Release of autocrine growth factor by primary and immortalized Schwann cells

(cell culture/laminin)

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ABSTRACT Schwann cells derived from neonatal rat sciatic nerve are quiescent in culture unless treated with specific mitogens. The use of glial growth factor (GGF) and forskolin has been found to be an effective method for stimulating proliferation of Schwann cells on a poly(L-lysine) substratum while maintaining their ability to myelinate axons *in vitro*. We find that repetitive passaging of Schwann cells with GGF and forskolin results in the loss of normal growth control; the cells are able to proliferate without added mitogens. The immortalized cells grow continuously in the absence of added growth factor and in the presence or absence of serum yet continue to express distinctive Schwann cell-surface antigens. The cells can associate with axons in culture, deposit a basal lamina, and ensheath axons, but they gradually lose their capacity to myelinate axons. The immortalized cells release growth-promoting activity into their culture medium. The released activity is effective in stimulating proliferation of primary Schwann cells that retain normal growth properties. Extracellular matrix molecules (laminin and fibronectin) augment the response of primary Schwann cells to the secreted mitogen. Quiescent primary Schwann cells also secrete a growth factor into their culture medium, but its activity is detectable only in the presence of added laminin or fibronectin. The results suggest that both normal and immortalized Schwann cells secrete an autocrine growth factor. Response to the autocrine factor appears to entail a multicomponent mechanism. Unlike primary cells, immortalized Schwann cells have the capacity to secrete all of the necessary components and to respond to them constitutively.

In the absence of neurons, rat Schwann cells in culture are quiescent, even in serum-containing medium (1–3). Contact with neurons (1, 3, 4) or with axolemma preparations derived from cultured neurons (4–6), PC12 pheochromocytoma cells (7), or brain (6, 8, 9) will induce Schwann cells to enter the cell cycle and undergo mitosis *in vitro*. In addition, a limited array of soluble growth factors are active in stimulating proliferation of cultured Schwann cells. A protein designated glial growth factor (GGF), isolated from brain and pituitary (10–12), is mitogenic for Schwann cells and astrocytes. A second protein purified from brain, glial maturation factor (13, 14), affects the morphology of Schwann cells and central glial cells and has weak mitogenic activity. Agents that increase intracellular cAMP are also mitogenic for Schwann cells (10, 15, 16). It is not known whether soluble polypeptide growth factors are relevant to the contact-mediated mitogenic stimulation of Schwann cells by neurons, and the role of cAMP in neuronal stimulation of Schwann cells remains to be established (7, 17). In addition, the extracellular matrix components laminin (18) and fibronectin (19), when added in soluble or substrate-bound (20) form, stimulate Schwann cell

proliferation. Because Schwann cells secrete laminin and form a basement membrane at an initial stage of neuronal ensheathment (21), it is important to establish the role of these components in the control of proliferation and differentiation.

We report that repetitive passaging of Schwann cells derived from neonatal rat can result in a population of immortalized cells that, depending upon their duration in culture, display all or most of the functional characteristics of a primary Schwann cell. It was found that both immortalized and primary Schwann cells secrete growth-promoting activity. Whereas the mitogen(s) secreted by primary cells are almost completely dependent upon supplementation with laminin or fibronectin, the immortalized cells secrete a product(s) whose activity is enhanced but not dependent upon exogenous laminin or fibronectin. We describe here some of the characteristics of the immortalized Schwann cells and the mitogenic activity that they secrete.

MATERIALS AND METHODS

Materials. One- to 3-day-old Sprague–Dawley-derived rats were obtained from Chappel Breeders (St. Louis), tissue-culture plasticware from Falcon, fetal bovine serum (FBS) from Hazelton Research Products (Denver, PA), and tissue culture media from the Media Center at Washington University School of Medicine. [*methyl*-³H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was acquired from New England Nuclear. Collagenase (type I), L-1-tosylamido 2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin), and rabbit complement were from Cooper Biomedicals (Malvern, PA). Poly(L-lysine) hydrobromide (*M_r* 30,000–70,000) was from Sigma. Anti-Thy-1.1 hybridoma cells were from the American Type Culture Collection. Laminin was from GIBCO and human plasma fibronectin was a generous gift from J. McDonald (Washington University, St. Louis).

Schwann Cell Culture. Rat sciatic nerve Schwann cells were prepared and purified according to the methods of Brookes *et al.* (2). In brief, sciatic nerves were dissected from neonatal rats, dissociated with 0.1% collagenase and 0.25% trypsin in Leibovitz' L-15 medium, and plated on tissue-culture plastic in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS. After 18–24 hr, the medium was changed to one containing 10 μ M 1- β -D-arabinofuranosylcytosine. Three to 5 days later, the cells were suspended with 0.05% trypsin and 0.02% EDTA in calcium- and magnesium-free Hanks' balanced salts solution and treated for 30 min with anti-Thy-1.1 hybridoma culture supernatant and then rabbit complement to remove contaminating fibroblasts. Treatment and passaging of cells in medium containing GGF and forskolin have been described (16). In brief, Schwann

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Abbreviations: GGF, glial growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CM, conditioned medium (media).

cells isolated as described and plated on a poly(L-lysine) substratum were treated continuously with a crude GGF preparation (final concentration 10 μ g/ml) and 2 μ M forskolin in DMEM with 10% FBS. Cells were passaged every 7–10 days.

Conditioned Medium (CM). Immortalized Schwann cells (1.17 cells) were plated at 8.5×10^4 per cm^2 in poly(L-lysine)-treated plastic tissue-culture dishes in serum-free medium (N2; ref. 22) without nerve growth factor. CM was collected three times per week and stored frozen at -70°C . Cells were passaged once a week.

Assay of Schwann Cell Proliferation. Primary Schwann cells that had been treated with arabinofuranosylcytosine and anti-Thy-1.1 but that had never been treated with mitogens were plated into poly(L-lysine)-coated 24-well culture dishes at 50,000 cells per well in DMEM plus 10% FBS. After 24 hr, the medium was replaced with 0.2 ml of CM diluted with DMEM and FBS to obtain the desired final concentration of CM protein and 10% FBS. Cells were treated with mitogen for 72 hr, fresh mitogen and medium being added every 24 hr, and [^3H]thymidine (2 $\mu\text{Ci}/\text{ml}$) was included for the last 24 hr. Medium was then aspirated, and cells were washed with medium and then treated for 30 min with 10% trichloroacetic acid. Cells were washed again, solubilized in 0.3 M NaOH/1% NaDodSO₄, and prepared for scintillation counting (8).

Immunocytochemistry. Cells plated on poly(L-lysine)-treated glass coverslips were washed once with L-15 medium plus 10% horse serum, incubated for 45 min with antibody [rabbit anti-laminin serum from J. Sanes, Washington University; rabbit Ran-1 antiserum (23) from K. Fields, Albert Einstein University; and mouse monoclonal antibody 217C (24, 25) from J. de Vellis, University of California at Los Angeles] diluted in L-15 plus serum, washed four times, incubated with goat anti-mouse or goat anti-rabbit immunoglobulin serum conjugated to either a rhodamine or fluorescein chromophore, and washed again. The cells were then fixed with acid/ethanol, and the coverslips were mounted on slides and viewed with a Zeiss microscope equipped for epifluorescence.

Dorsal Root Ganglion Neuron/Schwann Cell Coculture. Preparation of neurons and coculture with Schwann cells have been described (1, 16, 22, 26, 27).

Electron Microscopy. Neuron/Schwann cell cultures were fixed in 2% glutaraldehyde/46 mM phosphate buffer, pH 7.3/100 mM sucrose and then processed for thin-sectioning and visualization as described (22).

Protein Assay. Determination of protein was done according to the method of Peterson (28) to avoid interference by phenol red in tissue-culture medium.

Quantitation of Laminin and Fibronectin. Known quantities of primary and 1.17 cell CM protein were applied to nitrocellulose membrane through a Bio-Rad dot-blot manifold. The membrane (blot) was incubated for 2 hr with Tris/NaCl (50 mM Tris-HCl, pH 7.5/0.5 M NaCl) plus 3% nonfat dry milk and 0.02% Na₂S₂O₃ to block nonspecific binding sites and then incubated for 16 hr with primary antibody diluted in the same buffer. After washing in Tris/NaCl plus 0.1% Tween 20, the blot was treated with the Vectastain secondary antibody ABC Kit reagents (Vector Laboratories, Burlingame, CA) diluted in Tris/NaCl plus 0.1% Tween 20, and conjugated peroxidase activity was detected with 3,3'-diaminobenzidine (0.5 ng/ml) and H₂O₂ (0.009%) in phosphate-buffered saline. To quantitate the color development, the air-dried membrane was made transparent with mineral oil and then scanned in an LKB Ultrascan XL laser densitometer with automatic peak integration. The values from duplicate samples were compared to the linear portion of a standard curve produced with purified fibronectin or laminin. For the laminin assay, crossreactivity of the antiserum with entactin was accounted for by preparing two blots,

in which the incubation with anti-laminin was done in the presence or absence of excess purified laminin, and the degree of staining obtained in the presence of added laminin was subtracted from the value obtained in the absence of added laminin. The rabbit anti-laminin antiserum for these experiments was prepared in our laboratory by C. Eldridge and M. Ard, and the rabbit anti-fibronectin antiserum was provided by J. McDonald.

RESULTS

We showed previously (16) that primary Schwann cell cultures can be effectively expanded by using a combination of GGF and forskolin [an activator of adenylate cyclase (29)] on a poly(L-lysine) substratum. Schwann cells treated in this way for 2–3 months (6–9 passages) retain normal growth control. After GGF and forskolin are removed for 4 days, the cells return to quiescence in 10% FBS and will respond to re-addition of mitogens with the same kinetics as the original cell population. Further, after continuous passage for up to 8 months (24 passages), the cells still retain their ability to differentiate and to myelinate sensory axons *in vitro* (16).

Although the cells are still capable of differentiating, continuous treatment with GGF and forskolin for longer than 4 months (12 passages) causes the cells to become independent of exogenous mitogens. Unlike the original cell population, they proliferate in the absence of GGF and forskolin. Their growth is independent of serum; the rates of proliferation are comparable in serum-containing and serum-free media (Fig. 1). The doubling time of 2 days is the same as in cultures containing GGF plus forskolin or GGF plus cholera toxin.

The cells that have become mitogen-independent, called 1.17 cells[§], display some morphological heterogeneity (Fig. 2). Some are broad and flat, similar in shape to (though smaller than) fibroblasts. Most are bipolar and tripolar and thus look like typical early-passage Schwann cells (compare to figure 2, ref. 16). Despite the morphological heterogeneity, all of the cells can be labeled with antibodies directed to Schwann cell-specific surface antigens. The Ran-1 antiserum (23) is specific for rat Schwann cells, and the 217C monoclonal antibody (24, 25) recognizes an antigen similar to that of Ran-1 and is also specific for Schwann cells. Both antibodies label essentially all cells, regardless of morphology. In addition, all cells are recognized by anti-laminin antibodies and show the punctate pattern typical of primary cells (30) (Fig. 2). Faint immunofluorescent staining can be detected with antibodies to the myelin protein P₀, as well as with antibodies to glial fibrillary acidic protein and protein S100 (data not shown). Efforts to clone the cells have been unsuccessful because the cells grow very slow at low density.

Using the same techniques of repetitive passaging in medium with GGF and forskolin, we have independently generated a second population of immortalized Schwann cells (7.18 cells[§]) that also grow continuously in defined medium and are capable of myelination at low passage number. Thus the generation of the 1.17 cells was not an isolated occurrence and can be reproduced.

Continual passaging of the 1.17 cells in defined medium leads to a gradual decline in their ability to form myelin when combined with dorsal root ganglion neurons. After 2 months in culture and 6 passages with GGF and forskolin, the cells retained normal growth properties as well as the ability to myelinate neurons. Cell populations cultured for 8 months (24 passages) with GGF and forskolin still contained cells with the ability to differentiate and form myelin (16). We realized

[§]The numbers 1.17 and 7.18 refer to the dates that these cells were initiated in primary culture: January 17, 1985 and July 18, 1985, respectively.

FIG. 1. Growth curve of primary and 1.17 Schwann cells. Primary (■) and 1.17 (○, ●, △) Schwann cells were plated in poly(L-lysine)-coated 35-mm dishes in N2 medium without serum (open symbols) or with 10% FBS (filled symbols). At the indicated times, duplicate cultures were suspended with trypsin and cells were counted in a hemacytometer. 1.17 cells that had been maintained continuously in culture for 4 months were tested in N2 (○) and in N2 with 10% FBS (●) and then 5 months later in N2 (△).

at this point that the cells had become independent of exogenous growth factors. In retrospect, it became apparent that the cells had begun to display high background [³H]thymidine uptake after only 4 months of growth in medium containing GGF and forskolin. In generating the 7.18 cells, we have confirmed that within 4 months the cells had become mitogen-independent. After 16 months (56 passages), the 1.17 cells had lost their ability to form myelin. When seeded onto neurons, the cells display neuronal recognition in that they bind to and proliferate along the neurite processes. Unlike normal Schwann cells, however, late-passage 1.17 cells continue to proliferate, even after the

neurites are completely populated. Despite this abnormal growth behavior, the 1.17 cells are able to construct some basal lamina (often, but not always, continuous) and ensheath some axons (Fig. 3). No compact myelin formation was seen. However, 1.17 cells that had been frozen at early passage (8 months, 24 passages) were myelination-competent when thawed and reintroduced with neurons, although their growth properties were abnormal. The ability to freeze and maintain cells at different stages of competence to myelinate axons could prove useful for studying the essential events in Schwann cell differentiation.

The continued growth of 1.17 (and 7.18) cells in culture correlates with the detection of mitogenic activity in the medium conditioned by these cells (CM). When medium conditioned by 1.17 cells (1.17 CM) in the presence or absence of serum is added to primary Schwann cells, the previously quiescent cells are stimulated to proliferate (Fig. 4). When the CM is fractionated by gel-filtration chromatography, the activity is eluted in three peaks corresponding to estimated molecular weights of 150,000, 30,000, and <5000. However, the eluted mitogenic activity stimulates a maximal [³H]thymidine uptake that is only 20% of that attained with whole CM. We reasoned that two or more components of the mitogenic activity had been separated by chromatography. Supplementation of the fractionated CM with laminin or fibronectin results in regeneration of the mitogenic activity. Both laminin and fibronectin enhance the activity of whole 1.17 CM, but fibronectin has a smaller effect than laminin with both whole (Fig. 4) and fractionated CM (data not shown). Although laminin alone is mitogenic for Schwann cells, the mitogenic effect of the 1.17 secreted activity is significantly greater than that seen with laminin or fibronectin

FIG. 2. Immunofluorescent labeling of Schwann cell-surface antigens. 1.17 immortalized Schwann cells were plated onto poly(L-lysine)-coated glass coverslips and stained for surface antigens as described in *Materials and Methods*. These antibodies labeled 100% of the cells. At left are phase-contrast images; at right are the same fields under epifluorescent illumination. Phase image E provides a typical example of 1.17 cell morphological heterogeneity. (A and B) Anti-laminin antiserum. (C and D) Ran-1 antiserum, described by Fields *et al.* (23) as recognizing a Schwann cell-specific antigen. (E and F) Monoclonal antibody 217C, developed by Peng *et al.* (25), recognizing a Schwann cell antigen similar to Ran-1. [Bar (in A) = 20 μ m.]

FIG. 3. Electron micrograph of dorsal root ganglion neurons in coculture with primary or 1.17 Schwann cells. Primary (A) and 1.17 (B) Schwann cells were combined with separate cultures of dorsal root ganglion neurons in defined medium, as described (16). After 10 days the Schwann cells had repopulated the neuronal surfaces. The cultures were then provided for an additional 3 weeks with medium containing serum and ascorbic acid to promote myelination (31). Finally, cultures were fixed and processed for electron microscopy. (A, $\times 28,700$; B, $\times 20,300$.)

alone (Fig. 4). Furthermore, the CM mitogen is not active with primary cells in serum-free medium unless laminin is present. Even in the presence of laminin, the activity in serum-free medium is one-tenth that seen in 10% FBS. It appears, therefore, that the mitogen(s) secreted by 1.17 cells is dependent upon an extracellular matrix molecule for its activity. Laminin and fibronectin are synthesized and secreted by normal and 1.17 Schwann cells (30), but we do not know whether laminin or fibronectin is in fact the cofactor present in 1.17 CM. Whether the same mitogenic molecules are responsible for Schwann cell proliferation in the presence and absence of FBS remains to be determined. We compared the mitogenic activity in the culture medium from 1.17 to that of normal primary Schwann cells. Since these cells are quiescent in culture, we did not expect any mitogenic activity in medium conditioned by primary Schwann cells. In fact, the CM from normal cells does contain mitogenic activity, but unlike the 1.17 CM, the normal cell CM activity is almost completely dependent on exogenous laminin (Fig. 5).

These data suggest that the difference between primary and 1.17 Schwann cell CM is not in the concentration of mitogen but rather in the quantity of cofactor—i.e., laminin or fibronectin. We have therefore attempted to quantitate laminin and fibronectin secretion by quantitative immunodot-blot analysis. The secretion of these components by both the primary and the 1.17 cells is quite variable, falling in the range of 0.05–1.0 ng of laminin and 0.35–5.0 ng of fibronectin per μg of total secreted protein (data not shown). No

consistent difference has been detected between primary and 1.17 cells in laminin or fibronectin secretion. It is possible, therefore, that the cofactor in 1.17 CM is neither laminin nor fibronectin but rather a molecule whose function can be mimicked by these molecules.

DISCUSSION

We have shown that primary rat Schwann cells can be immortalized by repetitive mitogenic stimulation and that these immortalized cells retain functional properties of Schwann cells. The 1.17 (and 7.18) Schwann cells described here, although defective in growth control, retain most or all Schwann cell functional capabilities. They are able to myelinate neurons at low passage number and are able to construct a basement membrane and ensheath axons at high passage number (>50 passages), although they have lost their capacity to form myelin. Cultures of 1.17 cells have been carried continuously for longer than 1 year with no apparent decline in growth rate or viability. Unlike the RN-2 Schwannoma cell line (32), these cells were generated without the use of carcinogenic agents or viral infection. Their growth is serum-independent but anchorage-dependent. Autocrine growth regulation of other nontransformed cells has been described: e.g., regulation of activated T lymphocytes by interleukin 2 (33), and of smooth muscle cells (34, 35) and placenta (36) by platelet-derived growth factor (PDGF)-like peptides. It is interesting that in the case of T lymphocytes, proliferation is mediated or regulated via cell-cell contact, as in the neuron/Schwann cell system.

FIG. 4. Proliferation of primary Schwann cells in response to 1.17 CM, laminin, and fibronectin. N2 defined medium was conditioned by 1.17 Schwann cells and then diluted into DMEM with 10% serum, added to duplicate wells of primary Schwann cells, and assayed for mitogenic activity. Results are expressed as cpm of trichloroacetic acid-precipitable incorporated [^3H]thymidine. The concentration of CM is based on total CM protein prior to dilution. (A) Response to 1.17 CM alone (●) or plus laminin at 1 $\mu\text{g/ml}$ (○). (B) Response to laminin alone (●) or plus 1.17 CM at 100 $\mu\text{g/ml}$ (○) or to fibronectin alone (■) or plus 1.17 CM at 100 $\mu\text{g/ml}$ (□). Background [^3H]thymidine uptake in DMEM/10% FBS (4471 ± 990 cpm) has been subtracted.

FIG. 5. Comparison of mitogenic activity in medium conditioned by primary and 1.17 Schwann cells. Primary and 1.17 Schwann cells were plated as described for Fig. 1 and allowed to condition the medium for 48 hr. The two CM were diluted into DMEM with FBS (10% final concentration) and assayed for mitogenic activity with primary Schwann cells in the presence or absence of laminin (1 $\mu\text{g/ml}$). ○, Primary CM alone; ●, primary CM plus laminin; □, 1.17 CM alone; ■, 1.17 CM plus laminin. [^3H]Thymidine uptake in DMEM/10% FBS alone (1985 ± 280 cpm) has been subtracted.

The immortalized Schwann cells secrete an autocrine growth factor that appears to be also secreted by normal cells, suggesting that control of response rather than secretion of a mitogen is the major element that distinguishes immortalized cells from normal ones. Sporn and Roberts (37) have proposed that acquisition of the transformed phenotype (i.e., abnormal growth properties) may occur either as a result of the appearance or increase of a secreted growth factor or as a result of an enhanced cellular responsiveness. Change in cellular responsiveness could result either from a change in the number of growth factor receptors or from an altered affinity of these receptors. Alternatively, the change may be in the induction of mediators of intracellular mitogenic signal transduction. Since normal Schwann cells also secrete a growth factor, it appears that through repetitive passaging we may have selected for Schwann cells with an enhanced capacity to respond to that growth factor in culture, either because of an ability to secrete more cofactor (a laminin/fibronectin-like molecule) or because of an alteration of the receptor mechanism.

These data raise fundamental questions pertaining to the control of Schwann cell growth and its regulation by neurons. In particular, we need to understand why isolated Schwann cells fail to respond to a factor that they secrete. Furthermore, if we are to correlate these findings to the interactions of Schwann cells with neurons *in vivo*, then it will be necessary to determine whether the neuronal mitogen, shown to be associated with a heparan sulfate proteoglycan (38), plays a role in the responsiveness of Schwann cells to a secreted factor.

Laminin, or a laminin-like molecule, appears to play a regulatory function in the Schwann cell response to autocrine growth factor. Laminin is a secretory product of Schwann cells (30) and is central to the construction of a basement membrane by differentiating Schwann cells (21). We have shown here that laminin enhances the mitogenic activity of 1.17 CM for primary Schwann cells. CM from primary Schwann cells has very little activity in the absence of added laminin. A major difference between the mitogenic activity in the two CM is their relative dependence on laminin. Laminin alone has a mitogenic activity that begins to plateau at a concentration of 10 $\mu\text{g}/\text{ml}$ and reaches a maximum at 100 $\mu\text{g}/\text{ml}$. When CM is added to laminin, the proliferative effect is greater than that seen with laminin alone. Therefore, the mitogenic activity cannot be attributed to laminin in the CM. The mitogenic activity does not bind to laminin or fibronectin, and the activity cannot be precipitated from the CM by anti-laminin antibodies (data not shown). Instead, it seems likely that the mitogenic effect of laminin described by others (18) is due to its role as a cofactor for the secreted mitogen(s) present in the growth medium.

In an attempt to integrate these findings with the interaction that leads to proliferation when Schwann cells contact neurons in neuron/Schwann cell cocultures, we speculate that the mitogen on the axon regulates the ability of Schwann cells to respond to their autocrine growth factor. *In vitro*, laminin, in the presence of serum, can substitute for the neuronal mitogen. Alternatively, the effects of the neuronal mitogen *in vivo* may be mediated via laminin on the Schwann cell surface, and an excess of laminin *in vitro* can obviate the requirement for the axon.

In recent *in vivo* experiments, 1.17 cells were injected into rat central and peripheral nervous systems. Preliminary evidence indicates that in the peripheral nervous system the cells survive, continue to divide, and form an encapsulated mass similar in appearance to a Schwannoma (L. Langford and S.P., unpublished results). These observations suggest that Schwannomas may form by the same alteration of growth properties that has occurred in the 1.17 and 7.18 cells

and that these cells will be a valuable tool for studying the mechanism of induction of Schwann cell tumors as well as the mechanisms of Schwann cell growth control.

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