Plasminogen Activator Release at the Neuronal Growth Cone

Abstract. *The site of plasminogen activator release by differentiated neuroblastoma clonal cell lines was determined with a fibrin overlay assay. Release of plasminogen activator was seen at the growth cone in 72 percent of the cells bearing neurites. For 21 percent of these cells the growth cone was the predominant or exclusive site of this enzyme activity. Selective release of protease at the "trailblazing" tip of the neurite may be important in neuron migration and neurite growth in vivo.*

Cell migrations during morphogenesis have been described in detail (*1*), but the molecular control of cell movement remains largely unknown. Tissue culture of nerve cells offers a means for studying molecular and cellular aspects of cell migrations accompanying neurogenesis.

We recently demonstrated the production of plasminogen activator (PA) by granule neurons in dissociated cell cultures of postnatal mouse cerebellum (*2*). This protease is produced by other migratory cells (*3*), and we have been exploring its possible role in cell movement. Attempts with a histological technique (*4*) to determine whether PA might be selectively released at the tips of growing neurites were hampered in primary brain cultures by the small size of the neurons, their tendency to aggregate with other cells, and weak fibrinolysis compared to that occurring during growth of malignant cells.

Therefore, we used neuroblastoma clonal cell lines capable of neurite extension to explore enzyme release in cells of a highly specialized morphology. When C1300 neuroblastoma clone N18 (*5*) or neuroblastoma/glioma hybrid clone NG108cc15 (*6*) were grown in medium containing serum, 95 to 100 percent of the cells were positive for plasminogen-dependent proteolysis by the fibrin slide assay (Fig. 1a). Lytic zones were round and centered about the cell body, and they increased in diameter with time of incubation. Fibrinolysis was not seen in the absence of plasminogen (Fig. 1b) or when the cover slips were first fixed with formaldehyde or incubated with soybean trypsin inhibitor.

Clone NG108 was chosen for detailed study since it can be seeded at low density, grows attached to the substrate, and exhibits spontaneous neurite outgrowth in chemically defined serum-free N2 medium (*7*). The facility with which certain neuroblastoma clones extend neurites in synthetic medium may be due, in part, to the effects of plasminogen depletion on neurite development (*8*). Neurite outgrowth can be further augmented by the addition of dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) (*9*). When neurite formation was induced in N2 medium with or without the addition of dibutyryl cyclic AMP, 95 percent of the cells were again positive for fibrinolytic activity (Fig. 2). The majority of the cells (72 percent) displayed significant PA activity at the growth cone, the strongly adherent neurite tip. In 21 percent of the cells PA release was predominantly or exclusively restricted to this leading front (*10*) of neurite extension (Fig. 1, c to f, and Fig. 2). A prominent growth cone with microspikes is shown in Fig. 1f; very fine microspikes were visible on

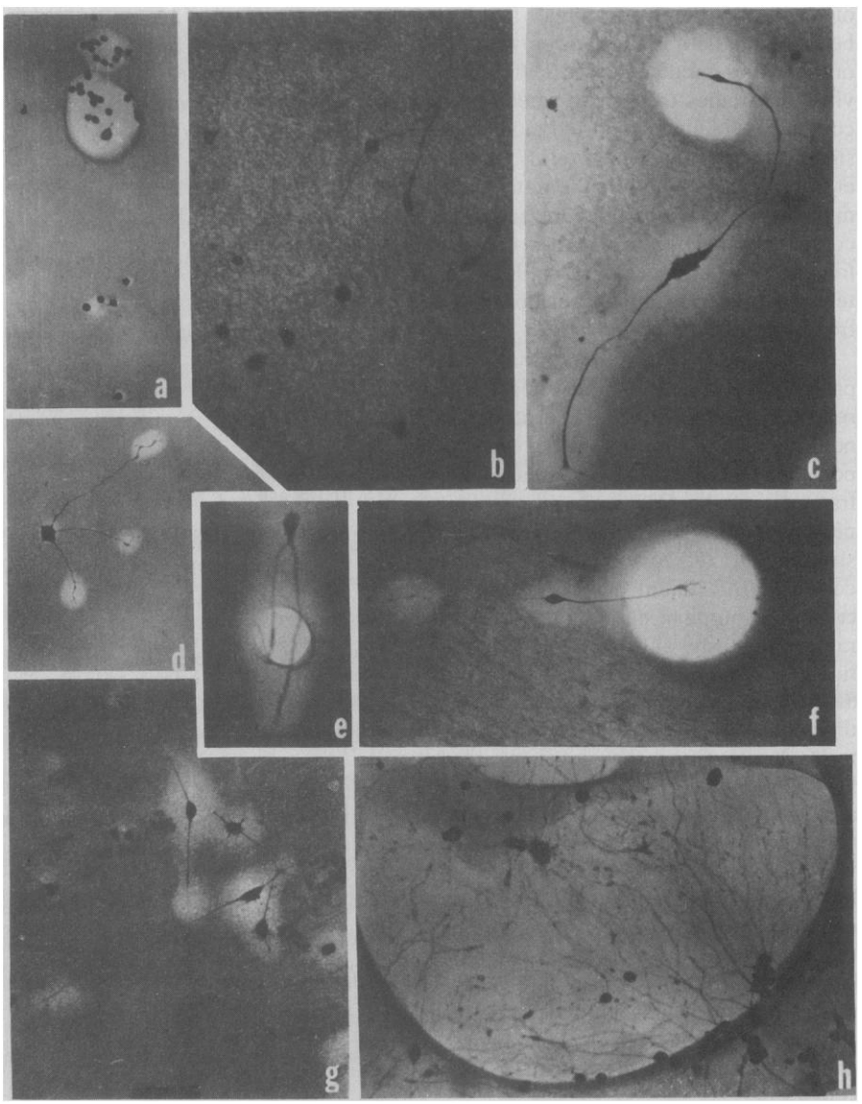


Fig. 1. Photomicrographs showing sites of PA release from N18 and NG108 cells. Cells were seeded onto uncoated or poly-D-lysine–coated cover slips and used after 1 to 5 days in the fibrin slide assay with plasminogen-containing fibrinogen (*19*), except for the slide shown in (b), in which the overlay was made with plasminogen-depleted fibrinogen (*20*). Cover slips were washed three times with phosphate-buffered saline, a drop (~ 25 μl) of bovine thrombin (Sigma grade III, 50 μl/ml) was applied and allowed to coat the surface, and the cover slips were drained. A drop (~ 40 μl) of the fibrinogen (10 mg/ml) was similarly applied and the cover slips were partially drained. The residual coating of fibrinogen clotted in 5 to 15 seconds. The cover slips were incubated at room temperature in a moist chamber to allow the fibrinolytic reaction to proceed, stained with Coomassie brilliant blue R, and photomicrographed. The cells shown are NG108 cells in serum-free N2 medium except for those shown in (a), which are N18 cells grown in medium containing 10 percent fetal calf serum, and those shown in (g), which are N18 cells grown in medium containing 0.3 percent fetal calf serum. The cells shown in (c) to (f) and (h) were treated to promote neurite outgrowth as described in text. The fibrin slide assay required less time for development of lytic zones than methods in which test substrates are embedded in agarose (*21*). Scale bar, 100 μm.

0036-8075/81/0925-1532$01.00/0

most cells at higher magnification. Selective enzyme release at the growth cone could be detected as far as 700 μm from the cell body.

An especially pronounced lytic zone was seen when two neurites (from the same or different cells) were in close apposition (Fig. 1e). This could be related to the rapid development of microspikes which occurs when a neurite "feels" its way along another process or cell body (*11*). Neurite outgrowth from clusters of NG108 cells was radial, and areas of high neurite density with relatively few cell bodies showed extensive fibrinolysis (Fig. 1h). A similar localization of PA at the growth cone was observed with clone N18, which extends neurites only when plated in medium containing 0.3 percent serum (Fig. 1g).

Possible artifacts resulting from the method were considered. A lytic zone might be detected at a neurite tip if the tip were in contact with a cell body that released enzyme but was then dislodged from the cover slip during fixation or staining. This was discounted by scoring individual well-separated cells before and after the assay. Possible tearing of the fibrin clot by neurite growth is not involved. Neurite length of several cells marked before the assay did not change significantly (that is, 1 μm) during the 2- to 4-hour incubation at room temperature.

To assess the degree to which PA release is limited to the growth cone, a higher power negative of the photograph in Fig. 1d was enlarged and the two-dimensional surface areas of cell body and growth cone (including microspikes) were compared with those of their respective lytic zones. The result suggests that enzyme release is 7.5 times greater at the growth cone than at the cell body. Although this value would be lower if the total surface area of the fine microspikes could be calculated, the point remains that a thin cellular process can display membrane properties that differ quantitatively from those of the cell body.

Since cells other than neurons can possess thin membrane extensions, often as part of a crawling movement on the substrate, it was necessary to examine other cell types. Early passage mouse embryo fibroblasts were uniformly negative for PA by the fibrin slide assay. Avian sarcoma virus–transformed fibroblasts (*12*) were positive for PA but exhibited a rounded morphology. Cell spreading and limited process formation could be induced by growing the transformed fibroblasts on surfaces coated with poly-D-lysine or by adding 1 m*M* dibutyryl cyclic AMP to the medium. In these cases PA release could be detected around all areas of the cell body, with significant amounts at the process. The fibrinolytic area seemed to be roughly proportional to membrane area. However, no pronounced or exclusive release of PA at the tips of the processes was observed.

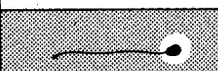
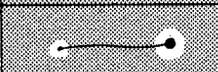
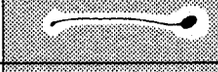
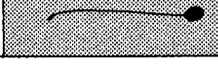
Site	Schematic	Percentage of cells
Soma only		23
Soma and growth cone		42
Soma and grow cone (growth cone predominating)		10
Growth cone only		11
Soma, growth cone, and neurite		9
No lytic zone		5

Fig. 2. Sites of PA release from NG108 cells. Methods were as described in Fig. 1. Cells evaluated were those not in contact with each other and possessing neurites longer than 100 μm.

Laug *et al.* (*13*), using the plate assay with ^{125}I-labeled fibrin, reported that dibutyryl cyclic AMP greatly stimulates the production of PA in uncloned C1300 neuroblastoma cells. We observed PA release at growth cones regardless of the method used to induce neurite outgrowth. The quantitative increase in PA activity with differentiation measured by Laug *et al.* probably reflects the increased number of neurites and growth cones in such cultures.

The mechanism of enzyme release at the growth cone is not clear. Most evidence suggests that PA is a tightly bound membrane enzyme (*14*) and that secretion may be regarded as "membrane shedding" (*15*). Such loss of membrane material may be potentiated by the rapid movement of microspikes. To test this possibility, NG108 cells were incubated for 24 hours with cytochalasin B (10 μg/ml) to prevent microspiking of actin-containing membrane projections while maintaining the neurite length (*16*). The cells were overlain with a fibrin clot also containing cytochalasin B. Release of PA was inhibited around the cell body as well as the growth cone. Thus no conclusion can be made about microspikes in particular, although actin-containing cytoskeletal components probably have a role in PA release. Vassalli *et al.* (*17*) reported that cytochalasin B does not inhibit secretion of macrophage PA into conditioned medium; the difference in these results may be due to the difference in cell type or fraction (soluble or insoluble) of PA being measured.

In other experiments we found that large amounts of PA can be deposited on the substratum by the cells used here as well as by other malignant cell lines. Thus activity measured at the neurite tip may be derived from both localized release and a trail of membrane material left behind by the advancing growth cone. PA secretion by other cell types may be involved in localized, regulated tissue remodeling accompanying normal developmental cell movements (*3*). Release of this enzyme may also be part of the mechanism allowing nerve fibers and cell bodies to move large distances in the tissue matrix of the developing nervous system. In preliminary studies with dissociated spinal ganglia cultures we observed PA release around neuronal growth cones.

The qualitative methodology described here allows elucidation of aspects of PA secretion to a degree not possible with the more widely used radiochemical method (*3, 13, 18*), and should prove useful in studies of protease release at other cellular processes during developmental interactions.

ALPHONSE KRYSTOSEK
NICHOLAS W. SEEDS
Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver 80262

References and Notes

1. J. P. Trinkaus, in *The Cell Surface in Animal Embryogenesis and Development*, G. Poste and G. L. Nicolson, Eds. (North-Holland, Amsterdam, 1976), p. 225.
2. A. Krystosek and N. W. Seeds, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
3. W. H. Beers, S. Strickland, E. Reich, *Cell* **6**, 387 (1975); S. Strickland, E. Reich, M. I. Sherman, *ibid.* **9**, 231 (1976); J. C. Unkeless, S. Gordon, E. Reich, *J. Exp. Med.* **139**, 834 (1974).

4. A. S. Todd, *J. Pathol. Bacteriol.* **78**, 281 (1959); T. Astrum, in *Proteases and Biological Control*, E. Reich, D. B. Rifkin, E. Shaw, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1975), p. 343.
5. N. W. Seeds, A. G. Gilman, T. Amano, M. W. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 160 (1970).
6. B. Hamprecht, *Int. Rev. Cytol.* **49**, 99 (1977).
7. J. E. Bottenstein and G. H. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 514 (1979).
8. P. R. Becherer and J. T. Wachsman *J. Cell Physiol.* **104**, 47 (1980).
9. A. Krystosek, in preparation.
10. Anuran and chick sensory ganglia neurons explanted into culture dishes extend axons along the substratum [R. G. Harrison, *J. Exp. Zool.* **9**, 787 (1910); D. Bray, *Proc. Natl. Acad. Sci. U.S.A.* **65**, 905 (1970); M. A. Luduena, *Dev. Biol.* **33**, 268 (1973); P. C. Letourneau, *ibid.* **44**, 77 (1975)]. Elongation occurs at the growth cone, which rapidly extends and retracts long microspikes. Axonal growth cones can also be seen in the developing nervous system by using histological techniques [S. Ramon y Cajal, *Studies on Vertebrate Neurogenesis*, L. Guth, Transl. (Thomas, Springfield, Ill., 1960)]. Growth of neurites from neuroblastoma cells is qualitatively similar, in terms of the cellular processes involved, to that described for primary neurons (*5*).
11. G. A. Dunn, *J. Comp. Neurol.* **143**, 491 (1971).
12. Fibroblasts of the European field vole (*Microtus agrestis*) transformed by the Schmidt-Ruppin strain of avian sarcoma virus were the kind gift of R. L. Erikson. Properties of the cells are described in Y. C. Chen, M. J. Hayman, P. K. Vogt, *Cell* **11**, 513 (1977); M. S. Collett and R. L. Erikson, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2021 (1978); and S. E. Nielson and T. T. Puck, *ibid.* **77**, 985 (1980).
13. W. E. Laug, P. A. Jones, C. A. Nye, W. F. Benedict, *Biochem. Biophys. Res. Commun.* **68**, 114 (1976).
14. J. P. Quigley, *J. Cell Biol.* **71**, 472 (1976).
15. P. H. Black, *Adv. Cancer Res.* **32**, 76 (1980).
16. K. M. Yamada, B. S. Spooner, N. K. Wessells, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 1206 (1970).
17. J.-D. Vassalli, J. Hamilton, E. Reich, *Cell* **8**, 271 (1976).
18. J. C. Unkeless, A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, E. Reich, *J. Exp. Med.* **137**, 85 (1973).
19. A. G. Ware, M. M. Guest, W. H. Seegers, *Arch. Biochem.* **13**, 231 (1967).
20. M. W. Mosesson, *Biochim. Biophys. Acta* **57**, 204 (1962).
21. Casein agarose and fibrin agarose overlay assays were previously used to detect PA [A. R. Goldberg, *Cell* **2**, 95 (1974); P. Jones, W. Benedict, S. Strickland, E. Reich, *ibid.* **5**, 323 (1975)]. Agarose overlay thickness was approximately 400 μm in the center of the plate, increasing to 2000 μm at the edges (2.3 ml of substrate per 60-mm dish). Typical development times for lytic zones around transformed cell colonies were 12 to 16 hours at 37°C. More than 24 hours was needed to detect fibrinolysis by single NG108 cells. With the fibrin slide method clots had an average thickness of 15 μm and could vary above and below this value on the same cover slip. Thickness of the overlay is decreased when the clot is dehydrated for microscopic viewing. Partial fibrinolysis was detected after as little as 30 minutes, and incubations of 2½ to 3 hours were used for the examples shown in Fig. 1.

5 May 1981

Asbestos Surface Charge Heterogeneity and Biological Effects

To accomplish the goal of Schiller *et al.* ["to minimize the undesirable occupational hazards" of amphibole particles (*1*, p. 1530)], it is important to relate biological effects to the reported surface charge heterogeneity, which is responsible for a smaller net surface charge for blocky amphibole cleavage fragments than for elongated asbestos fibers. The impact that long-term in vivo leaching has on net surface charge and surface charge heterogeneity should be considered when one is relating the results of Schiller *et al.* to long-term biological effects.

Net surface charge, as represented in terms of electrophoretic mobility (μ), is expressed in (*1*) as a function of basal (μ_1) and lateral (μ_2) electrophoretic mobilities and the aspect ratio for a cylindrical particle (γ, length divided by diameter)

$$\mu = \frac{\mu_1 + 2\mu_2\gamma}{2\gamma + 1} \quad (1)$$

Adsorption studies with amphibole particles have shown that in neutral aqueous media μ_1 is positive because of cation layers and μ_2 is negative because of silica ribbons (*2*). By statistically fitting empirical data to Eq. 1, Schiller *et al.* quantified μ_1 and μ_2 for amphibole particles.

In relating charges on fragments and fibers to long-term biological effects, a complicating factor is in vivo leaching (*3*), which for amphiboles would mainly decrease the positive basal charge by preferential dissolution of hydrated surface cations (*4*). For amphibole edge leaching, cation removal changes μ_1 to $\mu_1^*(t)$, where t is time; as μ_2 is essentially determined by less soluble silica, its value is relatively constant. The net electrophoretic mobility for leached fibers [$\mu^*(t)$] is thus a function of leaching time and is approximated by

$$\mu^*(t) = \frac{\mu_1^*(t) + 2\mu_2\gamma}{2\gamma + 1} \quad (2)$$

To simulate the rate of change for μ_1 in vivo, we leached fibers of the amphibole crocidolite for various times using Tyrode's (physiological buffer) solution (*5*). From measurements of $\mu^*(t)$ for fibers having a γ of 5, I estimate that $\mu_1^*(t)$ would equal zero after 33 days and that on further leaching, both edges and faces would become negatively charged. Since similar leaching would occur in vivo, its impact on the net surface charge and surface charge heterogeneity should be considered when one is relating the results of Schiller *et al.* to long-term biological effects. In addition to leaching, adsorption onto fibers of materials such as surfactants (*5*) would also affect the fiber surface charge in vivo.

Long-term leaching in vivo could obliterate fiber edge and face charge differences. Blocky cleavage fragments and elongated fibers would then display a net surface charge comparable to that for homogeneous materials such as silica, quartz, and glass and independent of γ. Certain fibers having homogeneous surfaces, such as glass fibers, are similar to crocidolite in that fibers with large values of γ induce a higher incidence of malignant pleural mesenchymal neoplasms than those with small values (*6*). As the net surface charge is independent of γ for fibers having homogeneous surfaces, factors other than fiber edge and face charge heterogeneity would account for the observed variation in biological activity.

WILLIAM GODDARD LIGHT
Walden Division of Abcor, Inc.,
Wilmington, Massachusetts 01887

References and Notes

1. J. E. Schiller, S. L. Payne, S. E. Khalafalla, *Science* **209**, 1530 (1980).
2. N. A. Prasad and F. D. Pooley, *J. Appl. Chem. Biotechnol.* **23**, 675 (1973).
3. A. Holmes and A. Morgan, *Nature (London)* **215**, 441 (1967); A. Morgan, A. Holmes, C. Gold, *Environ. Res.* **4**, 558 (1971).
4. J. Ralston and J. A. Kitchener, *J. Colloid Interface Sci.* **50**, 242 (1975); I. Choi and R. W. Smith, *ibid.* **40**, 253 (1972).
5. W. G. Light and E. T. Wei, *Nature (London)* **265**, 537 (1977); *Environ. Res.* **13**, 135 (1977); in *The In Vitro Effects of Mineral Dusts*, R. C. Brown, Ed. (Academic Press, London, 1980), p. 139.
6. M. F. Stanton, M. Layard, A. Tegeris, E. Miller, M. May, E. Kent, *J. Natl. Cancer Inst.* **58**, 587 (1977); M. F. Stanton and C. Wrench, *ibid.* **48**, 797 (1972).
7. I thank W. C. Cooper, E. T. Wei, E. T. Peters, and A. E. Rogers for reviewing the manuscript.

30 December 1980

The comments by Light raise an interesting area for further study, namely, to determine the relationship between electrophoretic mobility (μ) and aspect ratio (γ) for amphibole fragments and fibers that have been suspended in biological fluids. However, there is no basis for assuming that in biological systems basal electrophoretic mobility (μ_1) varies with time and lateral electrophoretic mobility (μ_2) is constant. One could test this hypothesis by obtaining data on μ and γ for leached fibers and fragments by the method that we used; μ data from fibers alone are not sufficient to permit one to determine the effects of leaching independently on μ_1 and μ_2. Equation 5 that we derived (*1*) shows that μ is a function of γ and charge densities on different regions of the particles. It is necessary to measure γ along with μ in order to evaluate μ_1 and μ_2.

J. E. SCHILLER
S. L. PAYNE, S. E. KHALAFALLA
Twin Cities Research Center,
Bureau of Mines,
Minneapolis, Minnesota 55417

References

1. J. E. Schiller *et al.*, *Science* **209**, 1530 (1980).

3 August 1981

0036-8075/81/0925-1534$01.00/0