Production of plasminogen activator in cultures of superior cervical ganglia and isolated Schwann cells
(protease/neuron–Schwann cell interaction)

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ABSTRACT Plasminogen activator has been implicated in tissue remodeling and cell migration during embryogenesis. In the developing nervous system, these processes are evident in the migration of neurons, axonal extension, Schwann cell migration, and the ensheathment and myelination of nerves. We have studied the production of plasminogen activator in cultures of superior cervical ganglia under conditions in which both neurons and glia are present. We have found that a principal source of the enzyme in these cultures is the glial cells and that the enzyme could not be detected at the growing tips of neurites. Plasminogen activator is also produced by Schwann cells isolated from neonatal rat sciatic nerve. The production of the enzyme by these cells is stimulated 6- to 10-fold by cholera toxin. Isolated Schwann cells and glial cells in the ganglion explant cultures produce the tissue form of plasminogen activator, a form of the enzyme not often found in nonmalignant cells. Preliminary experiments suggest that neuronal–glial interactions may regulate enzyme production by Schwann cells.

Studies on embryonic (1–3) and adult (4–6) systems have suggested that cell migrations and tissue remodeling may be facilitated by the localized secretion of the neutral serine protease, plasminogen activator (PA), and the subsequent conversion of plasminogen to plasmin (7). This view has been strengthened by the temporal correlations that have been demonstrated between the production of the enzyme and the migration of specific cells in the developing embryo (2, 8) or specific cases of remodeling (2, 9).

Cell migration and tissue remodeling are conspicuous events during the development of the nervous system, and several recent studies have implicated PA in these situations. For example, the production of PA during the migration of granule neurons in the cerebellum has been reported (10–12) and it has been suggested that there are changes in the levels of PA in different regions of the brain during postnatal development (13).

Both neurons and Schwann cells produce PA in vitro. The enzyme is produced by neuroblastoma (13, 14) and neuroblastoma/glioma (14) cell lines, and, when they exhibit some of the morphological properties of neurons in culture, it has been possible to localize the enzyme at the neuritic growth cones of a small percentage of these cells (13, 14). Schwann cells migrate along axonal extensions, proliferate, presumably in response to interactions with the neuron (15, 16), ensheath the nerve fiber and, in some cases, myelinate it. It has been suggested that Schwann cells produce PA in vitro (17) and that their proliferation (18) and perhaps the ensheathment of neurons (19) is regulated by the PA–plasmin system.

In the present communication, we report the results of initial investigations on PA production in primary cultures of neonatal rat superior cervical ganglia (SCG). We have used this system not only because several of the events integral to nerve morphogenesis are clearly displayed in vitro, but also because it has afforded us the opportunity to study neuronal and non-neuronal cells simultaneously in coculture. We show that PA is indeed produced in the cultures and that a principal source of the enzyme is the Schwann cell. Furthermore, we demonstrate that Schwann cells, both in the ganglion explant cultures and when isolated from rat sciatic nerve, produce the tissue form of PA, a form of the enzyme found in only a few nonmalignant cell types (20–23), and that enzyme production and secretion may be stimulated significantly by cholera toxin. We were unable, however, to detect the production of PA at the growth cones of neurites in the explant cultures, even when very sensitive methods were used.

MATERIALS AND METHODS

Cell Culture. Superior cervical ganglia were excised from newborn Sprague-Dawley rats (Taconic Farms, Germantown, NY) and cut into three pieces (''explants'') or minced into small fragments (''mini-explants''). Then, 4–6 explants, or 15–20 mini-explants, were placed in 35-mm culture dishes coated with bovine skin collagen (Vitrogen, Flow Laboratories) reconstituted according to the method of Elsdale and Bard (24). One milliliter of Dulbecco's modified Eagle's medium (DMEM medium; GIBCO) supplemented with acid-treated (25), heat-inactivated (56°C, 30 min) fetal bovine serum (10%; Flow Laboratories) and 66 ng of 7S nerve growth factor per ml (gift of Ira Black, Cornell University Medical College, New York) was added and the cultures were incubated at 37°C in an atmosphere of 5% CO2/95% air. Fluoroethoxyuridine (10 μM; Sigma) and 10 μM uridine (Sigma) were added as indicated to inhibit the growth of non-neuronal elements in the preparations (26). The culture medium was changed every other day.

Enriched populations of Schwann cells were obtained by a modification of the method of Brockes et al. (27). Sciatic nerves were dissected from 1- to 2-day-old rats and incubated in 0.03% collagenase (type IV, Worthington) in L-15 medium (GIBCO) for 30 min, followed by an additional 30 min in 0.2% trypsin (Difco) in L-15 medium. The nerves were dissociated in three passes through a 23-gauge needle. The resulting cell suspension was plated in 25-cm² tissue culture flasks in DMEM medium containing 10% acid-treated and heat-inactivated fetal bovine serum. One day after the cultures were initiated, the cells were treated with 10 μM cytosine arabinoside (Sigma) for 12–24 hr, and then the medium was replaced. One to 3 days later, the cultures were treated with rabbit anti-Thy-1.1 (New England Nuclear) and rabbit complement

Abbreviations: PA, plasminogen activator; tPA, tissue PA; uPA, urokinase; SCG, superior cervical ganglion.
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(Cappel Laboratories, Cochraneville, PA). This treatment removed most (>90%) of the rapidly dividing fibroblasts in the preparations. After an additional 4 days, the cultures were trypsinized and the cells were transferred either to 35-mm culture dishes or to 24-well dishes (Linbro) that had been coated with reconstituted collagen. Control cultures of fibroblasts were prepared from sciatic nerves that had been dissociated as described above, but the treatments with cytosine arabinoside and rabbit anti-Thy-1.1 were omitted and fibroblasts were permitted to overgrow the cultures. After a single passage, we estimate that at least 90% of the cells were fibroblasts.

**PA Assays.** A modification of the single cell overlay assay described by Jones et al. (28) was used to detect PA production by cells in SCG or Schwann cell cultures. The cells or explants were overlayed with casein agar (29) containing 580 μg of human plasminogen per ml, purified by the method of Deutsch and Mertz (30), 66 ng of 7s nerve growth factor per ml. DME medium and 10% acid-treated heat-inactivated fetal bovine serum; plasminogen was omitted from control plates. The preparations were incubated at 37°C for the indicated times, fixed, with formalin, and examined by phase contrast microscopy or stained with silver methenamine according to the method of De Martino and Zamboni (31).

PA secretion by cultured cells was measured by the 125I-labeled fibrin plate assay described by Strickland and Beers (4). Cells were trypsinized and plated at a density of 5 x 10⁴ cells per well on 24-well plates. After overnight culture the cells were washed, the medium was replaced, and aliquots were removed at various times for assay. Some cultures were treated with chola toxin (1 μg/ml) as indicated in the figure legends.

**Characterization of PA Produced by Cultured SCG, Schwann Cells, and Sciatic Nerve Fibroblasts.** The apparent molecular weights of the enzymes produced by cultured cells or SCG explants were determined by NaDodSO₄/polyacrylamide gel electrophoresis (32) followed by PA zymography according to the method of Granelli-Piperno and Reich (33). The identity of the enzymes was further confirmed by the use of rabbit antisera directed against tissue (tPA) or urokinase (uPA) forms of the enzyme. These antisera were the gifts of E. Waller and D. Belin, respectively, and had previously been shown to neutralize the activity of PA (22,35). In one set of experiments, IgG from anti-human tPA antiserum was incorporated into the indicator layers of the NaDodSO₄/PAGE zymograms or into the casein agar overlays as described in the corresponding figure legends. In a second set of experiments, conditioned medium from cholera toxin-stimulated Schwann cells containing 1–2 milliunits of PA, was incubated with increasing amounts of IgG from anti-human tPA or anti-mouse uPA for 60 min at 4°C in 0.25 ml of a buffer containing 0.1 M Tris-HCl, pH 8.0, and 0.05% Triton X-100. Aliquots of the reaction mixture were then assayed for PA activity by using the fibrin plate method. Control reactions included conditioned media containing known types of PA. HeLa cell conditioned medium, a source of tPA, was the gift of A. Piperno and MSV-3T3 conditioned medium was the gift of L. Ossowski (Rockefeller University).

### RESULTS

**PA Production in Explants of SCG.** When explants of rat SCG were cultured for 1 week in the presence of nerve growth factor, fluorodeoxyuridine, and uridine an extensive outgrowth of neurites was observed (Fig. 1A); these outgrowths contained very few non-neuronal cells. When these preparations were overlaid with casein agar, plasminogen-dependent zones of caseinolysis could be detected around the explant after 1–2 hr of incubation at 37°C (Fig. 1A); these zones of lysis grew concentrically, reaching the periphery of the neuritic outgrowth in 6–8 hr. Caseinolysis was not observed in any of the cultures in the absence of plasminogen.

In 14 separate experiments, in which >500 explants were examined, we were unable to detect the production of PA at the tips of the neurites, where growth cones were clearly visible (Fig. 1). Observations on mini-explant cultures confirmed that PA was not produced at the growth cones under these conditions. These preparations contained very few cells (<100), some of which produced very long neurites. Their use permitted us to measure PA production during the course of much longer incubations than those described above. In six experiments, in which a total of 120 mini-explants were analyzed, PA secretion at the growth cones was

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**FIG. 1.** PA production in SCG explants. SCG from neonatal rats were excised, placed in culture, and analyzed for the production of PA by the casein agar overlay technique. (A) SCG explant cultured for 1 week in the presence of fluorodeoxyuridine and uridine prior to overlay. After 2 hr of incubation, the preparation was fixed with 2.5% paraformaldehyde, stained with silver methenamine, and photographed using bright-field optics. Arrows indicate margins of the zone of plasminogen-dependent caseinolysis. (B) SCG mini-explant after 2 weeks in culture and 7 hr of incubation with a casein agar overlay. This preparation was photographed unstained using phase contrast optics. Arrow points to thickenings on some of the neurites where closely associated cells are found. (Inset) Phase contrast photograph of the cell indicated by the lower arrow in B.
undetectable even after 16 hr of incubation with the casein agar overlay (Fig. 1B).

Although zones of lysis were not detected at the growing tips of neurites, randomly distributed plasminogen-dependent plaques, similar to those recently described by Krystosek and Seeds (17), were observed along the nerve fibers (Fig. 1B). These zones of lysis, although sparse (2–5 per field), were present in each of the explant preparations previously discussed. Examination of these lytic zones (Fig. 1B, Inset) revealed the presence of spindle-shaped cells closely associated with the neurites, suggesting that non-neuronal cells, which had migrated out of the explant, might be the source of PA in these cultures. This interpretation was supported by two other observations: (i) non-neuronal cells grew extensively in cultures of SCG prepared in the absence of fluorodeoxyuridine and uridine. These cells migrated a significant distance from the explant and formed a network of spindle-shaped cells and neurites. The zones of plasminogen-dependent lysis produced in this case (Fig. 2A) were extensive and followed the contours of the cellular outgrowth. (ii) PA activity could be detected in only a small fraction (~10%) of the cells in cultures of neurons isolated from the SCG.

**PA Production by Schwann Cells.** The apparent morphology of the PA-producing cells and their close apposition to the neurons *in vitro* suggested that they might be Schwann cells. To investigate the production of PA by these cells, we established primary cultures of Schwann cells from rat sciatic nerve. These cultures were assayed first by the single cell overlay method. After 3 hr of incubation in casein agar, plasminogen-dependent zones of lysis developed around <10% (42/561) of the cells examined. However, 87% (488/561) of the positive cells had the spindle-shaped morphology characteristic of Schwann cells (Fig. 3). Similar results were obtained in secondary cultures (see Materials and Methods) enriched to >90% by immunosurgery with anti-Thy-1.1 and complement. Thus, only a minor population of the isolated Schwann cells appears to produce PA spontaneously *in vitro*.

We next tested the effects of several agents on the production of PA by these cells. Among those tested, only cholera toxin, a Schwann cell mitogen (34), had significant reproducible effects. Treatment of cultures of purified Schwann cells with 1 μg of cholera toxin per ml resulted in an increase in the percentage (88%; 253/288) of cells that produced PA, as determined with casein agar overlays (Fig. 3). To quantitate this effect, medium that had been conditioned by Schwann cells grown in the presence of cholera toxin was assayed for PA by using the 125I-labeled fibrin plate technique. Fig. 4 shows that the low basal level of PA secretion was stimulated 8- to 10-fold by this treatment. Cholera toxin did not significantly affect the production of PA by sciatic nerve fibroblasts. Other Schwann cell mitogens, including fibronectin (10–20 μg/ml) and pituitary extracts (100 μg/ml) (34), exerted only modest effects on enzyme secretion. Nerve growth factor (0.1 μg/ml) had no effect on PA production.

![Fig. 2](image-url) PA production by SCG explants cultured in the absence of antimitotic agents: Effects of rabbit anti-tPA. SCG explants were grown for 1 week in the absence of antimitotic agents and were then overlaid with casein agar. The preparations were fixed and stained with silver methenamine and photographed using dark-field illumination. (A) SCG explant overlaid and incubated for 2 hr. (B) SCG explant cultured as in A, but overlaid in the presence of 200 μg of rabbit anti-human tPA IgG per ml.

![Fig. 3](image-url) Casein agar overlay of sciatic nerve Schwann cells. Sciatic nerves were dissected from neonatal rats, dissociated with collagenase, and cultured as described. Schwann cell cultures were prepared after immunosurgery with anti-Thy-1.1, treated with cholera toxin (1 μg/ml), and overlaid with casein agar.
that the 40-kDa (Fig. 5, cultures NaDodSO4/polyacrylamide gels. nature cholera toxin-stimulated fibroblast preparations. enzyme the PA secreted by the toxin; because PA production by isolated sciatic nerve Schwann cells was determined by PA zymography on NaDodSO4/polyacrylamide gels. The PAs produced in these cultures (Fig. 5, lane A) consisted of a major species of 40 kDa and minor species of 67–70 kDa and 25 kDa. It seems likely that the 40-kDa band visible in lane C was produced by cholera toxin-stimulated Schwann cells that were present in the fibroblast cultures, but we cannot exclude the possibility that the 40-kDa enzyme is a product of selected subpopulations of fibroblasts or of yet a third cell type present in the preparations. Because the apparent size of the predominant species of enzyme in Schwann cell cultures (40 kDa) was much lower than that reported for tPA found in other rat tissues (22) (the 40-kDa species has been observed in other systems; refs. 22 and 35), it was necessary to confirm the identities of these enzymes by an alternative method. PA from cholera toxin-stimulated Schwann cells or fibroblast-conditioned medium was incubated with the IgG fractions derived from rabbit anti-tPA or anti-uPA antisera prior to assay by using the fibrin plate method. The curves shown in Fig. 6 demonstrate that >95% of the enzymatic activity was inhibitable by anti-tPA, while virtually no inhibition was obtained with an equivalent amount of anti-uPA IgG. The enzyme produced by rat embryonic sciatic nerve fibroblasts was inhibited 75.8% by anti-urokinase IgG and 15.7% by anti-tPA. Identical results were obtained in single-cell overlay assays in which cholera toxin-stimulated Schwann cells were incubated with casein agar containing plasminogen and anti-tPA or anti-uPA IgG. Furthermore, when SCG explants prepared in the absence of antimotics, were overlayed under the same conditions, anti-tPA completely inhibited the appearance of the large zone of lysis that generally surrounds the explant and the production of enzyme by Schwann cells that had migrated out along the neurites (Fig. 2).

DISCUSSION

It has been suggested that PA is involved in cell migration and tissue remodeling (2, 8, 9) and, specifically, in the case of the nervous system, in axonal extension (12, 17) and ensheathment of nerves by Schwann cells (18). A common thread in many of these studies is the inference that there is a relationship between PA production and interactions between neurons, glia, and perhaps the extracellular matrix. The experiments described in this report serve to establish the groundwork for further studies on the role of PA in nerve morphogenesis. Three major conclusions can be drawn from our studies on the SCG from the neonatal rat. First, by using sensitive single cell assays for PA, we have demonstrated that the enzyme is produced by SCG explants. This is also true of chicken embryo neural tube, dorsal root, and spinal cord explants (unpublished observations). Second, Schwann cells seem to be responsible for the production of PA in the SCG preparations. Third, we have been unable to detect PA production at the actively growing tips of neurites extending from the explanted ganglia. We cannot fully explain the disparity between our findings and those of others (12, 13), but we suggest that they may be due, in part, to the
use, in some cases, of tumor cell lines instead of normal primary cell cultures, or to differences in culture conditions (e.g., substrata, sera, etc.), which might potentiate or inhibit enzyme production. It should also be noted that only a small proportion of the cells described in these reports displayed enzyme activity at the growth cones: PA was observed in the cell bodies in at least as many cells. Therefore, the specificity of the site of secretion can be questioned. The sensitivity of the single cell assay, and the long incubations to which some of our preparations were subjected, make it unlikely that enzyme production at the growth cones would have gone undetected.

The production of PA by non-neuronal elements in the SCG cultures was especially evident in cells that had migrated along the neurites and that remained in close proximity to them. A precise identification of these cells was not possible (36), but the morphology of the cells and their association with neurons was typical of Schwann cells seen in similar preparations (26). The ability of Schwann cells to produce PA was confirmed by the demonstration that enriched populations of Schwann cells from neonatal rat sciatic nerve were able to produce the enzyme (17).

Production of PA by Schwann cells is stimulated by cholera toxin, a molecule that is mitogenic for Schwann cells (34) and that affects the levels of PA in many types of cells in vitro (37). This is of interest on several counts. First, the induction of PA in cultured cells has been shown to be related to the metabolism of cyclic nucleotides (38, 39). Second, Bunge and co-workers have demonstrated that the induction of cell division in Schwann cell preparations is accompanied by an increase in intracellular levels of cAMP (15). This is true of agents such as cholera toxin, but also of homogenates of neurites and partially purified proteins from the membranes of differentiated PC12 cells (15, 16). Finally, it has been reported that proteases, such as plasmin, are mitogenic for Schwann cells (18).

What, then, is the role of PA in nerve morphogenesis? A relationship between the induction of PA and interactions between neurons and glia, as indicated by the circumstantial evidence cited above, seems likely. Preliminary experiments involving cocultures of SCG and sciatic nerve Schwann cells have suggested that plasminogen-dependent zones of lysis are observed predominantly in those parts of the cultures in which neurites and Schwann cells were in contact (unpublished observations). We postulate, therefore, that the local production of proteases could occur as a consequence of neuronal-glia interactions. The enzyme that is produced, whether plasmin or PA itself (40), could be channeled during the early phases of nerve morphogenesis into a number of processes. These might include the generation of peptides that could be mitogenic for Schwann cells (41) or the creation of surfaces (42) upon which subsequent migration or remodeling could proceed.

Tissue and urokinase forms of PA are products of different genes (43) and, as such, might be expected to have separate functions and to be subject to different regulatory controls. In the case of the nervous system, this view is supported by the observation that sciatic nerve Schwann cells make tPA and are stimulated by cholera toxin, while sciatic nerve fibroblasts produce urokinase and are not induced by cholera toxin. In a similar situation, Marotti et al. have found that different types of mouse embryonic endoderm produce different forms of the enzyme (22). In light of these findings, and because of the relative rarity of this form of the enzyme in normal tissue, the production of tPA by Schwann cells might be used in future studies as a marker of gene regulation, which is controlled at least in part by cell-cell interactions.

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