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Regulation of neuronal migration and neuritogenesis by distinct surface proteases

Relative contribution of plasmin and a thrombin-like protease

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The relative contribution of two neuronal surface proteases, plasmin and a protease with thrombin-like specificity, on NB2a/d1 neuroblastoma migration and neuritogenesis were examined. Exogenous plasmin induced cell body rounding and increased cell migration, but did not prevent or reverse neurite outgrowth. Inhibition of endogenous plasmin by its specific inhibitor, aprotinin, suppressed migration but did not induce neuritogenesis. Removal or inhibition of the thrombin-like protease by serum deprivation or hirudin addition, respectively, induced neurite outgrowth, as shown in our previous studies, but did not suppress migration. By contrast, trypsin induced simultaneous cell rounding and neurite retraction. These findings indicated that plasmin may regulate cell migration, while the thrombin-like protease may regulate facets of neurite outgrowth. Although unable to induce de novo neuritogenesis, plasmin inhibition potentiated the otherwise transient neurites induced by simultaneous inhibition of the thrombin-like protease. Since cultured neuronal cells migrate primarily in the direction of newly elaborated neurites, this finding is interpreted to indicate that cessation of neuronal migration by plasmin inhibition enhances net neurite outgrowth by inhibition of the putative thrombin-like protease.

Plasmin; Thrombin; Neuronal migration; Neuritogenesis; Cell adhesion; Cell motility; Differentiation

1. INTRODUCTION

Proteolysis is instrumental at several stages during neuronal differentiation and neurite outgrowth (for review, see [1]). Neurons release proteases [2–10], certain of which regulate the turnover of specific membrane proteins, and the inhibition of these proteases suppresses neuroblast migration and fosters the establishment of minor neuritic processes [2,11–19]. Two proteases which play regulatory roles in these processes are plasmin and thrombin (or an undisclosed protease with thrombin-like specificity).

The cell-mediated conversion of the zymogen plasminogen to the active enzyme plasmin is accomplished by specific cell-associated and urokinase-type plasminogen activators (for review, see [20]), both types of which are expressed by a variety of cultured neuronal cells, including granule neurons [5], sympathetic and sensory neurons [21,22] and neuroblastoma [23]. Since plasmin is capable of hydrolyzing components of the extracellular matrix, the conversion of plasminogen into the active plasmin by these activators has been suggested to play a key role in the timing of cell migration [6,7,20].

Thrombin, or a thrombin-like enzyme, present on the outer plasma membrane surface or as a serum component, has been implicated in the regulation of neurite outgrowth, since the specific thrombin inhibitor, hirudin, induces neuritogenesis as effectively as serum depletion [16,24,25], and exogenous thrombin prevents or reverses neuritogenesis in some [11,25,26] but not all [16] neuroblastoma cultures. The mRNA for the thrombin zymogen, prothrombin, has been demonstrated in both rat and human brain [25]. Thrombin has also been shown to inactivate fibroblast growth factor, a known neurite-promoting agent [27], suggesting that it may regulate neuritogenesis at multiple levels.

While differential expression of surface proteases such as plasmin and thrombin undoubtedly accompanies neuronal differentiation, the localized secretion by glial cells of specific inhibitors of these proteases profoundly influences the timing of neuronal development and maturation. Glial cells secrete protease inhibitors, termed 'nexins' [28], which promote neurite formation and possess potent inhibitory activity against tissue plasminogen activator, urokinase, and thrombin [1,26,29–32]. Since nexins are 10-fold more effective inhibitors of thrombin than of plasmin [1], they may function chiefly to inhibit thrombin activity; non-nexin in-

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hibitors of plasminogen activators are also secreted by glial cells [33]. Glial cells also secrete extracellular matrix components (for review, see [34]) which, by providing a more adhesive substrate, foster neuritogenesis, and stabilize otherwise transient neurites elaborated by some neuronal cells in response to protease inhibitors [17,35]. Localized application of protease inhibitors [36] can also influence the direction of neurite outgrowth. Furthermore, secreted protease inhibitors can bind to the extracellular matrix and this binding increases their inhibitor activity and alters their specificity [37].

In some cell culture systems, increased plasmin activation enhances neurite outgrowth [2,12,35]. Moreover, plasminogen activators become localized to the growth cone [23], leading to the additional suggestion [1] that the growth cone may represent a structure that continues migrating despite the cessation of migration of the neuronal cell body. In other cell culture systems, however, neurite outgrowth was attributed to inhibition of thrombin (e.g. [16,17,32]). Inhibition of thrombin promotes the elaboration of transient neurites in neuroblastoma cells [17]; these transient neurites can be stabilized by an additional class of inhibitors, active against calpain but not thrombin, that are themselves incapable of inducing neuritogenesis [16].

In the present study, we demonstrate that plasmin activity modulates cell adhesion and migration in NB2a/d1 neuroblastoma cells, but does not prevent or reverse neurite outgrowth, while, as we have previously demonstrated, the activity of a thrombin-like protease exhibits the opposite behaviour. Our results, and those of the above studies, underscore the likelihood that the events of neurite initiation and continued elaboration are controlled by a balance of multiple proteases and their inhibitors.

2. EXPERIMENTAL

2.1. *Cells and culture conditions*

NB2a/d1 cells [17,35] were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 25 μg/ml Gentamycin (Sigma Chemical Co., St. Louis, MO) in a humidified atmosphere of 95% air and 5% CO_2.

2.2. *Treatment of neuroblastoma cells with proteases and inhibitors*

To assay the effect of exogenous proteases and protease inhibitors, cells were plated at a density of 10^4/plate in 35-mm^2 plates or 10^3/well in 24-well trays in 1 ml of the above medium. Twenty-four hours later, the medium was replaced with medium containing either 0.8% serum, which is sufficient to maintain cell division and prevent neurite outgrowth, but permissive for the effects of various proteases and inhibitors [16], or no serum, to induce the elaboration of axonal neurites [15]. At this time, triplicate cultures received one or more of the following: thrombin (0.1–10 U/ml), plasmin (0.1–10 U/ml), hirudin (5 U/ml), aprotinin (0.1–1.0 U/ml), trypsin (0.1–10 U/ml). All components were obtained from Sigma.

2.3. *Quantitation of neuronal morphology and neurite length*

At 4 or 24 h after the above treatments, duplicate cultures were rinsed in Tris-buffered saline (TBS; pH 7.4), fixed for 10 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 15 min, then rinsed and stored in TBS as described previously [15]. Between 100–200 cells in each of the fixed cultures were examined by phase-contrast microscopy for neuronal morphology and neurite length. The relative number of cells possessing rounded soma (defined, and readily detectable by, their characteristic phase-dark, refractile appearance versus the phase-lucent appearance of flattened cells) is determined by the following calculation: (mean number of cells with rounded, phase-dark soma/mean number of total cells scored) × 100. While rounded cells typically lack filopodia, our preliminary studies (below) demonstrate that soma roundness is independent of neurite elaboration: both flattened and rounded cells are routinely observed with and without neurites depending upon culture treatment.

The relative extent of neurite outgrowth was quantitated by comparison of neurite length with respective somal diameter (SD) as previously described [15]. Previous quantitative studies of NB2a/d1 neurite outgrowth [15,16] have shown that the majority of undifferentiated NB2a/d1 cells possess putative neurites of <1 SD, while the respective modal length of neurites induced by various treatments is 1–2 SD at 4 h, and 2–3 SD at 24 h. Accordingly, for analyses of neurite induction at 4 h, the percentage of cells/culture with neurites was determined by the following calculation: (mean number of cells with ≥1 SD neurites/ mean number of total cells scored) × 100. Similarly, the percentage of cells/culture with neurites at 24 h was determined by calculating (mean number of cells with ≥2 SD neurites/mean number of total cells scored) × 100. Values obtained in the above quantitations were statistically compared by Student's *t*-test as described [15,35].

2.4. *Quantitation of neuronal migration*

The influence of proteases and their inhibitors on migration was examined by a modification of the technique of Meissauer et al. [38]. Cells (100 cells/1 ml) were plated in BioCoat cell culture inserts with a Matrigel base (Collaborative Research, Bedford, MA) within 24-well trays. The cells were plated in 1 ml of medium containing either 0.8% serum or no serum, with or without one of the following: plasmin (10 U/ml), aprotinin (1.0 U/ml), thrombin (10 U/ml), and hirudin (5 U/ml). Twenty-four hours later, the inserts were removed, and the percentage of cells which had migrated through the Matrigel and had adhered to the culture tray substratum were quantitated. Duplicate wells from each of two trays were examined. Additional cultures were plated directly into wells without inserts in the presence of the above compounds; no deterimental effect on cell viability or attachment to culture plastic was observed (not shown). The 'fold change' in migratory cells was determined by dividing the mean percentage of cells which had reached the culture tray substratum under various conditions by the mean percentage obtained in the presence of serum without further additions.

2.5. *Cell-free assays of inhibitor specificity*

The specificity of aprotinin and hirudin for plasmin and thrombin, respectively, was determined by degradation of exogenous chromogenic substrates essentially as previously described [16]. Chromozym-TH (specifically hydrolyzed by thrombin) and Chromozym-PL (specifically hydrolyzed by plasmin) were incubated with 10 U of plasmin or thrombin and degradation was monitored spectrophotometrically in cell-free assays in the presence and absence of 10 U each of aprotinin (specific inhibitor of plasmin) or hirudin (specific inhibitor of thrombin) in duplicate according to the substrate manufacturer's instructions (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The percent inhibition of protease activity by each inhibitor was determined by the formula: [(mean amount of substrate hydrolyzed in the presence of each inhibitor/mean amount of substrate hydrolyzed in the absence of inhibitor) × 100]−100. We observed that, under these conditions, aprotinin inhibited plasmin activity by 97.6% but thrombin by only 4%, while hirudin inhibited 100% of thrombin activity but only 8.1% of plasmin activity.

3. RESULTS

3.1. *Effect of plasmin on neuroblastoma morphology*

A significant increase in the number of cells with rounded perikarya was observed within 4 h following addition of plasmin (0.1 U/ml) to NB2a/d1 cells in the presence of serum (Fig. 1). When cultures were deprived of serum to induce neurite outgrowth [15,16], plasmin still caused perikaryal rounding, but did not alter the rate and extent of neurite outgrowth (Table I; Fig. 1). The plasmin inhibitor, aprotinin [39], inhibited plasmin-dependent cell rounding, and, unlike hirudin [16,17], did not induce neurite outgrowth (Table I; discussed further below). These effects on soma morphology were specific since trypsin (0.1–1 U/ml) did not induce cell rounding within 4 h and extended (48 h) treatment resulted in neurite retraction simultaneously with perikaryal rounding (Table I). Alteration of soma, but not neuritic, morphology following manipulation of plasmin activity therefore suggested that plasmin was more active against components localized primarily on the perikaryal rather than neuritic surface.

3.2. *Plasmin enhances neuronal migration*

In the presence of serum, a relatively small percentage of NB2a/d1 cells were observed to migrate through a Matrigel barrier. Depletes of the thrombin-like protease by serum-deprival [16,17] did not alter this percentage (Fig. 2), nor did addition of the specific thrombin inhibitor, hirudin (not shown). However, the addition of plasmin to serum-containing or serum-free medium increased the percentage of migratory cells by approximately 5-fold, while the addition of aprotinin reduced this percentage by approximately 5-fold.

3.3. *Potentiation and enhancement of neurite outgrowth by inhibition of plasmin*

Aprotinin alone had no effect on neurite outgrowth (Tables I and II); neither did it influence the number of hirudin-induced neurites present at 4 h (Table II). As previously shown [17] culturing of cells for 24 h in the presence of serum induced an approximate doubling of neurites; identical results were observed in the presence

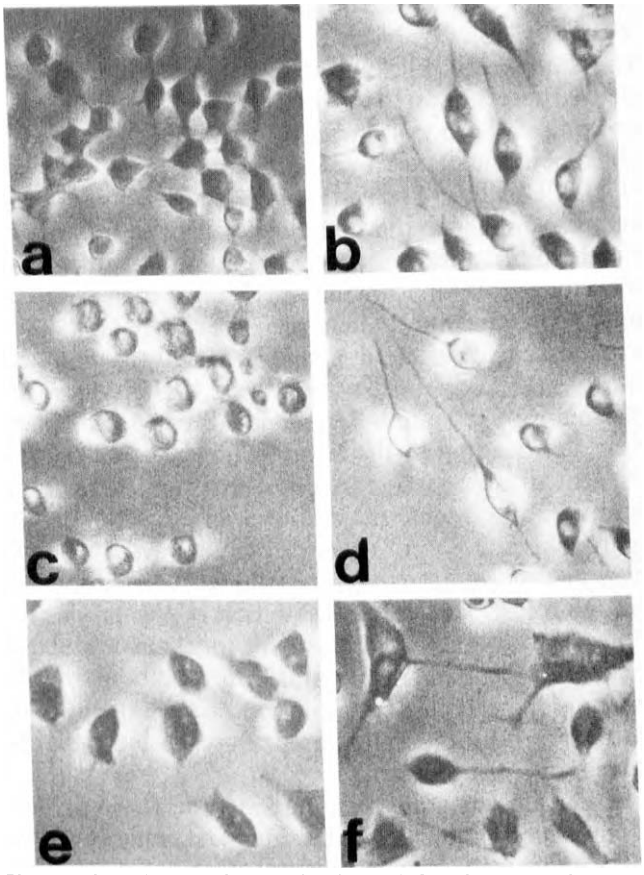

Fig. 1. Effect of manipulation of activity of plasmin and the thrombin-like protease on NB2a/d1 morphology. Cells were cultured for 4 h in medium containing 0.8% serum (panel a), no serum (panel b), 0.8% serum + plasmin (panel c), no serum + plasmin (panel d), 0.8% serum + aprotinin (panel e) and 0.8% serum + hirudin (panel f), then were fixed and photographed under phase-contrast microscopy. Note that plasmin induces rounding of cell bodies but does not retract neurites induced by serum deprivation (panels c, d). Note also that plasmin inhibition by aprotinin does not induce neurites (panel e) but inhibition of the thrombin-like protease by hirudin (panel f) induces neurites.

Table I

Effect of exogenous proteases and inhibitors on NB2a/d1 morphology

Conditions	Time	% Cells w. neurites	% Rounded cells
− serum	4 h	25.4 ± 7.3	24.7 ± 6.5
− serum + plasmin	4 h	19.8 ± 5.9	58.2 ± 15.3*
− serum + plasmin + aprotinin	24 h	24.5 ± 4.7	26.6 ± 6.8
− serum	24 h	24.7 ± 6.5	24.7 ± 10.1
− serum + plasmin	24 h	23.8 ± 4.7	54.7 ± 7.5*
− serum + trypsin	4 h	23.7 ± 6.3	26.1 ± 5.7
− serum	48 h	67.9 ± 4.4	22.7 ± 2.1
− serum + trypsin	48 h	41.8 ± 3.3*	71.3 ± 9.1*

* $P < 0.0005$ vs. −serum alone as compared by Student's t-test. All other values statistically equivalent to those obtained for −serum alone at respective incubation time. N.D., not determined (see Table II). Cells were deprived of serum and treated with plasmin or trypsin for the indicated times then fixed, and the % cells with neurites and the % rounded cells were separately quantitated under phase-contrast microscopy as described in section 2.

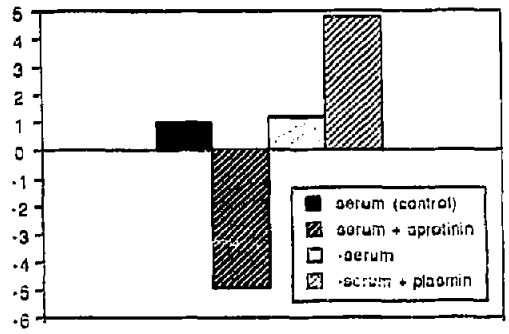

Fig. 2. Influence of plasmin activity on NB2a/d1 migration. Equivalent numbers of cells were plated in medium containing serum, medium containing serum + aprotinin, medium lacking serum, and medium lacking serum + plasmin into Matrigel-based inserts placed within 24-well trays as described in section 2. After 24 h, the number of cells which had migrated through the Matrigel base and attached to the culture tray substratum were quantitated by phase-contrast microscopy. These were considered migratory cells (see text); the number of migratory cells differed under each experimental condition. The 'fold change' in migratory cells induced by aprotinin, serum deprivation, or serum deprivation + plasmin vs. that observed under 'control' conditions (i.e. medium containing serum with no further additives) was determined by dividing the mean percentage of cells which had migrated through the Matrigel to the culture tray substratum under each condition by the mean percentage of observed under conditions.

of aprotinin. The transient neurites induced in NB2a/d1 cells by 4 h treatment with hirudin had retracted by 24 h (see also [16,17,35,40]). However, hirudin-induced neurites did not retract if aprotinin was also present. Similarly, aprotinin enhanced neurite outgrowth induced by serum deprivation.

4. DISCUSSION

The present study has examined the respective roles of two neuronal surface proteases, plasmin and a thrombin-like protease, in neuronal differentiation and neuritogenesis. Exogenous plasmin induced cell body rounding and increased cell migration, but neither prevented nor reversed neurite outgrowth. Rounding of cells following exposure to exogenous plasmin is likely to be a reflection of diminished adhesion, since the percentage of migratory cells was also increased. The effects of increases or decreases in plasmin activity were independent of the presence or absence of serum. Finally, inhibition of plasmin by aprotinin suppressed migration but did not induce neuritogenesis. The apparent specificity of plasmin for the soma, rather than neuritic, compartment is further underscored by the simultaneous withdrawl of neurites and rounding of somata in the presence of trypsin. These findings for plasmin are the opposite to those obtained following manipulation of the activity of the thrombin-like protease that regulates NB2a/d1 neuritogenesis (see also [16,17,35,40]). However, plasmin inhibition potentiated neurites elaborated in response to inhibition (by hirudin addition) or removal (by serum deprivation) of the thrombin-like protease. Recently, Liang and Crutcher [41] demonstrated preferential migration of cells in the direction of newly elaborated neurites, thereby lessening the apparent extent of neuritogenesis. NB2a/d1 cells have been shown to continue migration following neuritogenesis, and to continually elaborate and resorb multiple neurites and neurite-like processes during the first 24 h of induction by inhibition of the thrombin-like protease [16]. Accordingly, plasmin inhibition may enhance net neurite outgrowth by preventing cell bodies from migrating in the direction of neurites induced by hirudin or serum removal. Indeed, suppression of cell motility has been shown to be the major rate-determining event in neuritogenesis in NG108-15 cells [42].

Table II

Enhancement and potentiation of neurites by plasmin inhibition

Culture conditions	% Cells with neurites	
	4 h Incubation	24 h Incubation
Serum	6.9 ± 2.4	14.6 ± 1.9
Serum + aprotinin	6.5 ± 2.6	14.1 ± 2.7
Serum + hirudin	22.7 ± 5.3*	13.7 ± 2.0
Serum + both	23.2 ± 2.7*	28.0 ± 8.5*
− serum	N.D.	28.6 ± 5.4*
− serum + aprotinin	N.D.	40.0 ± 5.1**

* $P < 0.0005$ vs. values obtained for serum as compared by Student's t-test. ** In addition, $P < 0.0005$ vs. values obtained for −serum as compared by Student's t-test. All other values statistically equivalent to those obtained for serum or −serum at 4 or 24 h, respectively. N.D., not determined. Cells were deprived of serum and treated with aprotinin, hirudin or both for the indicated times then fixed, and the % cells with neurites were quantitated under phase-contrast microscopy as described in section 2.

The physiological role of surface protease inhibition in neurite outgrowth in the developing brain remains uncertain [43]. These studies on cultured neuronal cells demonstrate that plasmin and thrombin, or a thrombin-like protease [16,17,35,40], mediate experimentally separable aspects of neuronal maturation and neuritogenesis. In developing brain, certain glial-derived inhibitors of plasminogen activators that are not active against thrombin [33] may initially suppress neuroblast migration, thereby allowing glial-derived thrombin inhibitors to induce efficient neuritogenesis. Similar hierarchical effects on the early stages of neuroblastoma differentiation have been described: glial-derived extracellular matrix factors [40] including laminin [35], and inhibition of a protease with calpain-like specificity [16], potentiate and enhance the outgrowth of the otherwise transient neurites induced by inhibition of the putative thrombin-like protease. In this regard, plasminogen binds with high affinity to both laminin and fibronectin [43], indicating that particular areas rich in extracellular matrix components may serve as 'reservoirs' for plasminogen during neuronal migration. Furthermore, neural crest migration on fibronectin requires urokinase-type plas-

minogen activator activity [3]. Similarly, glial-derived nexin, once complexed with thrombin, can bind to components of the extracellular matrix [44]. Since thrombin adsorbs to the neuronal plasma membrane [24] a temporal physical linkage, mediated by a thrombin-nexin complex, may occur between the neuronal surface and the extracellular matrix.

Differentiation of neuroblastoma cells by treatments such as retinoic acid or dbcAMP, both of which induce neurite outgrowth in NB2a/d1 cells [15,45], is ultimately accompanied by specific decreases in plasminogen activators [46,47]. In some cell systems, however, rather than undergoing a uniform decrease, plasminogen activators become localized to neuronal growth cones [23], and are thought to facilitate growth cone migration following cessation of cell body migration [1]. Whether or not a similar localization of plasmin activity accompanies continued NB2a/d1 neurite outgrowth and maturation, and under what conditions such localization could be crucial for growth cone migration and ultimate synaptogenesis, remain to be determined.

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