Schwann cell proliferation and localized proteolysis: Expression of plasminogen-activator activity predominates in the proliferating cell populations

(Neuron system histogenesis/extracellular proteolysis/plasmin-generating system)

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ABSTRACT The role of the serum proteolytic system plasminogen/plasminogen activator as a biochemical tool used by the glia or neurons, or both, for maintaining their temporary and flexible cellular interactions during histogenesis of the nervous system is under study. The present report identifies a glia cell type, the Schwann cell, as one of the cellular components of the nervous system that uses extracellular proteolysis at the time of the tissue construction. Purified dividing Schwann cells in culture produce extracellular plasminogen activator. The levels of extracellular plasminogen-activator activity, as measured by the biochemical fibrinolytic assay, were directly related to the proliferation rates of the Schwann cells. The cellular plasminogen-activator specific activity at the maximal rate of cell proliferation was 3–4 times higher than that of the cells at low rate of mitosis. It is concluded that plasminogen-activator activity is expressed predominantly by the proliferating Schwann cell populations, suggesting that the extracellular proteolysis is used by the tissue at those stages when the cells divide.

In studies on developing nervous systems, attention is being given to the factors that are involved in the establishment of specific cellular interactions—e.g., cell adhesion factors (1). However, a crucial process, one that leads to the breakdown of temporary or unwanted cellular interactions, or both, has been almost overlooked. Any cellular interaction is mediated by specific proteins—for example, fibronectin mediates cellular adhesion (2); once the cells separate, the glue is broken down. Proteolytic enzymes are utilized in the degradation of biological structures. Hence, it might be expected that proteolysis is a major factor in the developing nervous tissue, the cytoarchitecture of which is in a transient state. The plasmin-generating system, which is found in extracellular fluids (e.g., in plasma), has been suggested to be one of the physiological proteolytic machineries that are used in tissue remodeling processes in early development (3). This system is elaborated in the histogenesis of chicken spinal cord in vitro (4, 5) and in vivo (unpublished data).

The plasmin-generating system is used by tissues and cells to generate localized proteolysis (6). This system is composed of plasminogen, which is a zymogen, and the enzyme plasminogen activator (PA), which activates plasminogen to generate the potent protease plasmin. Physiologically, plasmin is generated in a localized manner and is used to dissolve fibrin clots or to act at surfaces of cells to degrade extracellular matrix or some membrane proteins. The high circulating level of plasminogen represents a reservoir of potential proteolytic activity; this can be recruited by cells for any function requiring localized extracellular proteolysis. The activation of plasminogen is regulated by the amounts of PA available; this step is controlled by the cells that secrete the PA. This secretion of PA by cells seems to fluctuate with their physiological or developmental state (3, 6).

Schwann cells in vivo proliferate during development of the nervous system (7, 8) and in the adult nervous system upon degeneration as a result of nerve injury (9). It was established in studies in vitro that Schwann cell proliferation is dependent on and controlled by neuronal activity (10). Schwann cells in culture can proliferate either when in direct contact with neuronal axons or in response to several different mitogens. Schwann cells in culture divide at a low rate. However, direct contact of Schwann cells with neuronal cells is sufficient to promote their proliferation either in a serum-supplemented medium (11) or in a chemically defined medium (12, 13). It was found in a series of studies (14, 15) that some surface neureite component is mitogenic to the Schwann cell population. Recently, a glial growth factor (GGF) that enhances Schwann cell proliferation was identified and purified from bovine pituitary (16, 17); nevertheless, fetal calf serum is an essential component in the medium for its action (18). In the developing nervous tissue, the proliferating Schwann cells penetrate axonal bundles and segregate them by individually ensheathing portions of these axons (19). These events lead to gross morphological changes that entail the breakdown of preexisting cellular interactions as the new axonal–Schwann cell connections are formed.

The long-range goal of this study is to identify cell types of the nervous system that use extracellular proteolysis in the process of the tissue formation. One cell type that produces PA was identified. First, a purified mouse Schwann cell population was obtained, and then the expression of PA activity by these cells under different physiological conditions was monitored. PA activity was expressed predominantly in the proliferating Schwann cell populations, the significance of which in the developing and regenerating nervous system is discussed.

MATERIALS AND METHODS

Materials. Newborn Swiss white mice (Nelson Collins Stock) were obtained from The Rockefeller University breeding colony. The following items were used: Dulbecco’s modified Eagle’s medium (DME medium) and heat-inactivated fetal calf serum (GIBCO); 1-300 trypsin (hag pancreas, for tissue dissociation; ICN); collagenase (CLS III) and trypsin (TL, for affinity chromatography) ( Worthington); freeze-dried bovine anterior pituitary bodies (Pel-Freez); carboxymethylcellulose CM-52 (Whatman); serum albumin (bovine, fraction I; Sigma); Sepharose 4B (Pharmacia); monoclonal anti-mouse Thy-1.2 antibody (New England Nuclear); fluorescein-conjugated IgG fraction (Cappel Laboratories); 125I (100 mCi/ml; 1 Ci = 37 GBq) for protein iodination (Amer...
Dissociation of Schwann Cells. Purified Schwann cell populations were prepared from the sciatic nerves of newborn mice by the procedure of Brockes et al. (20). The sciatic nerves were dissected and collected in a CaCl2- and MgCl2-free Puck's saline G (called "saline G") (21) containing 1 mg of bovine serum albumin per ml and then incubated for 45 min at 37°C in 4 ml of saline G containing 0.25% trypsin and 0.03% collagenase. The digestase was centrifuged (Arthur Thomas Co. clinical centrifuge) at the maximal rate for 10 min at 24°C, the pellet was suspended in 1 ml of medium, and the cells were dissociated by trituration through a Pasteur pipette. The cells, suspended in DME medium containing 10% fetal calf serum supplemented with the appropriate amount of GGF, were plated at about 5 × 10^4 per 60-mm dish and maintained in a 5% CO2 incubator at 37°C. Cultured cells were passaged by trypsinization with 0.05% trypsin in saline G containing 2 mM EDTA (pH 7.3) for 10 min at 37°C. Before being plated, the fibroblasts were eliminated by treatment with the anti-Thy-1.2 antibody and rabbit complement (20, 22). Usually, three cycles of passage-fibroblast elimination were needed to obtain a purified (>95%) Schwann cell culture. The degree of purity of the cultures for Schwann cells was determined by counting the fibroblasts visualized by indirect immunofluorescence labeling (20, 23), when the antigen Thy-1.2 served as a marker for fibroblasts (22). Cell cultures and immunofluorescence-labeled cells were examined with a Zeiss inverted microscope equipped with phase-contrast and epi-illumination optics with a filter for fluorescein.

Preparation of GGF Fractions, GF1 and GF2. GGF fraction was purified from freeze-dried bovine pituitary extracts by the procedure of Brockes et al. (16). The fraction that was used as a mitogen in this study is the fraction eluted from the carboxymethylcellulose with phosphate-buffered 0.2 M NaCl and is designated fraction GF1. Each GF1 preparation was calibrated by its capacity to stimulate Schwann cell division ([3H]thymidine uptake), usually 5–10 μCi/ml. However, this fraction, contains protease inhibitory activity that obstructs the measurements of PA activity. Protease inhibitors were removed from fraction GF1 by the affinity chromatography technique—i.e., by using trypsin-agarose. Activation of the agarose beads (Sepharose 4B) was CNBr as described by Parikh et al. (24) to which the trypsin was bound as described by Chauvet and Acher (25). The trypsin-Sepharose beads were equilibrated with a phosphate-buffered solution (pH 7.3), then fraction GF1 was applied to the column, and the unbound material was collected and designated fraction GF2. Fraction GF2 was calibrated for its mitogenic activity on Schwann cells, usually 0.5–1 μg/ml.

[3H]Thymidine Incorporation into Schwann Cells. Schwann cells were plated in DME medium containing 10% fetal calf serum; on the following day, the medium was replaced by the appropriate medium supplemented with [3H]thymidine (1 μCi/ml). The medium was changed 22–24 hr later to nonradioactive medium for a 3-hr period, and then the cells were washed twice with saline G containing bovine serum albumin at 1 mg/ml. The cells were collected by trypsinization with 0.05% trypsin in saline G containing 2 mM EDTA (pH 7.3) for 10 min at 37°C and were centrifuged for 15 min in a Eppendorf microcentrifuge. Each of the pellets was solubilized in an equal volume of 0.2% Triton X-100 and samples were measured in a beta counter.

Fibrinolytic Assay. PA was assayed in [125I]-labeled fibrin (125I-fibrin)-coated multwell plates, by monitoring the fibrinolytic release of [125I]-labeled peptides into the medium as described by Unkeless et al. (26). Preparation of [125I]-fibrin-coated plates and acid treatment of serum and conditioned media to inactivate protease inhibitors were performed following the procedures summarized by Strickland and Beers (27). Plasminogen was purified from fetal calf serum as described by Deutsch and Mertz (28). The assay plates were incubated in a 37°C CO2 incubator, and activity was monitored by measuring samples in a gamma counter. Maximal or 100% activity was defined as the amount of [125I]-labeled peptides solubilized by an excess of trypsin.

PA Determination. To determine extracellular PA activity, we used the following procedures. (i) Cell cultures in DME medium supplemented with 5% plasminogen-depleted fetal calf serum were plated directly on the [125I]-fibrin-coated multwell units. The assay was started by replacing the plating medium with the appropriate medium at 2 ml per well. (ii) Conditioned media were collected, and their protease inhibitors were inactivated by acid treatment (27). These acid-treated media were assayed in the multwell units: either 1 ml of sample was assayed in DME medium containing 10% acid-treated fetal calf serum (final volume 2 ml) or 150 μl of sample was assayed in 0.05 M Tris (pH 8.1) containing 5 μg of plasminogen and 40 μg of bovine serum albumin in a final volume of 300 μl. All samples were assayed either in duplicate or triplicate.

RESULTS

The first goal in these studies was to obtain purified dividing Schwann cell populations in culture. To achieve this, the procedures developed by Brockes and his colleagues for obtaining Schwann cells from newborn rat sciatic nerve (16, 20) were adopted and used on newborn mouse sciatic nerve. Specifically, these procedures involve: (i) elimination of the fibroblast cells by their specific lysis in a complement reaction with the antibody anti-Thy-1.2 (20, 22) and (ii) stimulation of Schwann cell division by a GGF, GF1 fraction, which is purified from bovine pituitary and which stimulates mouse Schwann cell division in a similar manner to the one described by Raff et al. (18) for rat Schwann cells; usually, maximal enhancement of cell division induced by the GF1 fraction is about 3–4 times that of the rate observed in the absence of this mitogen (Fig. 1). Schwann cell populations, at least 95% pure, were used for the studies described in this report (Fig. 2).

Production of Extracellular PA Under Different Growth Conditions. In media supplemented with acid-treated fetal calf serum. In experiments that examined the presence of extracellular PA activity in the growth medium of Schwann cell populations, a protease inhibitory activity was detected. This protease inhibitory activity was identified later as a component of the GF1 fraction that routinely was added to the Schwann cell cultures. Li and Chung (30) have reported that bovine pituitary contains a protease inhibitor. Presumably, the inhibitor in GF1 fraction is identical to the one described by Li and Chung, although no attempt was made in the present study to characterize this inhibitor. To pursue the experiments of this study, it became imperative to remove the protease inhibitory component from the GF1 fraction, which was accomplished by trypsin affinity chromatography, yielding fraction GF2.

Purified dividing Schwann cells produced extracellular PA
Fig. 1. Effect of GF₁ fraction on Schwann cell proliferation. Schwann cells were plated in a multiwell unit at 5 x 10⁵ cells per well, and the mitogenic effects of GF₁ fraction were determined by the amounts of [³H]thymidine incorporated into the cells. Note that the mitogen induced a maximum increase of ~3 times in the proliferation rate of the cells.

as monitored with the fibrinolytic assay (Fig. 3). Schwann cells were plated on the ¹²⁵I-fibrin-coated multiwell unit. The assay was started by replacing the plating medium with DME medium supplemented with one of the following: (i) 10% acid-treated fetal calf serum (without protease inhibitors); (ii) 10% acid-treated fetal calf serum with increasing amounts of GF₂; (iii) 10% acid-treated fetal calf serum with increasing amounts of GF₁; or (iv) 10% fetal calf serum.

Extracellular PA activity in the growth medium was directly related to the concentration of the mitogen GF₂ (Fig. 3). In the absence of fraction GF₂, the levels of PA activity were low, and they increased by approximately 3, 4, and 5 times with the increasing GF₂ at 0.2, 0.5, and 0.8 μg/ml.

However, a different pattern of extracellular PA activity was observed when the mitogen was fraction GF₁ (Fig. 4). At early stages, 1–3 days, higher activity was monitored with the lower amounts of GF₁. Only at later stages, 4–6 days, the pattern of PA activity reversed and was higher in cultures that showed the maximal rate of cell division—i.e., high concentration of the mitogen GF₁ (Fig. 4). This paradoxical extracellular PA production probably is due to the presence of protease inhibitor in fraction GF₁ as established by experiments described below. No fibrinolytic activity was detected in cultures maintained in medium supplemented with 10% fetal calf serum with or without the mitogen.

In media supplemented with fetal calf serum. As noted by Raff et al. (18), the GGF can exert its mitogenic effect only in the presence of fetal calf serum; namely, it requires some serum components for its action. Since the measurements of extracellular PA were carried out in media supplemented with acid-treated fetal calf serum, it was necessary to determine the pattern of Schwann cell division when the cells were maintained in medium supplemented with this modified serum. With the same concentration of GF₁ present, Schwann cells proliferated faster in medium that was supplemented with fetal calf serum than in that supplemented with acid-treated fetal calf serum, with a ratio of 1.75 ± 0.1. Presumably, the acid-treatment of the serum inactivates some factors that are required for Schwann cell division.

Since Schwann cells seem to proliferate better in medium supplemented with untreated fetal calf serum, the production of extracellular PA by the cells maintained in DME medium containing 10% fetal calf serum and increasing concentrations of the mitogen fractions, either GF₁ or GF₂, was monitored. The conditioned media were collected, the protease inhibitors were inactivated by acid treatment, and then the media were assayed for PA activity. Schwann cells produced or expressed PA activity in a dose–response manner proportional to the amount of GF₂ in the medium (Fig. 5), similar to the pattern of their mitotic rate that was induced by the mitogen (Fig. 1). PA activity reached a maximum value with fraction GF₂ in the range of 0.2–0.4 μg/ml, and then there was a decline in the activity at higher concentrations of GF₂ (Fig. 5). However, a different pattern of PA activity in the conditioned media was obtained when cell proliferation was induced with GF₁—the growth factor fraction that con-

Fig. 2. Purified Schwann cells in culture. (A, ×156; B, ×250.)

Fig. 3. Production of extracellular PA by Schwann cells as a function of increasing concentration of GF₁ fraction in the growth medium. The Schwann cells were plated at 10⁵ cells per well, and the fibrinolytic assay was started by removing the plating medium and adding the assay media. The assay was performed in DME medium containing acid-treated fetal calf serum without GF₂ (control (○)) or with increasing amounts of GF₂: 0.2 μg/ml (●), 0.5 μg/ml (▲), and 0.8 μg/ml (△).
tains the protease inhibitor (Fig. 6). The measured PA activity increased in an accelerated manner with increasing concentrations of GF1 fraction, even though maximal cell division was achieved (for this GF1 preparation) at ~5 μg/ml. The most logical explanation for the difference between the two patterns in extracellular PA production (Fig. 5 versus Fig. 6) in response to fractions GF2 and GF1 is that the same amounts of enzyme are secreted in both cases. However, the protease inhibitor in fraction GF1 presumably protects and stabilizes for the PA molecules; hence, the apparent higher activity in the presence of GF1 fraction.

**Cellular PA Activity.** The increasing activity of PA in the proliferating cell population could be due either to the increasing number of cells producing this enzyme or to augmentation in the specific activity of the enzyme. Therefore, the cellular PA specific activities of quiescent and proliferating cell populations were determined: PA specific activities of Schwann cells maintained at a low rate of cell division (i.e., in control medium) and at a maximal rate of cell division induced either by GF1 or GF2. Cells were collected, solubilized, and assayed for PA activity at the same protein concentration. The specific activity of proliferating Schwann cells was 3–4 times higher than that of a population that proliferated at a low rate without the growth factor (Fig. 7). However, the specific activities of the cultures that divide at the same rate when GF1 or GF2 are the mitogens were identi-
cal (Fig. 7), thus leading to the conclusion that the proliferating population is the one that expresses or uses PA activity. No fibrinolytic activity was detected when Schwann cell samples were assayed without plasminogen.

**DISCUSSION**

This study reports two novel characteristics of Schwann cells: (i) these cells produce extracellular PA and, hence, probably utilize extracellular proteolysis, and (ii) it is mostly the proliferating Schwann cells that express PA activity, thus identifying an enzymatic machinery used by the tissue presumably at those stages when the cells divide. Schwann cell division and differentiation is controlled and dictated by the neurons, presumably by physical cell-contact (10, 31). This implies that, even though the Schwann cells proliferate and neuronal processes are extended, neuronal–Schwann cell interaction persists throughout the histogenesis process of this nervous system. The logistics of establishing and maintaining the correct cellular interaction, while each of the two cell types performs opposing/separating functions, is the focus of this study. It was established in a series of studies by Kalderon (4, 5, 32) that the plasmin-generating system is involved in the regulation of neuronal–glial cell interaction in embryonic chicken spinal cord cells in culture. In this study, a purified glial-cell type was obtained and examined. Advantage was taken of the fact that reproducible procedures exist for obtaining purified proliferating Schwann cells. Also, the availability of a growth factor that is a specific mitogen for Schwann cells provides a good tool to manipulate Schwann cell division.

It was established in this study that Schwann cells produce extracellular PA and that the expression of PA activity predominates in the proliferating Schwann cell populations. These results lead to the conclusion that the nervous tissue uses the extracellular proteolytic machinery, the plasmin-generating system, during the proliferating stages of the Schwann cells. This extracellular proteolysis presumably is used in the process of neuronal–Schwann cell matching, while the Schwann cells migrate or envelop neuronal processes, or both. Neuronal–Schwann cell interaction would be established by one or more proteins, cell adhesion proteins. This cellular interaction then would be dissolved (the cells would detach) when these adhesion proteins are cleaved; allowing for new attachment to be formed, the cleavage is performed by extracellular proteolysis (e.g., plasmin). At the same time, the possibility exists that the plasmin-generating system functions as a mitogen, as a regulator of Schwann cell division. The mitogenic signal from the neurons to the Schwann cells could be a membrane neuronal protein as suggested by Salzer et al. (14, 15); this protein could be part of the cell adhesion protein, a proteolytic fragment. It is intriguing to speculate that a single protein can serve two functions: (i) the complete molecule for the cell adhesion function and (ii), after the proteolytic cleavage, one of the proteolytic fragments for the initiation of Schwann cell division. This hypothetical possibility posits a mechanistic clue, for example, to the process of initiation of Schwann cell division upon nerve injury as a response to Wallerian degeneration. In other biological systems, experimental evidence suggests that such a protein exists. Fibronecin is a cell adhesion protein; its proteolytic fragments are mitogenic to fibroblasts (33), and it can promote Schwann cell proliferation (23).

Among the effects of the presence of protease inhibitors in the growth medium on the expression of the extracellular PA activity, the most intriguing is the apparently higher activity revealed when the medium was supplemented with fetal calf serum and fraction GF1—namely, in the presence of both the serum and the pituitary protease inhibitors (Fig. 6). This result focuses our attention on the possible role of protease inhibitors in regulation of Schwann cell division or migration, or both. These inhibitors may act in a concerted manner with the plasmin-generating system, adding another dimension to the fine control of localized extracellular activities.

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