell behavior. Schwann cells given exogenous basement membrane form myelin segments (*arrows* in *d*) in medium containing 1 mM β-xyloside.

ing of HS-PG to soluble laminin or collagen type IV could be a rate-limiting step in the assembly process, perhaps inducing a conformational change in these molecules that accelerates their self-assembly. Indirect evidence for an effect of HS-PG binding on laminin conformation comes from the observation that antisera to laminin do not block the biological activity of laminin complexed to HS-PG (22). Finally, HS-PGs may be involved in the attachment of basement membranes to the cell surface. In vivo basement membranes assemble only adjacent to specialized surfaces of epithelial and other cells. The attachment is often assumed to occur via interactions between laminin and specific cell surface receptors (15). A putative laminin receptor has been identified in several laboratories as a M_r = 70,000 cell surface glycoprotein (3, 25, 27, 31). It is conceivable that integral cell surface HS-PGs, which have been identified in some basement membrane producing cells (18, 19, 32, 33), including Schwann cells (9), also function as receptors for laminin or other basement membrane proteins (23). A conclusion as to which of these hypotheses correctly identifies the role of HS-PGs awaits further investigation.

Our results also indicate that the cell surface PG is not required for basement membrane contact to elicit its developmental effects on Schwann cells. Under conditions in which the accumulation of this protein was reduced to 10% of control levels exogenous basement membrane was still able to induce spreading onto neurites and myelin formation by Schwann cells. Previously we had presented evidence that

this HS-PG is tightly but noncovalently associated with the Schwann cell cytoskeleton (9), which suggested a role for this molecule in mediating these developmental effects. Several possible explanations of the data can be considered. The most likely is that the association of this HS-PG with the cytoskeleton has another functional consequence (perhaps related to regulation of basement membrane assembly). Alternatively, it is possible the low numbers of cell surface HS-PGs in β-xyloside-treated cells were sufficient to carry out this signalling function. However, in this case it is likely there would have been quantitative differences in the extent or rate of cell spreading or myelin formation. Such differences, if they existed, were not apparent in our studies. Finally, Schwann cells may have redundant and functionally overlapping sets of receptors for transducing the effects of basement membrane contact on Schwann cell behavior. A definitive answer will require additional investigation into the identity and properties of basement membrane receptors in Schwann cells.

If the cell surface HS-PG does play a role in regulating basement membrane assembly in Schwann cells, then its association with the cytoskeleton could potentially explain the asymmetric nature of this process. In developing peripheral nerves, basement membrane assembly occurs on regions of the Schwann cell surface not contacting axons (42). Nerve cell contact is required, however, since only Schwann cells that are in contact with nerve cells assemble basement membrane (4). Thus, basement membrane assembly, plus the concomitant shape changes induced by basement membrane contact (10) result in the Schwann cell and its ensheathed axon becoming encased within a sleeve of basement membrane (5, 42), and impose a structural and functional polarity on the Schwann cell surface. Obviously, some process must regulate spatially the process of basement membrane assembly. The association of the cell surface HS-PG with the cytoskeleton suggests one mechanism by which specialized cell surface domains could be organized from within the cell in response to specific cues, such as adhesion of the cell to the surface membrane of an axon.

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