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Modulation of morphological differentiation of human neuroepithelial cells by serine proteases: independence from blood coagulation

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We have previously shown that a serum protein, termed differentiation reversal factor (DRF), is responsible for neurite retraction in differentiated cultures of an adenovirus 12 (Ad12) transformed human retinoblast cell line. Data is presented here to show that DRF is identical to the serine protease prothrombin. Both proteins have been immunoprecipitated using an antibody raised against purified prothrombin and have been shown to hydrolyse a specific thrombin substrate only after activation by the snake venom ecarin. Following addition to Ad12 HER 10 cells, which had previously been differentiated by culture in the presence of 2 mM dibutyryl cAMP in serum-free medium, thrombin and prothrombin caused half-maximal retraction of neurites at concentrations of 0.5 ng/ml and 20 ng/ml respectively. Interestingly, activation of prothrombin was shown to be unnecessary for biological activity. Using the inhibitor di-isopropylfluorophosphate (DIP), we have shown that abrogation of the proteolytic activity of thrombin also results in a loss (>2000 fold) of differentiation reversal activity. Thrombin and its zymogen both stimulated the mitosis of differentiated Ad12 HER 10 cells to a similar extent. In addition, differentiation reversal was highly specific since, at physiologically significant concentrations, closely related serine proteases did not cause neurite retraction. Prothrombin and thrombin also reversed morphological differentiation in the SK-N-SH neuroblastoma cell line and in heterogeneous cultures of cells from various regions in the human foetal brain.

Key words: differentiation/neurites/neuroepithelium/prothrombin/thrombin

Introduction

The outgrowth of neuritic processes is one of the first cellular events leading to the differentiation of the neuronal cell. An elucidation of the complex interaction of factors controlling this outgrowth is of crucial importance for an understanding of the development, maintenance and pathology of the nervous system. A number of studies using tissue culture systems have shown that cultures of various cell types of neuroepithelial origin can be induced to extend neurites by the addition of chemicals which increase intracellular concentrations of cAMP (Prasad, 1980; Pahlman *et al.*, 1981; Rupniak *et al.*, 1984; Grabham *et al.*, 1988). Spontaneous *in vitro* differentiation has also been observed in foetal brain (Ahmed *et al.*, 1983; Massacrier *et al.*, 1988), retinoblastoma (Kyritsis *et al.*, 1984) and neural crest cells (Ziller *et al.*, 1983), following culture in serum-free defined medium without the addition of stimulatory chemicals. Interestingly, serum has a strong inhibitory effect on the outgrowth of neurites in neural crest cells (Ziller *et al.*, 1983). Similarly the re-addition of serum to previously differentiated cultures of an adenovirus transformed human retinoblast cell line (Ad12 HER 10) causes the retraction of neuritic processes (Grabham *et al.*, 1988). These observations support the hypothesis that morphological differentiation *in vitro* is the expression of a balance between stimulatory and inhibitory signals and that a serum factor(s) is responsible for inhibition. We have recently purified to homogeneity, a serum protein responsible for the inhibition and reversal of cAMP-induced differentiation in serum-free cultures of Ad12 HER 10 cells (Grabham *et al.*, 1989). Differentiation reversal factor (DRF) which has a mol. wt of 72 000 (72K), is effective at physiological concentrations and is capable of stimulating cell proliferation. It has also been shown to reverse morphological differentiation in primary cultures of human foetal retinoblasts.

Various studies have implicated serine proteases, known constituents of serum, in the control of neurite outgrowth. The bioregulatory enzyme thrombin, a serine protease of central importance in haemostasis, has been shown to bind specifically to murine spinal cord cultures (Means and Anderson, 1986) and human brain and spinal cord tissue (McKinney *et al.*, 1983). Furthermore, thrombin has been reported to inhibit morphological differentiation in serum-free cultures of neonatal mouse dorsal root ganglia (Hawkins and Seeds, 1986) and mouse neuroblastoma (Gurwitz and Cunningham, 1988). Consistent with the theory that neurite outgrowth is governed by an interplay of positive and negative signals, inhibition of protease activity has been shown to stimulate neurite extension in neuroblastoma cells (Monard *et al.*, 1983). Also, it has been demonstrated that rat glioma cells release a neurite promoting factor (Guenther *et al.*, 1985) which binds and inhibits the protease activity of thrombin (Stone *et al.*, 1987).

In view of these observations, we investigated the possibility that DRF is a serine protease. Immunoprecipitation and quantitative assays of biological and proteolytic properties revealed DRF to be identical to prothrombin (factor II). Thus far, the reversal of differentiation by thrombin and its zymogen has not been reported in human cells of neuroepithelial origin. We therefore extended our examination of the action of these enzymes on transformed human retinoblasts to include human neuroblastoma cells and heterogeneous cultures derived from various tissues in the normal human foetal brain. Since factor II belongs to a family of closely related blood clotting factors, we used a biological assay (Grabham *et al.*, 1989) to determine the reversal activity of other serine proteases and related

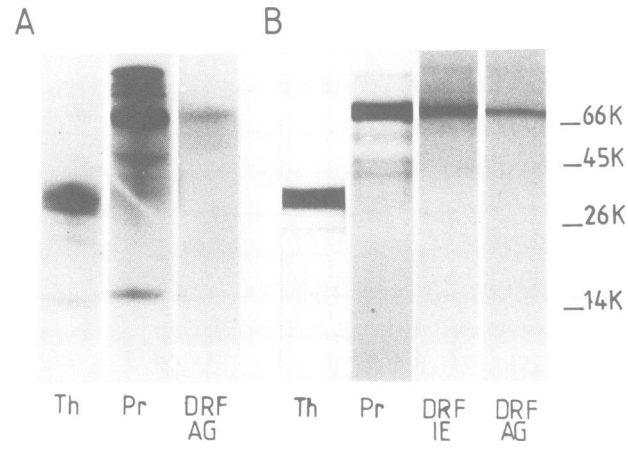

Fig. 1. Identity of differentiation reversal factor with prothrombin.
A. Autoradiograph of SDS polyacrylamide gel of purified proteins labelled with [^{125}I]Na using the chloramine T procedure.
B. Autoradiograph of SDS polyacrylamide gel of ^{125}I-labelled proteins immunoprecipitated with an antibody raised against human prothrombin as described in Materials and methods. Th, human thrombin; Pr, human prothrombin; DRF AG, differentiation reversal factor after preparative gel electrophoresis; DRF IE, differentiation reversal factor after ion exchange chromatography. The position of migration of standard proteins is indicated in the final track.

Table I. Proteolytic and biological activities of DRF, prothrombin and thrombin

	Specific proteolytic activity, pM of substrate hydrolysed/ng/min	Specific biological activity[a] units/μg
DRF	≤0.001	40
DRF[b]	0.12	40
Prothrombin	≤0.001	50
Prothrombin[b]	1.1	50
DIP prothrombin[b]	2.3	50
Thrombin	10	2000
DIP thrombin	≤0.01	≤1

Proteolytic and biological activity were assayed as described in Materials and methods, each value represents the mean from at least three assays. One unit of biological activity is defined as the quantity of protein required to give half-maximal reversal of differentiation after 2 h.
[a]Between experiments the minimum variation of this activity is 10%.
[b]Denotes pretreatment with ecarin for proteolytic determination.

enzymes. In addition, the well documented mitogenic activity of thrombin on fibroblasts (Chen and Buchanan, 1975; Carney *et al.*, 1978) and epithelial cells (Reddan *et al.*, 1982; Bruhn *et al.*, 1983; Medrano *et al.*, 1987) prompted us to investigate the mitogenic potential of factors II and IIa on differentiated Ad12 HER 10 cells.

Results

Identity of DRF with bovine prothrombin

In view of the evidence that thrombin and protease inhibitors are involved in the control of neurite outgrowth (Monard, 1988), we examined the possibility that the polypeptide (DRF), responsible for reversal of cAMP-induced differentiation of Ad12 HER 10 cells (Grabham *et al.*, 1989), might be a member of the serine protease family. Based on the mol. wt and acidic nature of purified DRF it was considered that it might be identical to prothrombin. A number of experiments were performed to investigate this possibility.

(i) *Immunoprecipitation studies.* Using ^{125}I-labelled thrombin, prothrombin and DRF, immunoprecipitation studies were performed with an antibody raised against purified human prothrombin. From the autoradiograph shown in Figure 1 it can be seen that the major radiolabelled protein band in the most highly purified DRF preparation (mol. wt 69K), was immunoprecipitated in good yield by the antibody to prothrombin, as were thrombin and prothrombin. A protein of similar size was also immunoprecipitated from the rather less pure DRF fraction obtained after ion exchange chromatography (DRF IE) (Grabham *et al.*, 1989). No proteins were immunoprecipitated with the control normal rabbit serum.

(ii) *Proteolytic studies.* Prothrombin has no inherent proteolytic activity but after it is cleaved to thrombin it has the ability to hydrolyse a limited number of peptide bonds. This activity may be assayed using several different synthetic substrates – such as 2 AcOH.H-D-CHG-Gly-Arg-pNA which has been employed here. The conversion of prothrombin to thrombin may be achieved by incubation of the protein with the prothrombin activator ecarin (Morita and Iwanaga, 1981). It can be seen from the data presented in Table I, that this treatment results in an increase in proteolytic activity of at least 1000-fold. Like prothrombin, purified DRF has virtually no activity in this colorimetric assay, but again after incubation with ecarin there is a very marked increase in the ability of the protein to hydrolyse the specific synthetic peptide substrate, indicating the presence of a thrombin-like activity. On the basis of these observations and the immunoprecipitation study described above, we conclude that DRF is identical to bovine prothrombin.

The results shown in Table I indicate a similar specific activity for prothrombin and DRF when assayed for their ability to reverse cAMP-induced differentiation in Ad12 HER 10 cells, but some difference in their ability to hydrolyse the peptide substrate after ecarin treatment. No explanation for this discrepancy is apparent at present, but it is possible that proteolytic activity could have been reduced by partial denaturation of the protein during the prolonged purification procedure.

Thrombin contamination of purified prothrombin

Whilst the observation that prothrombin, either in purified form or as DRF, is capable of reversing cAMP-induced differentiation of Ad12 HER 10 cells is of considerable importance, the proposition that these results might be explained by the presence of small amounts of contaminating thrombin had to be considered. Two sets of experiments were therefore performed to exclude this possibility. Firstly, a comparison of the differentiation reversal activity of purified thrombin and prothrombin was made using the assay system described in Materials and methods. The results of this determination are presented in Table I and it can be seen that thrombin is ~20-fold more active than its zymogen on a molar basis (40-fold more active based on the use of equal weights of protein) in the biological assay. However, when the two proteins are compared for their abilities to hydrolyse the synthetic substrate it was found that thrombin was at least 10 000-fold more active than prothrombin. Indeed it was

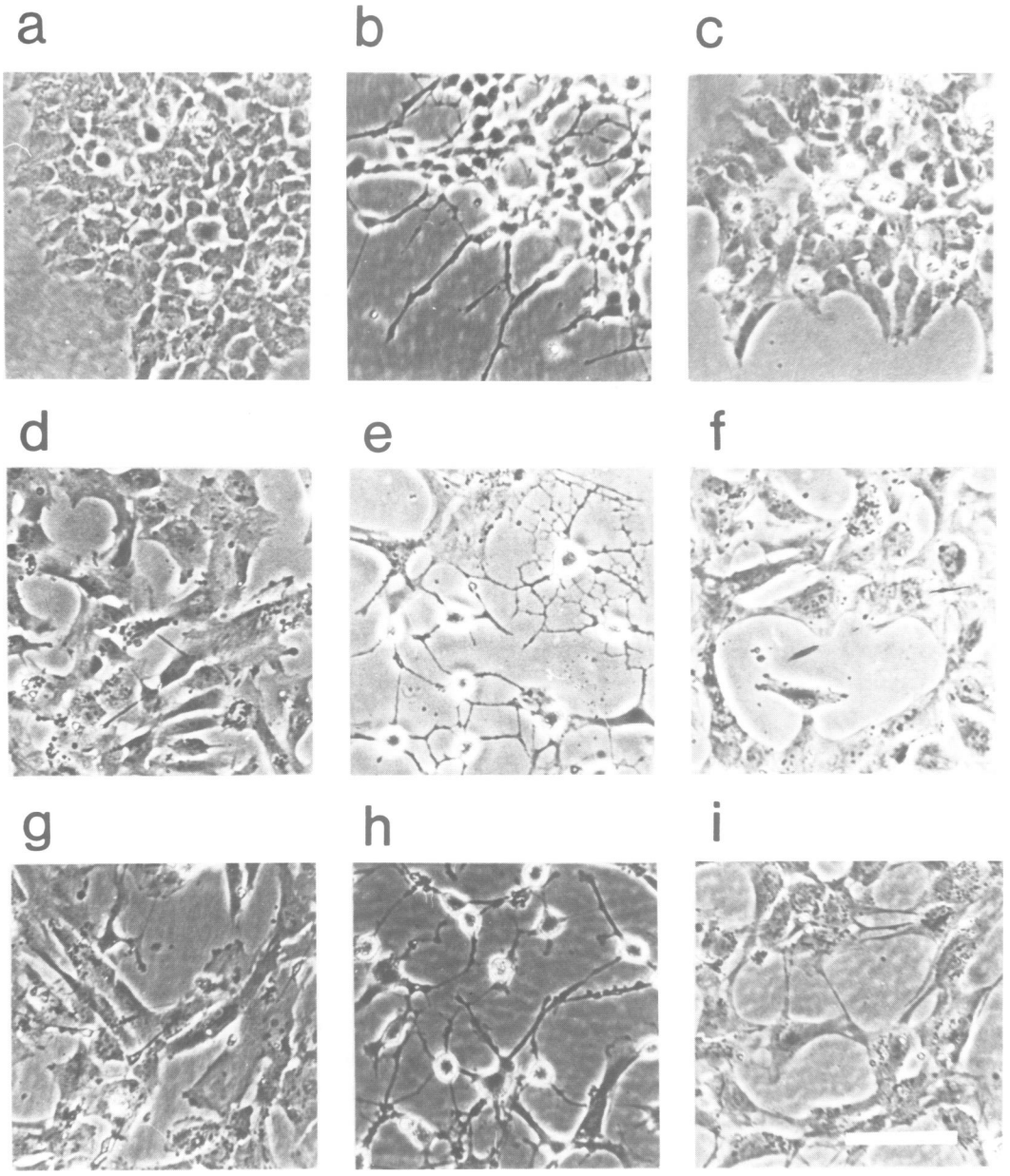

Fig. 2. Morphological differentiation and induced reversal of differentiation in human neuroepithelial cell types. Panels **a, b, c**, Ad12 HER 10 cells; **d, e, f**, primary culture of human foetal cerebellum and **g, h, i**, primary culture of human foetal hippocampus. The cells were cultured in DMEM supplemented with 20% FCS (a, d, g); differentiated with 2 mM dbcAMP (Materials and methods) (b, e, h) and reversed by treatment with 100 ng/ml DRF (c); 50 ng/ml thrombin (f) and 100 ng/ml prothrombin (i). All three proteins effect a similar morphological reversal of differentiation. Bar represents 100 μm.

difficult to detect any substrate hydrolysis in the case of the latter protein except after prolonged incubation (4 h) and with very large amounts of enzyme (100 μg per assay). Similarly when serum-free medium and medium conditioned by differentiated Ad12 HER 10 cells, were incubated with prothrombin no proteolytic activity was observed.

Secondly, a set of experiments was performed to confirm that the observed biological activity of prothrombin was not due to thrombin impurities. It has long been established that incubation of thrombin with the inhibitor di-isopropylfluorophosphate (DIP) results in a loss of enzymic activity. Therefore both DIP-treated thrombin and prothrombin were included in the biological and proteolytic assay systems. It can be seen from the data presented in Table I that treatment of thrombin with the inhibitor results in a virtual total loss of its ability to reverse differentiation and to hydrolyse the synthetic substrate, whereas similar treatment of prothrombin appears to have no detrimental effect as measured in either system. When [^{3}H]DIP was used in comparable experiments we have found appreciable radioactivity associated with thrombin, but none either bound to prothrombin or any other proteins in the prothrombin preparations (e.g. thrombin impurities). Similarly, [^{3}H]DIP did not bind to any polypeptides in DRF preparations.

On the basis of these data, we have concluded that the ability of purified prothrombin (and DRF) to reverse the cAMP-induced differentiation of human cells in culture is due to an inherent activity, and not to small amounts of

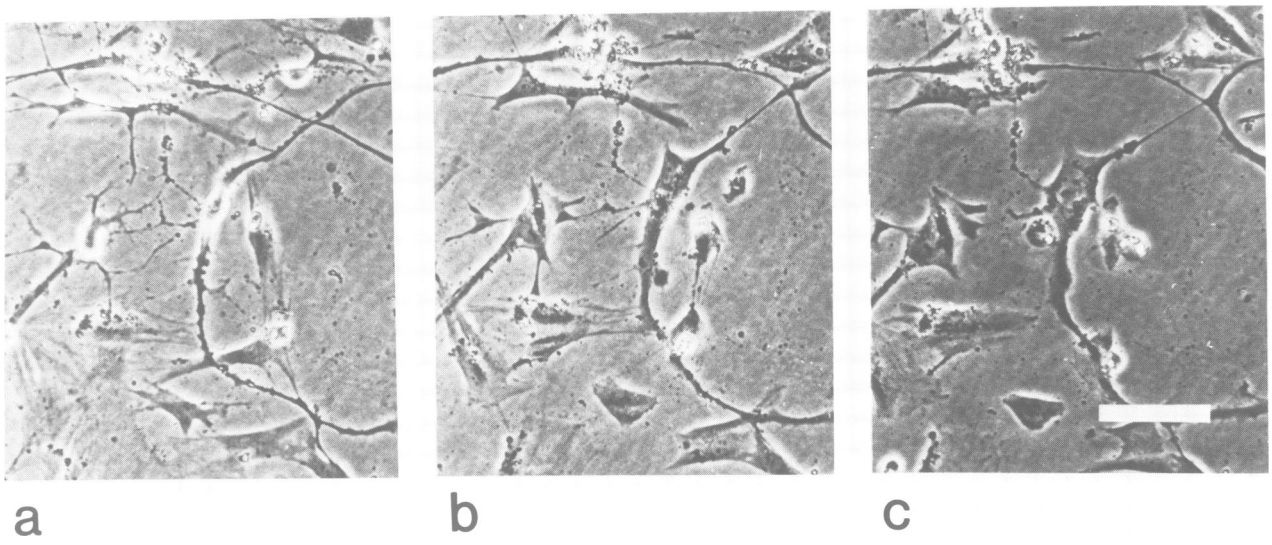

Fig. 3. Effect of DRF on previously differentiated primary cultures of human foetal mid-brain cells. (**a**) Cells differentiated by exposure to 2 mM dbcAMP in serum-free defined medium; (**b**) and (**c**) the same cell 1 and 2 h respectively after the addition of 100 ng/ml purified DRF. It can be seen that DRF has caused the retraction of cellular processes and a reversal to an epithelioid morphology. Bar represents 100 μm.

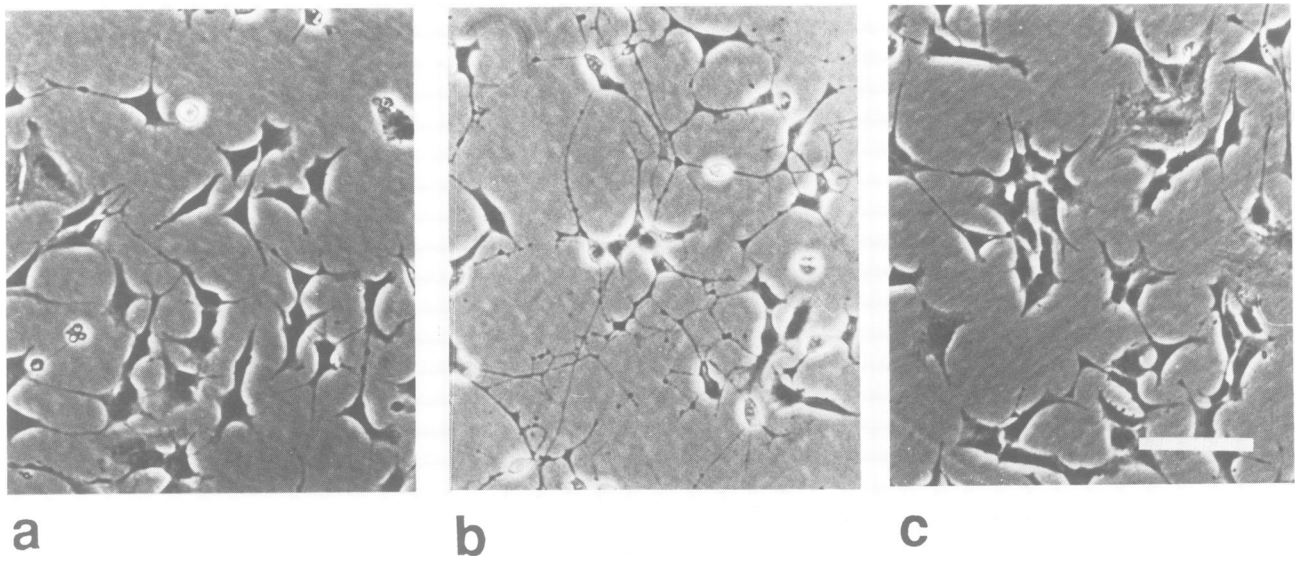

Fig. 4. Morphological differentiation and induced reversal of differentiation in SK-N-SH human neuroblastoma cells. (**a**) Cells cultured in DMEM supplemented with 8% FCS. (**b**) Cells differentiated after 48 h exposure to 2 mM dbcAMP in serum-free medium. (**c**) Reversal of differentiation 4 h after the addition of 100 ng/ml prothrombin. Bar represents 100 μm.

thrombin present as a contaminant or a degradation product in the prothrombin preparation.

Reversal of cAMP-induced differentiation of human neuroepithelial cells by thrombin and its zymogen

The morphological changes associated with differentiation and its reversal as exemplified by Ad12 HER 10 cells (developed into an assay for quantitative determination of reversal activity), are shown in Figure 2. When grown in serum-supplemented DMEM these cells have an epithelioid morphology (Figure 2a), but after the removal of serum and addition of 2 mM dbcAMP, virtually all (98%) cell bodies round up and extend neuritic-type processes (Figure 2b). The reversal of differentiation is shown in Figure 2c, in this case 100 ng/ml DRF has caused the retraction of neuritic processes and flattening of cell bodies.

We have previously shown that a proportion of cells in heterogeneous cultures of primary human foetal retinoblast cultures (the normal counterpart to the Ad12 HER 10 cell line), can be morphologically differentiated and subsequently reversed using DRF (Grabham *et al.*, 1989). In the present report we have extended this study to include other tissues of the human central nervous system. Primary cultures of human foetal cerebellum (Figure 2d) and hippocampus (Figure 2g) were subjected to differentiating conditions (Materials and methods). As with primary cultures of retinoblasts, a proportion of these cells exhibited the type of cell body rounding and neuritic process extension associated with neuronal differentiation (Figure 2e and h). However, at present there is no evidence to suggest that the cells are neurons (the multipolar cell in Figure 2e appears to be an oligodendrocyte). Regardless of the cell type it can be seen that all cells which morphologically differentiated (including bipolar and multipolar types), could be induced

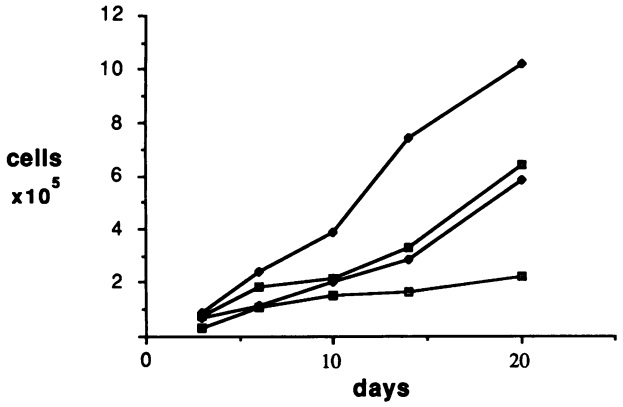

Fig. 5. Relative growth rates of Ad12 HER cells in prothrombin, thrombin and FCS. Cells plated in 24 well multidishes were differentiated (Materials and methods) then treated with 10% FCS (—◆—), 100 ng/ml prothrombin (—■—), 50 ng/ml thrombin (—◆—) and serum-free medium alone (—□—). Treatments of 2 mM dbcAMP and test substances were made at 3 day intervals. Each point represents the mean count from three separate tissue culture wells.

to retract processes and flatten cell bodies by the addition of 50 ng/ml thrombin (Figure 2f) or 100 ng/ml prothrombin (Figure 2i).

In Figure 3, a time lapse reversal of differentiation of individual cells of the human foetal midbrain is shown. Again, this culture is heterogeneous and the cell types are unknown. In the differentiated cultures (Figure 3a), at least two morphological types can be seen to have interconnecting and varicose cellular processes. One hour after the addition of 100 ng/ml DRF, many cell connections have broken (Figure 3b). After 2 h (Figure 3c) the cells have almost fully returned to an epithelioid morphology. In all the tissues examined, DRF, thrombin and prothrombin reversed morphological differentiation in a similar manner.

Evidence of the reversal of morphological differentiation of a well defined neuronal cell type is shown in Figure 4. Although the SK-N-SH cell line derived from a human metastatic neuroblastoma (Biedler *et al.*, 1973) exhibits short neurites when grown in serum-supplemented DMEM (Figure 4a), after 2 days treatment with 2 mM dbcAMP in serum-free defined medium, the cells produced longer varicose neurites (Figure 4b). Subsequent treatment with FCS, thrombin or prothrombin (Figure 4c) caused a retraction of neurites and a return to a morphology similar to that seen in Figure 4a.

Effects of thrombin and its zymogen on growth of Ad12 HER 10 cells

We have previously shown that dbcAMP, in the absence of serum inhibits the growth of Ad12 HER 10 cells in a dose dependent manner (Grabham *et al.*, 1988), and that DRF can partly restore cell growth at a concentration similar to that needed for reversal of differentiation (Grabham *et al.*, 1989). In Figure 5 the growth rates of differentiated Ad12 HER 10 cells in the presence of FCS, prothrombin and thrombin are shown. Prothrombin and thrombin stimulate cell growth at a similar rate when compared on a molar basis. 10% foetal calf serum (FCS) however, increases the growth rate further, possibly due to additional growth factors which are present in serum.

Reversal of differentiation in Ad12 HER 10 cells by various serine proteases

Using the assay system described, (Materials and methods) to determine the concentrations of protease required for half-maximal reversal of differentiation in Ad12 HER 10 cells prothrombin and DRF were found to have similar activities of 20 ng/ml and 25 ng/ml respectively. Thrombin, with a half-maximal value of 500 pg/ml, is 20-fold more active on a molar basis. At present we have no explanation for this difference, although it may be of significance that the kinetics of reversal activity differed, in that thrombin appeared to act in a more rapid and transient fashion.

Since thrombin and its zymogen belong to a closely related family of blood clotting factors (Neurath, 1984), we investigated the ability of other serine proteases to reverse differentiation. None of the vitamin K dependent serine proteases (factors VII, IX and X up to concentrations of 1 U/ml, 0.5 U/ml and 1 U/ml respectively), other components of haemostasis (factor XII, tissue plasminogen activator and kallikrein up to concentrations of 10 μg/ml, 0.5 μg/ml and 100 μg/ml respectively) or thrombin-like enzymes (ancrod, acutase and agkistron up to concentrations of 0.2 U/ml, 1 U/ml and 0.02 U/ml) exhibited differentiation reversal activity. We conclude therefore that the protease–cell interaction leading to the retraction of neurites in Ad12 HER 10 cells is specific to thrombin and prothrombin.

Discussion

The first series of observations described in this report have established that DRF, a serum protein purified on the basis of its ability to reverse neuronal differentiation (Grabham *et al.*, 1989), has many physical and biological properties in common with prothrombin. Both proteins exhibit the same molecular weight after PAGE (Figure 1) and are eluted from DEAE 52 cellulose columns by a similar salt concentration (Miletich *et al.*, 1981; Grabham *et al.*, 1989). More significantly, purified and partially purified DRF can be immunoprecipitated using an antibody raised against purified prothrombin (Figure 1B). Neither DRF nor prothrombin can hydrolyse a synthetic thrombin substrate (2 AcOH.H-D-CHG-Gly-Arg-pNA), unless the proteins are first incubated with the prothrombin activator ecarin (Table I). Thus, DRF is able to perform the same substrate specific hydrolysis as prothrombin.

A comparison of biological activities using Ad12 HER 10 cells in a neurite retraction assay (Materials and methods), revealed that prothrombin has a similar specific activity (50 U/μg) to that of DRF (40 U/μg) (Table I). When tested in the above assay thrombin was also active in the reversal of morphological differentiation (Table I). Although this observation suggests that the differentiation reversal activity of prothrombin resides in the catalytic portion of the molecule, the higher specific activity of thrombin (20 times on a molar basis), raises the possibility that the activity of prothrombin is due to a contamination with small amounts of thrombin derived from spontaneous cleavage of the proenzyme. However, the failure of uncleaved DRF and prothrombin to hydrolyse the specific chromogenic substrate or become catalytically inactivated by the inhibitor di-isopropylphosphofluoridate DIP (Table I), clearly demonstrates that thrombin is not present. Following cleavage by

ecarin, prothrombin did not increase its specific proteolytic and biological activities to a level similar to that of thrombin. At present we have no explanation for this anomaly, but it is possible that the presence of both ecarin and the non-catalytic portion of prothrombin may have an inhibitory effect.

Aside from its role in haemostasis, thrombin has been attributed with the ability to stimulate mitosis in serum-free cultures of fibroblasts (Chen and Buchanan, 1975; Carney *et al.*, 1978) and epithelial cells (Redden *et al.*, 1982; Bruhn *et al.*, 1983; Medrano *et al.*, 1987). Studies which have investigated the requirements necessary for thrombin stimulated cell division, have revealed that the proteolytic activity of the enzyme is essential (Glenn and Cunningham, 1979). When derivatized at the catalytic site serine (residue 205 of the thrombin B chain) with a di-isopropyl-phospho-group, thrombin is not mitogenic in mouse, hamster, chick and human fibroblasts (Glenn *et al.*, 1980). In the present report we have shown that the proteolytic activity of thrombin is also necessary for neurite retraction. A reduction in the specific enzyme activity (> 10 000-fold) of thrombin treated with DIP resulted in a comparable reduction (2000-fold) in specific biological activity. Prothrombin however, is not susceptible to cataytic inactivation by DIP and retains its differentiation reversal activity (Table I). There appear to be two possible mechanisms by which the proenzyme could act directly on Ad12 HER 10 cells: firstly, a cell derived protein could cleave the prothrombin and generate activated enzyme (thrombin) or secondly, prothrombin could cause neurite retraction via a novel mechanism not involving cleavage, e.g. receptor occupation. In any event, the observation that prothrombin can act without blood coagulation may be physiologically significant. Currently, thrombin stimulated mitogenesis is thought to occur at the site of a blood clot as a mechanism of wound healing and tissue repair (Carney *et al.*, 1985; Cunningham and Farrell, 1986). The biological activity of prothrombin described here would not, in theory, require a clotting response, since prothrombin normally circulates in human plasma at a concentration of ~150 μg/ml (Mann *et al.*, 1981).

The relationship between neurite modulation and mitotic stimulation in neuroepithelial cells is unclear. In the Ad12 HER 10 cell line, prothrombin and thrombin were found to stimulate both. Addition of either protein to cells maintained under differentiating conditions caused an increase in cell number appreciably above that of control cultures (Figure 5). Therefore, in this model, proenzyme and enzyme are both multifunctional. Furthermore, their role as mitogen suggests a function in neuronal development and possibly in neoplasia. The relative physiological importance of the control of neurite outgrowth is dependent on the number and types of neuroepithelial cells which respond to serine proteases. Studies using animal models have shown that neurite outgrowth in neonatal mouse sensory ganglia (Hawkins and Seeds, 1986) and mouse neuroblastoma (Gurwitz and Cunningham, 1988) is inhibited and reversed by exposure to thrombin. In the present report neurite outgrowth in the SK-N-SH neuroblastoma cell line, induced by treatment with 2 mM dbcAMP in serum-free DMEM, is reversed by exposure to FCS, thrombin and prothrombin (Figure 4). In addition, this reversal of morphological differentiation was found to occur in cells from different regions of the human foetal brain. Those cells which produced cellular processes in heterogeneous cultures of cerebellum, hippocampus and mid-brain returned to a morphology similar to that seen in serum-supplemented cultures after treatment with either thrombin or its zymogen (Figures 2 and 3). It is unlikely that under normal conditions, blood-born clotting factors ever come into direct contact with nerve cells in the brain, therefore thrombin may have a regenerative function following any trauma of the central nervous system which involves a compromised blood–brain barrier (Snider, 1986). However, the possibility that one or more proteases with thrombin-like specificity might be synthesized by tissue in the CNS, has not been eliminated, and it is significant to note that the mRNA for prothrombin has recently been detected in rat and human brain (Cunningham and Gurwitz, 1989).

A protease inhibitor (nexin) has been found to be synthesized from glial cells (Guenther *et al.*, 1985), and this glial derived nexin (GDN) is thought to participate in the regulation of neurite outgrowth by binding and inactivating serine proteases, particularly thrombin. Indeed, kinetic studies indicate that GDN has a 10-fold higher affinity for thrombin than another serine protease:plasminogen activator (Stone *et al.*, 1987). We have shown here that neurite retraction in Ad12 HER 10 cells is also specific to thrombin and its zymogen. A large number of related serine proteases (including plasminogen activator) have also been tested in this assay system and have been found to be incapable of reversing differentiation.

In conclusion, we have shown that DRF is in fact prothrombin and, in both enzyme and proenzyme forms is able to mediate neurite retraction and stimulate growth in differentiated cultures of Ad12 HER 10 cells. The fact that the proenzyme is active without prior cleavage is likely to be of physiological importance, since it provides a mechanism of control at the cellular level independent of blood coagulation. The activity of thrombin in the reversal of differentiation is dependent on its proteolytic activity and is highly specific to the enzyme and its zymogen. The importance of this protease and its interaction with neuroepithelial cells has been confirmed by the observation that reversal of morphological differentiation also occurs in SK-N-SH neuroblastoma cell line. Furthermore, this response is not restricted to neuronal cell types since it is widespread in heterogeneous cultures derived from various regions of the developing human brain.

Materials and methods

Highly purified thrombin, prothrombin, factors VII, IX, X and XII were a generous gift from Dr Peter Esnouf (Radcliff Infirmary, Oxford, UK). Tissue plasminogen activator and kallekrein were obtained from Boehringer (FRG). Ancrod, acutase and agkistron were obtained from Sigma Biochemicals Limited (Poole, UK). Thrombin substrate (2 AcOH.H-D-CHG-Gly-Arg-pNA) was obtained from Channel Diagnostics (UK).

Cells

Ad12 HER 10 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 8% foetal calf serum. Primary human foetal brain cells were obtained by section from 15–19 week old human foetuses (therapeutically aborted), dispersed by pipette aspiration and distributed into 5 cm plastic tissue culture dishes in Hepes buffered RPMI medium supplemented with 20% FCS. Prior to differentiation, cells were 'serum boosted' for 24 h in 20% FCS. All cells were differentiated for 24 h by treatment with 2 mM [^{6}N, ^{12}O]dibutyryl adenosine 3′,5′-cyclic mono-

phosphate (dbcAMP) (Sigma) in serum-free defined DMEM supplemented with 5 μg/ml insulin, 10 μg/ml transferrin, 6.6 ng/ml progesterone, 8.8 ng/ml putrescine and 4 μg/ml sodium selenite. Reversal of differentiation was initiated by the addition of serine proteases. After 2 h, retraction of neuritic processes and cell body flattening were observed by phase contrast microscopy.

Using the Ad12 HER 10 cell line, this method was adapted for quantification of differentiation reversal activity as described previously (Grabham *et al.*, 1989). Briefly, serial dilutions of samples were added to differentiated Ad12 HER 10 cells in 24 well multidishes, one unit of activity was defined as the amount of protein which caused half-maximal reversal after treatment for 2 h.

The effect of serine proteases on the growth of differentiated Ad12 HER10 cells was determined by the addition of thrombin, prothrombin or FCS to cells differentiated as for visual assays. Treatments (every 3 days) coincided with a change of serum-free medium containing 2 mM dbcAMP. Cell counts were made after 3, 6, 10, 14 and 20 days using a haemocytometer.

Immunoprecipitation of serine proteases

Bovine DRF (~1 μg) purified by the method described previously (Grabham *et al.*, 1989), human thrombin (10 μg) and human prothrombin (10 μg) were labelled with ^{125}I using a standard chloramine T procedure (Hunter and Greenwood, 1962). Unbound ^{125}I was removed from the protein by chromatography on a column of Sephadex G25 eluted with 0.2 M Tris–HCl pH 7.5 containing 2% (w/v) BSA. Fractions containing radiolabelled proteins were collected and stored at −20°C until needed.

Aliquots (50 μl) of ^{125}I-labelled protein were mixed with immunoprecipitation buffer (10 mM Tris–HCl pH 7.2, 0.7 M NaCl, 1% NP40) and immunoprecipitated as described by Paraskeva *et al.* (1982) using a rabbit monospecific antiserum raised against human plasma proteins and reactive against human prothrombin (Boehring) or normal rabbit serum (included as a negative control). Precipitated proteins were subjected to PAGE after which the gels were dried and autoradiographed.

Assays of thrombin activity

Thrombin activity was assayed using the synthetic substrate AcOH.H-D-CHG-Gly-Arg-pNA. Aliquots of protein were added to 50 mM Tris–HCl pH 7.2, 0.1 M NaCl (500 μl) and incubated at 37°C for 5 min in the presence or absence of ecarin (2×10^{-4} U) as appropriate. The reaction was initiated by the addition of substrate (32 nmol) and terminated by the addition of 4.4 M acetic acid (200 μl), the optical density (OD) was then read at 405 nm against a water blank. The OD produced by compete hydrolysis of the substrate was determined by allowing the reaction to go to completion in the presence of excess thrombin.

Binding of di-isopropylphosphofluoridate (DIP) to serine proteases

Purified human thrombin (20 μg) and prothrombin (200 μg) were dialysed against 0.1 M Tris–HCl pH 7.9, 0.3 M NaCl and DIP added to each to a concentration of 13.5 mM. After 45 min samples were dialysed exhaustively against PBS at 4°C and stored at −20°C until required.

Reaction of proteins with [^3H]DIP (Amersham International) was accomplished using a similar protocol except that the radioactive reagent (20 μCi) was added 30 min before the unlabelled DIP.

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