UNIVERSITY OF CALGARY

THE REGULATION OF PLASMINOGEN ACTIVATOR AND INHIBITOR ACTIVITY DURING DIFFERENTIATION IN CULTURED HUMAN NEUROBLASTOMA CELLS

BY

LAURIE ANN BENJAMIN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE
STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

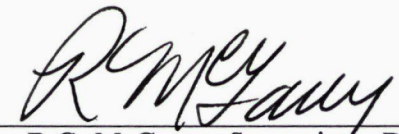
CALGARY, ALBERTA
SEPTEMBER, 1988

ISBN 0-315-46551-4

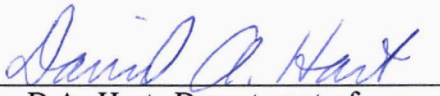
THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

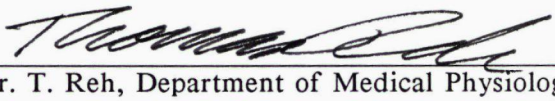
The undersigned certify that they have read , and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "The Regulation of Plasminogen Activator and Inhibitor Activity During Differentiation in Cultured Human Neuroblastoma Cells" submitted by Laurie Benjamin in partial fulfillment of the requirement for the degree of Master of Science.



Dr. R.C. McGarry, Supervisor, Departments of Pediatrics and Pathology



Dr. D.A. Hart, Department of Microbiology and Infectious Diseases



Dr. T. Reh, Department of Medical Physiology

Dr. M. Bentley, Department of Biological Sciences

ABSTRACT

The fibrinolytic enzyme profile of human neuroblastoma (NB) cell lines was assessed and found to vary during the differentiation process. Serum-free conditioned media (CM) from ten human NB cell lines were analyzed for plasminogen activator (PA) and plasminogen activator inhibitor (PAI) activity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography. All NB cell lines were found to constitutively secrete urokinase (UK)-type PA and all except two also produced tissue PA (tPA). PAs were identified based on molecular weight and sensitivity to inhibition by anti-UK and anti-tPA antibodies. Eight NB cell lines expressed PA inhibitory molecules; in five of these cell lines, a 35 kD form was observed, while a 40 kD form was found in the other lines. In addition, heparin binding affinity differed between these two molecular weight forms of PAI, suggesting that they are distinct PA inhibitors. Complex formation of both inhibitors with iodinated proteases revealed specific binding with UK and trypsin, but not plasmin, thrombin or kallikrein.

NB cell lines were induced to differentiate with a well known maturational agent, retinoic acid (RA). After six days of treatment with RA, six of the NB cell lines exhibited an increase in cell-associated and/or secreted tPA activity corresponding to morphological differentiation of the cells as manifested by extensive neurite outgrowth. A depression in UK secretion which correlated with differentiation was observed in four of these cell lines. Three cell lines exhibiting no detectable morphological alterations with RA treatment showed no dramatic changes in PA and PAI activity. In one cell line, SK-N-SH, RA induced the conversion of a mixed neuronal/flat cell phenotype to a predominantly flat cell phenotype, a change that was correlated with an enhancement

of cell-associated and secreted UK activity.

To further investigate differentiation-related changes in PA and PAI activity, one cell line (SMS-KAN) was chosen for more extensive study. Five maturational agents- RA, dibutyryl cAMP (dbcAMP), 5-bromodeoxyuridine (5-BrDU), sodium butyrate (NaB) and phorbol myristate acetate (PMA) were tested for their effects on cellular morphology, DNA synthesis and fibrinolytic enzyme activity.

Treatment of SMS-KAN cells with 1 uM RA resulted in an inhibition of cellular proliferation and extension of neurite-like processes indicative of differentiation. Morphological changes corresponded to a switch from secretion of UK to tPA, a reduction in PAI secretion and an increase in cell-associated tPA activity. Treatment of cells with 2 mM dbcAMP, 10 uM 5-BrDU, and 1 mM NaB caused neurite process formation which was correlated with an increase in tPA evident in both CM and lysates. PMA had no detectable effect on cell growth and induced little change in fibrinolytic enzyme profile and morphology.

Findings presented here suggest a complex interplay of both PAs and inhibitors occurs during differentiation and that tissue PA expressed by neuronal cells may play a critical role in this process.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Ron McGarry for his generous support during my time in his laboratory. My sincere appreciation to Dr. David Hart for his continued expertise and guidance during the course of this project. Thanks also to Dr. Tom Reh for his helpful suggestions and interest in my research.

A special thanks to Dr. Jitendra Gohill, Rohit Joshi, Dolores Freigang, Katayoun Khorrami and Nigel Beckford, whose friendship and encouragement have been invaluable to me. I am grateful to Sheilagh Armitage for her technical assistance at various stages of this project. I would also like to express my appreciation to the Gohill family for their enthusiastic support of my endeavors.

The financial support from the Alberta Heritage Foundation for Medical Research during the course of my studies is gratefully acknowledged.

DEDICATION

To my parents, Barry and Peggy, whose pride in this achievement has made it all worthwhile

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-BrDU	5-bromodeoxyuridine
CHAPS	3-[(3-Cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate
CM	Conditioned medium
CNS	Central nervous system
cpm	Counts per minute
CRABP	Cytoplasmic retinoic acid binding protein
Cu	Curie
dbcAMP	Dibutyryl cyclic AMP
DFP	Diisopropylfluorophosphate
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic Acid
FACS	Fluorescent activated cell sorter
Gd-NPF	Glial-derived neurite promoting factor
HMW-UK	High molecular weight urokinase
LMW-UK	Low molecular weight urokinase
MW	Molecular weight
NaB	Sodium Butyrate
Na_3PO_4	Sodium Phosphate
NB	Neuroblastoma
O.D.280	Optical Density at 280 nm
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis

PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PMA	Phorbol myristate
PN	Protease nexin
RA	Retinoic acid
SDS	Sodium dodecyl sulfate
tPA	Tissue plasminogen activator

INTRODUCTION

1.1 Neuroblastoma

Neuroblastoma is a common solid tumor of childhood, arising from the sympathetic part of the autonomic nervous system. One of the interesting features of this tumor is its high rate of spontaneous regression, which occurs most frequently in infants with localized disease. This group, left untreated or treated with minimal therapy, has a 70 % survival rate (D'Angio et al., 1972; Evans et al., 1976). NB may also mature spontaneously to benign ganglioneuroma which occurs more frequently in patients over three years of age (Everson, 1964; Adam and Hockholzer, 1981). Histologically, the differentiated tumor resembles mature sympathetic ganglion neurons surrounded by glial and Schwann cells (Triche and Askin, 1983).

Unfortunately, children with disseminated tumor and children older than one year at the time of diagnosis have a very low survival rate (Berthold, 1985). Multimodal treatment regimens including surgery, chemotherapy and radiation in children with advanced disease have had little success (Kumar et al., 1970; Helson et al., 1976; Imashuku et al., 1982). For this reason, attempts have been made to develop agents which might initiate the regression and maturation of neuroblastoma which is observed naturally in other patients.

The treatment of cultured NB cells with various agents such as retinoic acid and dibutyryl cyclic AMP induces differentiation of the cells, typically characterized by extensive neurite outgrowth and cell growth inhibition. The morphological changes induced by maturational agents in cultured cells appear to parallel the clinical observations. Thus, NB cell lines provide an experimental model for the investigation of naturally oc-

curing features of the tumor. In addition, the effects of differentiation agents on these cells can be tested in the hopes of finding therapeutic agents for the treatment of neuroblastoma.

1.1.1 Retinoic Acid-Induced Differentiation

Vitamin A and its metabolites alter the growth and tumorigenicity of a number of malignant cell types in vitro (Breitman et al., 1980; Lotan and Lotan, 1980). The effects of retinoic acid (RA) on the morphology and growth characteristics of neuroblastoma cells have been studied extensively. In a number of NB cell lines, RA induces morphological differentiation as manifested by neurite outgrowth and the inhibition of both anchorage-independent proliferation and growth in monolayer culture (Sidell, 1982; Sidell et al., 1983). Although differentiation to a neuronal phenotype is a common feature of many RA treated NB cells, maturation to both Schwannian and melanocytic phenotypes has been demonstrated (Tsokos et al., 1985; Tsokos et al., 1987). In these studies the different cell types have been identified based on cell surface antigens, production of extracellular matrix proteins, morphological characteristics, and the presence of pigmented granules (present in melanocytes). In fact, certain cell lines such as SH-SY5Y appear to differentiate to all three phenotypes (Pahlman et al., 1984). These observations have led to the suggestion that the cell of origin for neuroblastoma may be a primitive neuroectodermal stem cell since neuronal, Schwannian and melanocytes are all neural crest derived.

There is some evidence to suggest that certain functions of RA are mediated by cytoplasmic retinoic acid binding proteins (CRABP) (Haussler et al., 1984). In five human neuroblastoma cell lines, comparable levels of CRABP were detected although the presence of binding proteins did not correlate with the ability of retinoic acid to in-

hibit growth of the cells in monolayer culture or to induce morphological differentiation (Haussler et al., 1983). However, CRABP levels in the NB cell lines did correlate with the ability of cells to form anchorage-independent colonies in soft agar. Furthermore, Jetten and coworkers (1987) failed to find a direct correlation between biological activity and binding to CRABP in a number of cell lines. Although the binding of retinoic acid to cytosolic RA-binding proteins may be one of several events leading to RA-induced differentiation of neuroblastoma, a specific receptor for retinoic acid has recently been identified (Giguere et al., 1987).

Tumors of neuroectodermal origin (neuroblastoma and retinoblastoma) and freshly biopsied tumors generally have high levels of n-myc expression. However, cells showing features of differentiation have decreased levels of n-myc mRNA (Thiele et al., 1985). In the human NB cell line SMS-KAN, reduced n-myc expression occurs as early as six hours after treatment with retinoic acid and precedes both cell cycle changes and morphological differentiation which occur after 24-72 hours (Amatruda et al., 1985).

The differentiation of NB cells has been associated with increases in neurotransmitter enzymes such as acetylcholinesterase (Sidell et al, 1984), neuron specific enolase (Pahlman et al., 1984) and a decrease in ornithine decarboxylase (Mattsson et al, 1984). Other differentiation associated phenomena include increased membrane excitability (Robson and Sidell, 1985; Sidell and Horn, 1985) and a variety of cell surface antigen modulations (Reynolds and Maples, 1985; Gross et al., 1987).

In light of the ability of RA to induce the differentiation of NB cells in vitro, clinical trials of retinoic acid have been initiated for the treatment of patients with Stage IV disease (Dr. P. Reynolds; personal communication). Recently, Sugimoto and coworkers (1987) reported the development of a synthetic polyprenoic acid RA derivative which is

less toxic than RA and equally effective in causing morphological differentiation of cultured NB cells. This agent is presently being tested in experimental mouse models to determine its efficacy as a therapeutic agent for the treatment of neuroblastoma.

1.1.2 Other Maturational Agents

A variety of natural and synthetic molecules induce the differentiation of neuroblastoma cells. Although retinoic acid has been studied most extensively, other agents such as dibutyryl cAMP (dbcAMP), 5-bromodeoxyuridine (5-BrDU), and phorbol esters cause the morphological differentiation of these cells. Reynolds and Maples (1985) described the effects of several agents on SMS-KAN and SMS-KCNR neuroblastoma cell lines in terms of morphological and growth changes, as well as cell surface antigenic alterations. In this study, RA, dbcAMP and 5-BrDU induced neurite outgrowth of the cells although the time course of the differentiation response varied with the treatment. In addition, a heterogeneous mixture of cell morphologies resulted from the inducers, including a neuroblast-like cell, a flat cell with a glial/Schwannian phenotype and a neuronal neurite-extending cell. The ratio of these cell types depended on the inducing agent. These results correlate with those of Pahlman et al. (1984), who demonstrated that simultaneous treatment of the NB cell line SH-SY5Y with RA and a phorbol ester (12-O-tetradecanoyl-phorbol-13-acetate or TPA) led to a synergistic effect on morphological differentiation, although the resultant differentiated cells showed phenotypic variability. The ability of NB cells to undergo tripartite differentiation is thus affected in part by the agent chosen to induce the effect.

The intracellular mechanisms by which these agents induce the differentiation of tumor cells are poorly understood. Retinoic acid has been shown to increase the activity of cAMP-dependent kinases in several tumor systems, although the intracellular cAMP

levels remain unchanged (Ludwig et al., 1980; Plet et al., 1985). In murine neuroblastoma cells, dibutyryl cAMP and RA were found to exert opposite effects on the level of a cAMP binding protein, with RA decreasing the concentration of the protein (Prashad et al., 1987). Littauer et al. (1985) have suggested that agents such as retinoic acid and dimethyl sulfoxide (DMSO) may change membrane fluidity directly inducing neurite extension or act indirectly by influencing cellular division.

1.2 Fibrinolytic Enzymes

Fibrinolytic enzymes are best known for their physiological role in the maintanence of homeostasis in the circulation. Formation of a blood clot occurs through activation of the coagulation cascade leading to sequestering of platelets and fibrin. The fibrinolytic system is directly involved in the eventual dissolution of the clot through the action of enzymes such as plasminogen activators (PA). PAs are also important extravascularly in many physiological processes such as cellular migration and tissue remodeling (Hart and Rehemtulla, 1988).

Dysregulation of the fibrinolytic proteinases may be a contributing factor in various pathological disorders. For instance, the ability of transformed cells to degrade extracellular matrix and migrate through tissues is in part due to the aberrant secretion of proteinases by these cells. Compared to their normal counterparts, many types of tumor cells secrete high levels of proteases such as collagenases, cathepsins and plasminogen activators (Dano et al., 1985). The role of PAs in the behavior of malignant cells is a subject of extensive study.

1.2.1 Plasminogen Activators

Plasminogen activators are specific serine proteases which convert the zymogen plasminogen to the active protease plasmin. Plasminogen is a ubiquitous glycoprotein which is cleaved at an arginine-valine bond by PAs to yield the two-chain disulfide-linked plasmin. Plasmin is a general protease which degrades both fibrin and extracellular matrix components, as well as activating latent enzymes such as elastase and collagenase.

Two main types of biochemically and immunologically distinct plasminogen activators have been described- urokinase-type (UK) and tissue-type (tPA). Urokinase was first identified in urine and is found in conditioned medium from a number of tumor cell lines and in small amounts in plasma (Larsson et al., 1984). UK is secreted as a single chain proenzyme that can be converted to a two-chain active form by proteolytic cleavage (Blasi et al., 1987). Cell surface receptors for UK have been described which may serve to localize and enhance fibrinolytic activity (Vassalli et al., 1985). In addition to the active form of the enzyme (high molecular weight UK; MW= 52 kD), a partial degradation product can be found in conditioned medium (CM) from certain cell lines (low molecular weight UK; MW= 33kD). This latter form maintains catalytic activity but does not have the receptor binding fragment and is probably not relevant physiologically. Urokinase is important in processes requiring localized proteolysis, including cell migration and tissue degradation.

Tissue-type PA differs from UK in that it is a fibrin dependent enzyme, making it an important mediator of vascular homeostasis. The direct binding of tPA to fibrin leads to the activation of plasminogen to plasmin which occurs at the site of a blood clot. Tissue-type PA is released by endothelial cells as well as melanoma/melanocytes and is found in many vascularized tissues. Like UK, tPA exists in a single or a double chain form. Both forms have similar fibrinolytic activity, but their reaction with inhibitors is

very different (Kruithof et al., 1985). Evidence has been presented to suggest the presence of tPA receptors on hepatocytes (Bakhit et al., 1987). These receptors may play a role in the rapid clearance of tPA from the circulation by the liver.

1.2.2 Plasminogen Activator Inhibitors

Regulation of the fibrinolytic system occurs through specific protease inhibitors acting on plasmin and plasminogen activators. Plasminogen activator inhibitors (PAI) can be classified into four immunologically distinct types: the endothelial cell type (PAI-1), the placental type (PAI-2), the urinary type (PAI-3) and protease nexin (PN) (Sprengers and Kluft, 1987). PA inhibitors, though best known for their role in maintaining homeostasis of the circulation, appear to be involved in a plethora of physiological and pathological processes.

Of the four serine protease inhibitors listed above, PAI-1 is the most predominant in plasma and the most efficient inhibitor of UK and tPA (Sprengers et al., 1985). In addition to endothelial cells, other sources of PAI-1 include hepatocytes, hepatoma cells, fibrosarcoma cells, smooth muscle cells and platelets. PAI-1 is important in regulating fibrinolytic activity in the vasculature, particularly that of tPA. In fact, high levels of PAI-1 are associated with many disease states including coronary heart disease (Mehta et al., 1987) and deep vein thrombosis (Kruithof et al., 1988).

PAI-2 is found in placenta extracts and in conditioned medium from macrophage/monocyte cells and cell lines. This inhibitor forms SDS-stable complexes more efficiently with UK than tPA and can exist in an unglycosylated intracellular form (MW = 47 kD) and a secreted glycosylated form (MW = 60 kD) (Kruithof et al., 1986;

Wohlwend et al., 1987). PAI-2 is believed to be important in inflammatory processes and also in pregnancy, since levels of PAI-2 are enhanced in late pregnancy and rapidly decrease post-partum (Kruithof et al., 1988).

A PA inhibitor found in urine (Hart et al., 1984; Cieplak and Hart, 1985; Hart et al., 1986) is distinct from PAI-1 and PAI-2 and has been designated PAI-3 (Stump et al., 1986). This inhibitor appears to be identical to urinary Protein C inhibitor and may not be a functionally active inhibitor of PA in vivo (Heeb et al., 1987).

Protease nexins are broad spectrum serine protease inhibitors that have been isolated from fibroblasts, fibrosarcoma cells, heart muscle cells, kidney epithelial cells and astrocytes (Eaton and Baker, 1983). Three PN molecules have been described which differ in molecular weight and proteinase specificity: PN-1 complexes trypsin, thrombin, plasmin, UK and tPA; PN-2 complexes epidermal growth factor; and PN-3 binds the nerve growth factor gamma subunit (Knauer et al., 1983; Scott et al., 1985). Protease nexin secreting cells bear surface receptors which mediate the endocytosis and lysosomal degradation of protease-protease nexin complexes (Scott and Baker, 1983). Furthermore, PN-1 shares some homology with heparin cofactor II and antithrombin III and, like these molecules, has a high affinity heparin binding site (Baker and Gronke, 1986). Protease nexins are present only in very low concentrations in vascular areas and are believed to function in local control of serine proteases at the tissue and cellular level.

1.3 PA and PA Inhibitors in Neoplasia

Increased activity and expression of plasminogen activators have been found in tissues and cell lines of malignant origin (Tissot et al., 1984; DeBruin et al., 1987; Sappino et al., 1987; DeBruin et al., 1988). Urokinase is believed to be important in initiat-

ing pericellular proteolysis contributing to invasive growth and metastatic potential of tumor cells (Dano et al., 1985). The ability of antibodies to PA to block metastases supports this role for urokinase (Ossowski and Reich, 1983).

The interaction between tumor cells and the extracellular matrix is also important in the processes of invasion and metastasis. Tumor cells must first adhere to ECM components, degrade them, and then migrate through the degraded matrix (Varini et al., 1987). Tumor cell adhesion to the ECM involves specific binding of ECM components by cell surface receptors, synthesis of ECM molecules by the tumor cells, and incorporation of these materials into the matrix (Varani et al., 1983). Proteases produced by the malignant cells are critical in all of these processes. The action of plasminogen activators has been implicated in extracellular matrix-tumor cell interactions. PAs, particulary urokinase, are extremely effective at degrading ECM constituents and appear to be crucial in the adhesion steps as well.

Furthermore, protease inhibitory molecules interfere with tumor cell-extracellular matrix interactions. Protease nexin-1 blocks the degradation of fibroblast-produced ECM by human HT-1080 fibrosarcoma cells and thus may help to maintain the integrity of connective tissue matrices (Bergmann et al., 1986). Similarly, endothelial cell ECM contains functionally active PAI-1 which may play a role in protecting the ECM and ECM-associated fibrin from degradation through specific inactivation of PAs (Mimura and Luskatoff, 1987).

1.3.1 Alterations in PA Activity During Differentiation

Alterations in plasminogen activator activity occur during the differentiation process in various tumor systems. Treatment of renal carcinoma cells with the differentiating agents dimethylsulfoxide (DMSO), sodium butyrate and RA led to a decrease in PA syn-

thesis (Prager et al., 1986; Nelson et al., 1987). Similarly, Ossowski and Belin (1985) observed a reduced production of UK-type PA in human epidermoid carcinoma HEp-3 cells induced to differentiate with DMSO. Gross and coworkers (1988) demonstrated a reduced expression of PA when glioma cells were differentiated with sodium butyrate. In this latter study, PA inhibitory activity remained unchanged during the maturational process. These findings have led to the suggestion that induction of a more benign phenotype in certain tumor cell lines is correlated with decreased fibrinolytic activity.

In contrast to the tumor cell types described above, neuroblastoma cells appear to express higher levels of fibrinolytic activity when induced to differentiate by exogenous agents (Laug et al., 1976; Soreq et al., 1983) or plasminogen-depleted medium (Becherer and Wachsman, 1980). However, these cells undergo dramatic morphological alterations in addition to the cell growth inhibition and expression of enzymatic markers typical of the differentiation process. With the availability of numerous human NB cell lines and advanced techniques to analyze fibrinolytic activity, it is possible to further investigate the regulation of these enzymes during neuroblastoma maturation.

1.4 Fibrinolytic Activity in the Nervous System

The regulation of fibrinolytic enzymes has been investigated in many normal and tumor systems (for review see Hart and Rehemtulla, 1988). However, relatively little is known about PA/PAI interactions in cells of neural origin. The majority of recent work has focused on the role of PAs in normal brain development by examining primary cultures of rat central and peripheral nervous system neurons and glia. Several groups have investigated the type and cellular origin of PAs produced by neuronal cells in order to characterize the components of the fibrinolytic system in the normal brain (Alvarez-

Buylla and Valinsky, 1985; Moonen et al., 1985; Baron-Van Evercooren et al., 1987). These studies have revealed the presence of both urokinase and tissue PA in CNS and PNS cell cultures (Selak et al., 1986).

In addition, the PA-plasmin system is involved in many aspects of neuronal migration and differentiation (Kalderon, 1982; Moonen et al., 1982). PA release by granule neurons is enhanced at the time of granule cell migration in the developing cerebellum (Krystosek and Seeds, 1981b). In the PNS, Schwann cells produce PA which is believed to be important in their proliferation and ability to ensheath neurons (Alvarez-Buylla and Valinsky, 1985; Hawkins and Seeds, 1986).

1.4.1 Protease Inhibition and Neurite Outgrowth

Supportive cells such as astrocytes and Schwann cells secrete numerous molecules such as neuronotrophic and neurite-promoting factors which affect the behavior of neurons (Varon et al., 1986). One of these, recognized more than a decade ago for its ability to induce dose dependent neurite outgrowth in mouse neuroblastoma cells, has protease inhibitory activity (Monard et al., 1973; Monard et al., 1983). This 43-kilodalton (kD) glial-derived neurite promoting factor (Gd-NPF), isolated originally from rat C6 glioma cells and most recently from cultured astrocytes, forms SDS-stable complexes with UK, tPA, thrombin and trypsin (Monard et al., 1983; Guenther et al., 1985). The inhibition of thrombin is accelerated by Gd-NPF over 40-fold in the presence of heparin (Stone et al., 1987). Protein and cDNA sequencing of Gd-NPF has revealed its identity as a protease nexin (Gloor et al., 1986; Rosenblatt et al., 1987; Sommer et al., 1987). Therefore it is evident that inhibitory molecules play an important role in neurite outgrowth.

Neurite promoting activity is detected in primary cultures of rat brain at a critical developmental stage (3-5 days post-natal) at which time the burst of glial cell proliferation occurs and neuronal migration ceases (Schuerch-Rathgeb and Monard, 1978). In fact, purified Gd-NPF has been demonstrated to reduce the rate of migration of granule cell neurons in cultured explants from early postnatal mouse cerebellum in a dose-dependent manner (Lindner et al., 1986). Furthermore, this factor induces neurite outgrowth and promotes the survival of cultured rat adrenal medullary chromaffin cells (Unsicker et al., 1984). These findings indicate that a PN-1-like molecule is able to influence processes leading to establishment and maintenance of the nervous system.

The mechanism by which protease nexin influences neural cell activities is a subject of intensive study. Other serine protease inhibitory molecules, hirudin and the synthetic tripeptide D-Phe-Pro-ArgCH_2Cl, are as effective as Gd-NPF at promoting neurite extension but do not modify neuronal cell migration (Monard et al., 1983; Monard, 1985). This finding suggests that the binding specificity of a protease inhibitor is critical to its effect on neuronal cell behavior. The neurite-inducing activity of protease nexin and the other inhibitors mentioned above may depend on the ability of these molecules to inhibit thrombin. Thrombin, but not urokinase or plasmin, is able to block neurite outgrowth and produce neurite retraction in NB cells (Gurwitz and Cunningham, 1988).

Monard (1987) has proposed a model to explain how protease inhibition regulates cellular migration and neuritic growth. He suggests that inhibitors such as Gd-NPF may promote neurite outgrowth by functioning in two capacities : (i) direct inhibition of cell-associated proteolytic activity leading to interference with the deadhesion phase of the migrating cell, and (ii) simultaneous interaction with proteases at the cell surface and

components of the extracellular matrix, contributing to anchorage of the cell. Clearly, regulation of extracellular fibrinolysis through protease/protease inhibitor interactions is an important factor contributing to neural cell growth, migration and differentiation.

1.4.2 Extracellular Matrix

Components of the extracellular matrix are secreted by a number of nervous system-derived cell types and include laminin, fibronectin, collagens, and glycosaminoglycans (Carbanetto et al., 1983). Interactions between neurons and ECM proteins are believed to be important in a number of processes including the establishment of nerve-muscle junctions (Festoff, 1985), and neurite extension (Rogers et al., 1983; Lander et al., 1985).

Neuronal cells may be induced to morphologically differentiate by plating on various natural and synthetic substrates. For instance, laminin is a major basal lamina glycoprotein which promotes rapid and extensive axonal outgrowth of central and peripheral neurons (Manthorpe et al., 1983; Rogers et al., 1983), retinoblastoma (Kyritsis et al., 1986) and neuroblastoma (Luckenbill-Edds and Kleinman, 1988). The heparin-binding domain of this glycoprotein is responsible for its effects on neurite outgrowth and neuronal survival (Edgar et al., 1984). Since protease nexin also binds heparin, Lindner et al. (1986) have suggested that this characteristic may be relevant to the ability of PN but not other protease inhibitors to induce neural process formation in NB cells.

1.5 Research Objectives

Clearly, a delicate balance between proteolytic activity and inhibition is important in many neuronal processes. This research project was undertaken to characterize the fibrinolytic enzymes produced by human neuroblastoma cells and to explore interactions between PAs and their inhibitors during the differentiation process. Using a zymographical technique to detect PA and PAI, conditioned medium and lysates from ten human NB cell lines were analyzed before and after induction of differentiation with retinoic acid. Alterations in fibrinolytic enzyme activity were correlated with the effects of RA on cell growth and morphology of the NB cell lines. A more extensive analysis of one NB cell line was performed to examine the effect of other differentiation agents on these same parameters.

This work will provide insights into the role of fibrinolysis in the malignant behavior of neuroblastoma cells, as well as the growth and differentiation of neuronal cells.

2.0 MATERIALS AND METHODS

2.1 Cell Culture

Neuroblastoma cell lines utilized in this study included SMS-KAN and SMS-KCNR (courtesy of Dr. P. Reynolds, Bethesda Naval Research Hospital, Bethesda, Md); IMR-5 and NMB/N7 (both courtesy of Dr. F. Gilbert, Mt Sinai Hospital, New York, NY); LA-N-1 and LA-N-2 (courtesy of Dr. R. Seeger, UCLA, Ca.); CHP-126 (courtesy of Dr. J. Roder, Mt Sinai Research Institute, Toronto, Ont.); IMR-32, SK-N-SH and SK-N-MC (American Type Culture Collection, Rockville, Ma.). All cell lines were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10 % heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Ut.) and were mycoplasma free. The cells were maintained in a humidified atmosphere of 95 % air- 5 % CO_2 at 37^0 C. The NB cells were grown as adherent monolayers in 75 cm^2 tissue culture flasks (Corning). Subculturing was carried out by shaking flasks to remove all adherent cells or using a Trypsin-EDTA solution (Gibco). Cells were split twice a week in a one in two or a one in three dilution.

2.2 Preparation of Cells For PA Assays

To prepare conditioned medium (CM), 60-70 % confluent monolayers were washed twice with serum-free medium and were placed in culture with 10 mL of RPMI-1640. After incubation for 24 hours, the medium was removed and concentrated 10-fold by ultrafiltration using UM10 filters with a molecular weight cut-off of 10 kD (Amicon, Danvers, Ma.). The samples were concentrated under nitrogen pressure at room temperature. Lysates were prepared by treating adherent cells with 1 mL of 20 mM CHAPS (Sigma Chemical Co., St.Louis, Mo.) dissolved in phosphate-buffered saline (PBS, pH 7.4). Lysates were then centrifuged at 1500 rpm for 10 minutes to pellet cell debris.

2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared as described by Laemmli (1970). Stacking and resolving gels were formulated to yield final concentrations of 4 % and 10 % acrylamide, respectively. All solutions for gels and buffers, except 10 % SDS, were stored at $4^{o}C$ and ammonium persulfate was freshly prepared. Gels were cast with dimensions of 120 x 170 x 1.5 mm in a Bio-Rad Protean II System and all reagents for SDS-PAGE were obtained from Bio-Rad Laboratories, Richmond, Ca.

2.3.1 Sample Preparation for SDS-PAGE

Samples were prepared for electrophoresis by incubation of 100 uL of concentrated CM or detergent lysate with 20 uL sample buffer (4 % SDS, 20 % glycerol, 0.12 M Tris-HCl, pH 6.8, 0.1 % bromophenol blue) for 10 minutes in a $37^{o}C$ water bath. For detection of UK-binding proteins, samples were pre-incubated with 1 unit low molecular weight urokinase (Alpha Therapeutics, Los Angeles, Ca.) for 30 minutes at $37^{o}C$ prior to addition of sample buffer. As references and standards, 10 uL dialyzed human urine (freshly prepared within one month of use), 1 unit tPA (American Diagnostics, Greenwich, Ct.) or 1 unit high molecular weight urokinase (Alpha Therapeutics) was included on each gel.

2.3.2 Electrophoresis of SDS-PAGE Gels

The electrode buffer was prepared from a 10-fold concentrated stock solution to give a final concentration of 50 mM Tris, 0.38 M glycine, 0.1 % SDS. The gels were run at $4^{o}C$ and 75 V constant voltage overnight with bromophenol blue as the tracking dye.

2.4 Analysis of Fibrinolytic Activity

2.4.1 Preparation of Plasminogen

Plasminogen was purified from human plasma by a method originally described by Deutsch and Mertz (1970). Blood (200 ml) was collected in heparinized syringes from healthy volunteers and centrifuged at 2000 rpm for 20 minutes at 4°C to separate plasma and red blood cells. The upper plasma fraction was removed and diluted with an equal volume of 0.3 M sodium phosphate, pH 7.4 (Na_3PO_4). Plasma was then treated with diisopropylfluorophosphate (DFP)(0.5 M stock in anhydrous isopropanol) to yield a final concentration of 5 mM DFP and mixed with gentle stirring for 4 hours at room temperature. The above procedure was performed in a fume hood with extreme caution since DFP is a highly toxic chemical. To remove excess DFP, the plasma was dialyzed overnight against PBS at 4°C.

To purify plasminogen, DFP-treated plasma was applied to a Lysine-Sepharose column (Sigma) that had been previously equilibriated with 0.1 M Na_3PO_4. The column was then washed thoroughly with 0.3 M Na_3PO_4 to elute unbound plasma proteins. Elution of plasminogen was performed using 0.2 M epsilon aminocaproic acid in 0.3 M Na_3PO_4 and eluate with an O.D. 280 reading of greater than 0.05 was collected. To remove excess epsilon aminocaproic acid, purified plasminogen was dialyzed overnight against PBS at 4°C. and then concentrated by Amicon ultrafiltration (UM 10 filters) to approximately 3 mls. The concentration of plasminogen was determined by a Bradford protein assay (Bradford, 1976) obtained from Bio-Rad. Aliquots of 100 ug plasminogen (10mg/ml) were stored at -70°C for use in zymography.

2.4.2 Zymography and Reverse Zymography

Plasminogen-dependent and -independent fibrinolytic activity of enzymes separated by SDS-PAGE were detected by the fibrin overlay technique (Granelli-Piperno and Reich, 1978). After electrophoresis, slab gels were washed in 2.5 % (w/v) Triton X-100 (Fluka Fine Chemicals, Ronkonkoma, NY) for 2 hours using gentle agitation to remove the SDS.

During this time, zymography indicator gels were made using freshly prepared solutions of plasminogen-free human fibrinogen (Miles Laboratories, Montreal, Qu.), human thrombin (Sigma) and purified human plasminogen. Noble agar (Sigma) was prepared immediately prior to use by boiling in distilled water (2.5 % w/v). Fibrinogen (10 mg/ml) was dissolved in PBS, pH 7.4 containing 0.1 % thimerosal. All ingredients were mixed in a tube equilibriated to 55^{o}C in a water bath in the following amounts: 4.0 ml agar, 1.6 ml fibrinogen(16 mg), 2.5 ml plasminogen (50 ug), 10 ul (10 units) thrombin. To demonstrate plasminogen dependence of fibrinolytic activity, plasminogen was omitted from some indicator gels.

The mixture was poured into a lid of a 96-well tissue culture plate (Falcon), also warmed to 55^{o}C. The lid was removed from the water bath and allowed to stand at room temperature for 5 minutes to allow fibrin polymerization and solidification of the agar.

After two hours of washing in Triton, gels were thoroughly rinsed with numerous washings of distilled water to remove detergent, blotted with paper towels, and placed on the fibrin indicator gels. Any excess water was drained and air bubbles removed. The plates were wrapped in Saran wrap and incubated at 37^{o}C in a humidified chamber.

Development of lysis zones was checked at various time intervals from 12-36 hours and the plates were placed at 4°C to halt further fibrinolysis when suitably developed. At this time, the polyacrylamide gels were discarded and the indicator gels stained with 0.1 % amido black in methanol:acetic acid: water (70:10:20). The gels were destained in the same solvent, coated with 2 % glycerol and then air dried.

Using this method, areas of PA activity appeared as clear zones on a blue stained background with the size of the zone of lysis being proportional to the amount of activity in a given volume of sample loaded on the SDS-PAGE gel. PA-PAI complexes were detected by zymography since SDS denaturation and Triton X-100 treatment enabled complexes to regain PA activity. Free PAI activity in the sample was detected by formation of a complex between endogenous PAI and exogenously added LMW-UK appearing at a higher molecular weight (33 kD + PAI).

Alternatively, the presence of PAI was detected by reverse zymography (vanMourik et al., 1984). SDS-PAGE gels were prepared and electrophoresed as described above and then washed in 100 ml of 2.5 % Triton X-100 containing 75 units of LMW-UK for 6 hours. Zymography plates were prepared as above. Using this technique, low levels of PAI could be visualized as a dark zone on a clear indicator gel (Rehemtulla et al., 1987).

2.4.3 Identification of PA Activity

In order to identify PA activity in CM and lysates, indicator gels were prepared containing 50 ul rabbit anti-human tPA IgG (American Diagnostics, Greenwich, Ct.) or 80 ul rabbit anti-human UK IgG (Alpha Therapeutics, Los Angeles, Ca.). When analyzed alongside control indicator gels, the disappearance of bands of PA activity indicated the presence of UK or tPA.

2.4.4 Fibrinolysis in Gel Assay

Direct assessment (without prior electrophoresis) of PA activity was made using the fibrinolysis in gel assay described by Saksela et al. (1985). Indicator plates were prepared as outlined above except that the volumes were doubled . Circular wells (2mm) were cut into the gel and 10 ul aliquots of CM or cell lysates were pipetted into the wells. The gels were then sealed, incubated and stained as described previously. The diameter of the zone of lysis was measured after incubation and results were calculated in terms of UK activity by reference to LMW-UK standards measured simultaneously.

2.5 Specificity of PA Inhibitor

2.5.1 Preparation of Iodinated Proteases

The following proteases were tested for their ability to form complexes with proteins in CM from the two cell lines SK-N-SH and SMS-KAN: LMW-UK, thrombin, trypsin, plasmin and pancreatic kallikrein (Sigma).

For the labelling experiment, each protease (100 ug) was diluted in 100 ul distilled water and incubated with 1 uCi Na-$^{[125]}$-I (Amersham, Arlington Heights, Il.) and 2 iodobeads for 30 minutes at room temperature. The reaction was terminated by removing the mixture from the iodobeads.

To fractionate the labelled proteins from free Na-$^{[125]}$-I a spun column was prepared in a 1 ml plastic syringe. Glass wool was placed in the bottom of the syringe and it was filled with Sephadex G50 suspended in PBS, pH 7.4 (Pharmacia, Upsala, Sweden). The filled syringe was placed in a plastic test tube and centrifuged at 2900 rpm for 5 minutes to pack the Sephadex. The syringe was refilled and spun twice more and then washed twice with 100 ul PBS. The mixture was then applied to the spun

column and centrifuged at 3000 rpm for 5 minutes to separate unlabelled salt from the iodinated protease. An eppendorf tube inserted under the syringe, which contained the labelled protease, was removed with tweezers and capped. The contents of the eppendorf were then counted on the gamma counter. An appropriate volume of labelled protease was then calculated to yield 20,000 cpm per lane for electrophoresis.

2.5.2 SDS-PAGE and Autoradiography

To detect the presence of protease-binding proteins in CM from NB cell lines, each iodinated protease (20,000 cpm) was incubated for 30 minutes at 37^{o}C with 100 ul CM and subjected to SDS-PAGE as described above. Alternatively, $^{[125]}$-I-labelled proteases were added to cell cultures concomitantly with serum-free medium and 24 hour CM was analyzed by SDS-PAGE and autoradiography.

After electrophoresis, the polyacrylamide gels were removed and stained with 0.25 % Coomassie Blue in isopropanol: acetic acid: water (10:10:80). Gels were destained in the same solvent with frequent washings for several hours. The gels were then dried for two hours in a Bio-Rad gel drier and autoradiographed using Kodak X-Omat film for 48-72 hours.

Adjacent lanes on the SDS-PAGE gels contained CM with or without the labelled protease. Complex formation was demonstrated by a shift in radioactivity from the molecular weight of the protease to that of the protease- inhibitor complex. Molecular weight markers for electrophoretic separations were obtained from Bio-Rad and included: phosphorylase b (92,000), bovine serum albumin (66,200), ovalbumin (92,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

2.6 Heparin-Sepharose Chromatography

200 ml CM from each of SK-N-SH and SMS-KAN cell lines was tested for binding to heparin-Sepharose (Pharmacia). The column (2.5 x 20 cm) was equilibriated with 0.3M NaCl in 20 mM Na_3PO_4 (pH 7.4) prior to loading CM. CM was applied to the column and non-adherent proteins were collected. Elution of heparin-binding proteins was performed with 1.0 M NaCl in the same buffer and protein fractions were collected (O.D. 280 greater than 0.05). PA binding activity (PAI) was detected by SDS-PAGE followed by zymography.

2.7 Retinoic Acid Treatment of Ten NB Cell Lines

2.7.1. Preparation of Cell Cultures

Preliminary experiments were performed to determine the concentration of all-trans retinoic acid (Sigma) yielding optimal morphological differentiation. The concentration of RA which induced maximal formation of neurites in these cell lines was 1 uM RA. Parallel cultures of each cell line were prepared and treated with either 0.1 % ethanol (control) or 1 uM RA (dissolved in ethanol) for 3 or 6 days. Medium was removed after 3 days and replaced with fresh medium (RA or ethanol control) and CM and lysates were prepared as described above after 6 days. To assess morphological differentiation, cells were examined daily with a phase contrast microscope. Cells were counted as morphologically differentiated if they possessed one or more neurites at least twice as long as the soma diameter (Sidell, 1982).

2.7.2. [^{3}H]-thymidine Incorporation in Four NB Cell Lines

To evaluate the time-dependent effects of RA on [^{3}H]-thymidine incorporation, 10^4 cells/well were plated in quadruplicate in 96-well tissue culture plates (Falcon) with separate plates set up in parallel to be used for each time point. One day after plating, medium containing 1 uM RA or 0.1 % ethanol (solvent control) was added to each well. At 1, 3 and 6 days after adding RA, the plates were pulsed with 1 uCi of [^{3}H]-thymidine (42 Ci/mM) (Amersham) per well after various time intervals. After incubating for 18 hours at 37^{o}C, the cells were harvested on strips of fiberglass filter paper and placed into plastic scintillation vials. Five ml of scintillation fluid (Fisher Scientific, Fair Lawn, NJ) was added to the vials and they were incubated at room temperature for several hours. Incorporated [^{3}H]-cpm was determined by a beta- counter (Beckmann). Results were expressed as the percentage of [^{3}H]-thymidine incorporation into RA-treated cells compared to control cells that were pulsed and harvested on the same day.

2.8 Treatment of SMS-KAN Cells With Five Differentiation Agents

2.8.1 Preparation of Cell Cultures

To induce morphological differentiation, SMS-KAN cells were plated in 75 cm^2 flasks, allowed to adhere for 24 hours, and then treated with the following agents: 1 uM RA, 1 mM and 2 mM dbcAMP, 1 mM NaB, 10 uM 5-BrDU, and 100 ng/ml PMA (all agents from Sigma). Control cultures consisted of medium (RPMI) alone, medium containing 0.1% ethanol (solvent for RA) or 0.1% DMSO (solvent for PMA). This concentration of ethanol or DMSO had no detectable effect on cell growth, differentiation or PA activity (data not shown). Parallel cultures of SMS-KAN cells were treated with differentiating agents for 3 or 6 days, at which time morphological differentiation was assessed, the cells photographed, and samples obtained for PA analysis.

An additional time course experiment was performed with RA treatment, where these same parameters were measured daily over a course of six consecutive days.

2.8.2. [^{3}H]-thymidine Incorporation

The effect of the various agents on DNA synthesis was determined by measuring the incorporation of [^{3}H]-thymidine over a specific time interval as described above. Cells were treated with the differentiation agents and control solvents for 3 and 6 days.

3.0 RESULTS

3.1 Cell Culture

Nine human neuroblastoma cell lines derived from different patients and one subclone, IMR-5, were utilized in this study. Table 1 outlines several features of the cell lines such as site of origin of the tumor, adrenergic/cholinergic activity and their morphological characteristics in vitro. In addition, these cell lines varied dramatically in terms of growth rate with the most strongly substrate-adherent cells (IMR-32, SK-N-SH, NMB/N7,IMR-5, CHP-126) reaching confluence most quickly.

3.2 PA and PAI Activity of Ten NB Cell Lines

3.2.1 Identification of PA Activity

Serum-free CM from the ten human NB cell lines was analyzed by SDS-PAGE followed by zymography (Figure 1). As indicated in Table 2 and shown in Figure 1, all cell lines secreted a 52 KD protein which comigrated with human high molecular weight urokinase (HMW-UK) and was inhibited by the inclusion of rabbit anti-human urokinase antibodies into the fibrin overlay gel. CM from eight of the cell lines also contained a 65 kD PA which comigrated with human tPA and was inhibited by rabbit anti-human tPA antibodies. Figure 2 illustrates inhibition experiments with the IMR-5 NB cell line. Additional experiments revealed that the enzymatic activities in the CM were plasminogen-dependent, further supporting the identification of these bands as UK and tPA (data not shown).

TABLE 1

GENERAL CHARACTERISTICS OF HUMAN NEUROBLASTOMA CELL LINES

CELL LINE	SITE OF ORIGIN	ADR/CHOL[a]	MORPHOLOGICAL FEATURES
LA-N-1	bone marrow	adr	-mixture of neuronal and substrate adherent phenotypes (Seeger et al., 1977)
LA-N-2	bone marrow	mixed	-predominantly neuronal, tear-drop shaped cells (Seeger et al., 1977)
SMS-KAN	ovarian tumor	adr	-small, polygonal cells; grow in clumps (Reynolds et al., 1986)
SMS-KCNR	bone marrow after chemotherapy	adr	-similar to SMS-KAN, except cells smaller (Reynolds et al., 1986)
IMR-32	abdominal mass	adr	-single cells; neuronal with long processes (Tumilowicz et al., 1970)
IMR-5	abdominal mass	adr	-small, neuronal shaped cells in large clumps (subclone of IMR-32
NMB/N7	?[b]	?	-identical morphologically to IMR-32, but derived from a different patient (Brodeur et al., 1973)
SK-N-SH	bone marrow	adr	-mixture 50% flat fibroblast-like cells/50% neuronal (Biedler et al., 1973)
SK-N-MC	supra-orbital	chol	-predominantly small, round neuroblastic cells with 20% flat cells (Biedler et al., 1978)
CHP-126	bone marrow	?	-identical morphologically to IMR-5, but derived from a different patient (Schlesinger et al., 1981)

[a] ADR = Adrenergic
CHOL = Cholinergic
[b] ? = no data available

Figure 1: Zymographic analysis of conditioned medium (CM) from ten human neuroblastoma cell lines. CM was incubated with (right lane) and without (left lane) LMW-UK prior to electrophoresis and zymography.

PANEL (A) SK-N-MC; (B) LA-N-2; (C) SMS-KAN; (D) CHP-126; (E) LA-N-1; (F) IMR-5; (G) SMS-KCNR;(H) NMB/N7 (left and right lanes reversed); (I) IMR-32; (J) SK-N-SH; (K) H Ur-dialyzed human urine.

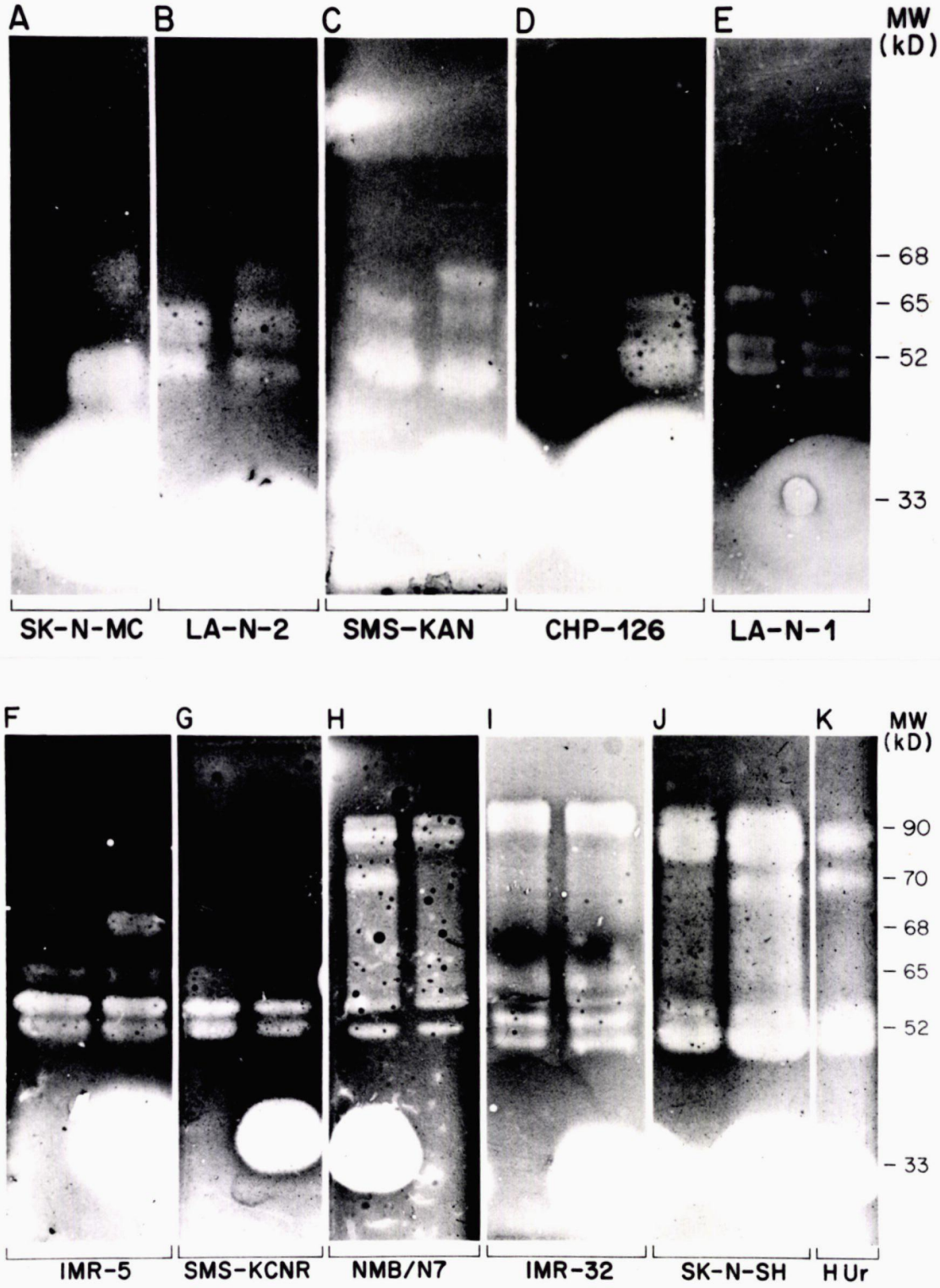

A
B
C
D
E
MW (kD)
68
65
52
33
SK-N-MC
LA-N-2
SMS-KAN
CHP-126
LA-N-1
F
G
H
I
J
K
MW (kD)
90
70
68
65
52
33
IMR-5
SMS-KCNR
NMB/N7
IMR-32
SK-N-SH
H Ur

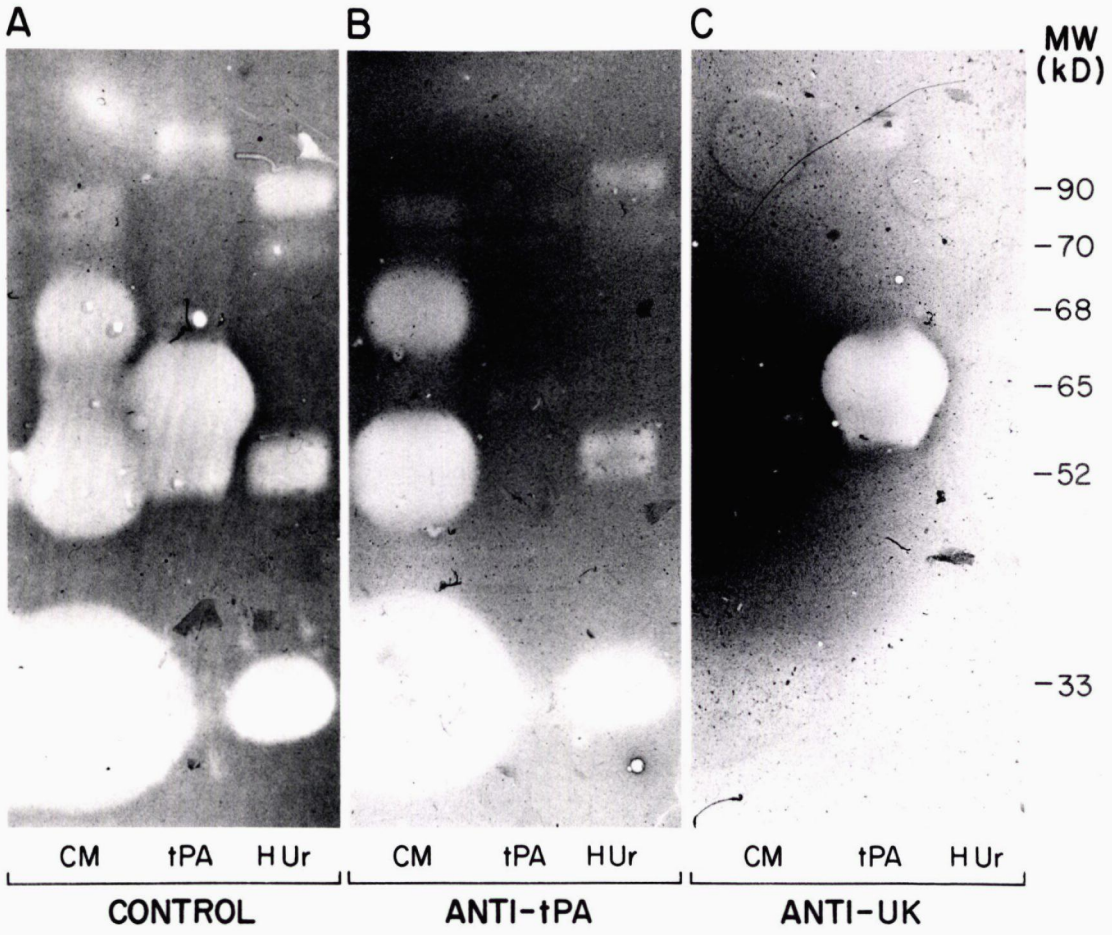
TABLE 2

PLASMINOGEN ACTIVATOR AND INHIBITOR ACTIVITY IN CONDITIONED MEDIUM FROM HUMAN NEUROBLASTOMA CELL LINES

CELL LINE	HMW-UK (52 kD)	tPA (65 kD)	PAI-complex (68 kD)[a]	PAI-complex (75 kD)[a]	PAI-complex (90 kD)[b]
SK-N-MC	+[c]	-	+	-	-
LA-N-2	+	+	+	-	-
SMS-KAN	+	+	+	-	+/-
CHP 126	+	+	-	-	-
LA-N-1	+	+	+	-	-
IMR-5	+	+	+	-	+/-
SMS-KCNR	+	+	-	-	-
NMB/N7	+	+	-	+	+
IMR-32	+	+	-	+	+
SK-N-SH	+	-	-	+	+

[a] PAI + exogenous LMW-UK
[b] PAI + endogenous HMW-UK
[c] Conditioned medium from each cell line was prepared and assayed as described in the Materials and Methods. The presence (+) or absence (-) of PA and PAI-complex activity in repeated analyses of the cell lines is indicated.

Figure 2: Identification of PA activity in conditioned medium from the NB cell line IMR-5. Indicator gels contained no antibodies (PANEL A); anti-human UK (PANEL B); or anti-human tPA (PANEL C). Standards included purified human tPA and dialyzed human urine (H Ur).


A
B
C
MW
(kD)
-90
-70
-68
-65
-52
-33
CM
tPA
HUr
CM
tPA
HUr
CM
tPA
HUr
CONTROL
ANTI-tPA
ANTI-UK

3.2.2 Detection of PA Inhibitors

The presence of plasminogen activator inhibitor (PAI) activity in the CM was detected by preincubating 100 uL of CM with 1 unit of low molecular weight urokinase (LMW-UK: MW= 33 KD), followed by SDS-PAGE and zymography. Previous experiments have revealed that the 70 kD and 90 kD zones of lysis observed zymographically in human urine concentrates are complexes of LMW-UK and HMW-UK with a UK-inhibitor of MW approximately 40 kD (Cieplak and Hart, 1985). In five of the cell lines (SMS-KAN, IMR-5, LA-N-1, LA-N-2, SK-N-MC), preincubation with LMW-UK yielded a fibrinolytically active band at MW= 68kD, suggesting the presence of a UK binding protein in the CM with a MW= 35 kD. A LMW-UK complex of slightly higher molecular weight (75 kD) was detected in CM from SK-N-SH, NMB/N7 and IMR-32. Addition of anti-UK antibodies to the indicator gels inhibited the detection of these complexes (see Figure 2). Higher molecular weight bands (MW approximately 95 and 105 kD) were detected in CM and lysates from several cell lines as well. These bands probably represent complexes of PA inhibitor with endogenous HMW-UK and tPA, respectively (reviewed in Hart and Rehemtulla, 1988).

3.3 Specificity of Inhibitor from SK-N-SH and SMS-KAN

The PAI molecules and protease nexin (PN) can be distinguished in part by their differing affinities for certain proteases. Plasminogen activator inhibitors are specific for UK and tPA, while PN also binds trypsin, plasmin and thrombin (Sprengers and Kluft, 1987). Inhibitors of plasma and pancreatic kallikrein have also been described (Lammle and Griffin, 1985; Scott et al., 1986).

To determine the specificity of the NB-derived PAI(s), radioactively labelled proteases were prepared (UK, thrombin, trypsin, plasmin and kallikrein) and tested for complex forming ability with CM from several of the NB cell lines. When iodinated UK was added directly to the cells or CM, a shift in radioactivity from MW= 33kD to Mr= 68kD was observed with SMS-KAN and IMR-5, supporting the above described indication that these cells secrete a 35 kD UK-binding protein (see Table 3). The inhibitor secreted by SK-N-SH formed a complex with UK which zymographically ran slightly higher than that of the previous two cell lines (PAI MW= 40 kD); this molecular weight was confirmed by the iodination experiments. Of the other proteases tested, only trypsin formed complexes with CM from all three cell lines. In contrast to the single band of complex observed with UK (Figure 1, Lanes A,B,C,E,F), multiple bands were present in the [CM/cells + trypsin] lanes.

3.4 Heparin-Sepharose Chromatography

CM from SK-N-SH and SMS-KAN cells were tested for the presence of heparin-binding PA inhibitors. When analyzed by zymography and reverse zymography, PAI was detected in both CM and eluate from a heparin-Sepharose column (see Figure 3) indicating that the SK-N-SH derived PAI is able to bind heparin. PAI present in conditioned medium from SMS-KAN cells did not bind to heparin-Sepharose. Comparison of the biochemical characteristics of the two NB-derived PAI molecules with other previously defined PAIs is presented in Table 3. Although the binding of the SK-N-SH PAI to heparin suggests that it may be protease nexin or PAI-3 (Scott and Baker, 1983; Sprengers and Kluft, 1987), protease specificity experiments indicate that this is prob-

TABLE 3

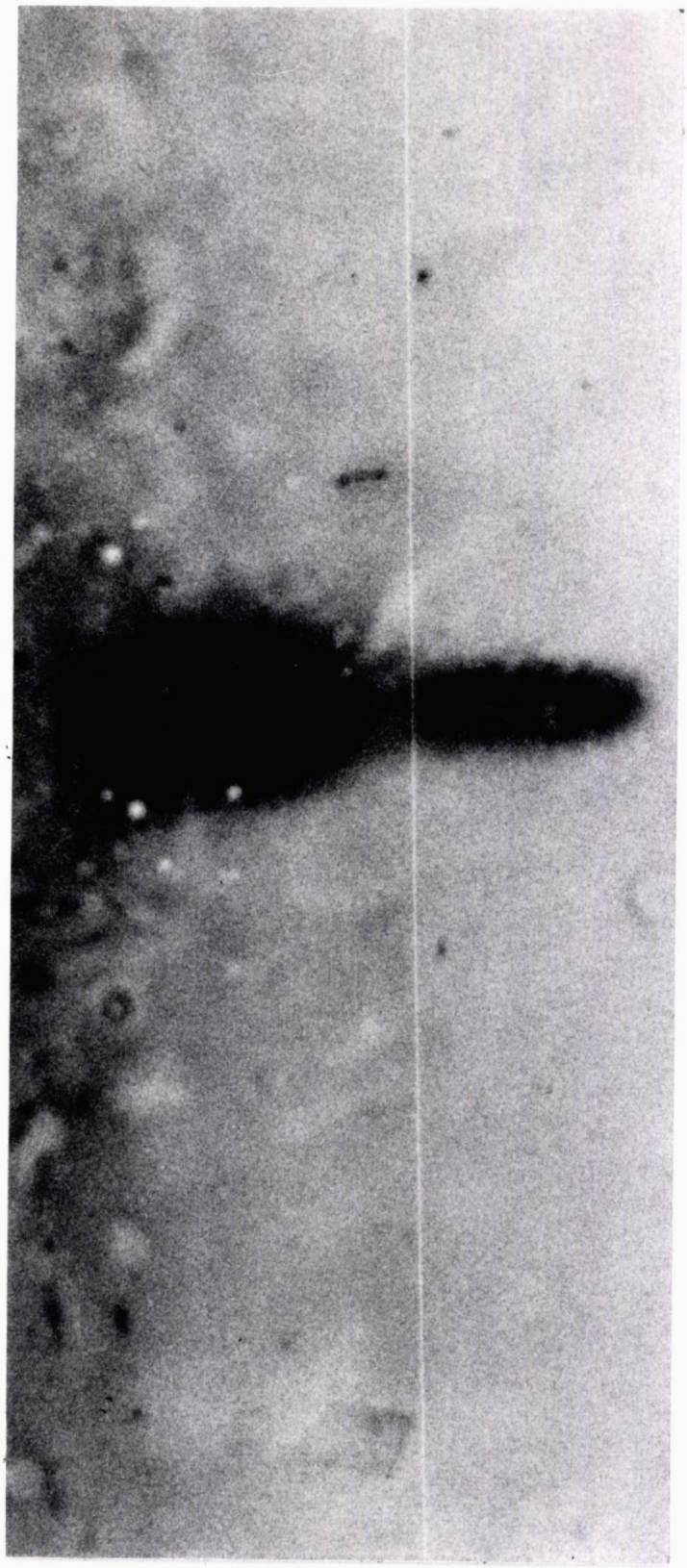
BIOCHEMICAL CHARACTERISTICS OF PA INHIBITORS

	PAI-1[a]	PAI-2[a]	PAI-3[a]	PN[a]	SK-N-SH	SMS-KAN
SOURCE	plasma platelets hepatocytes endothelium	placenta macrophages monocytes leukocytes	urine plasma	fibroblasts astrocytes	neuroblastoma cell lines	
MOL WT (kD)	54	47	50	51	40	35
HEPARIN-BINDING	weak	no	yes	yes	yes	no
PROTEASE SPECIFICITY						
UK	yes	yes	yes	yes	yes	yes
tPA	yes	slight	yes	yes	ND	ND
TRYPSIN	no	no	ND	yes	yes	yes
THROMBIN	no	no	no	yes	no	no
PLASMIN	no	no	yes	yes	no	no

[a] Reviewed in Sprengers and Kluft, 1987.

Figure 3: Reverse zymogram of SK-N-SH CM and eluate from heparin-Sepharose column demonstrating presence of PA inhibitor that binds to heparin.

(A) CM; (B) heparin-Sepharose eluate.



A B

ably not the case. The SMS-KAN PAI also appears to be different from the reported PAI molecules when both heparin binding affinity and protease specificity are considered.

3.5 Effect of Retinoic Acid on Ten NB Cell Lines

3.5.1 Morphology

Retinoic acid induces differentiation of NB cell lines to several different phenotypes (Reynolds and Maples, 1985). Typically, differentiation is indicated by extensive neurite outgrowth and inhibition of cell proliferation (Sidell et al., 1983), although in some cell lines differentiation to a Schwannian/melanocytic 'flat cell' is observed. Table 4 outlines the changes in morphology observed in the NB cell lines after treatment with RA for 6 days. In several of the cell lines, spontaneous neurite outgrowth was observed (5-25% differentiated cells observed prior to treatment). Photographs of both control and RA-treated cells (four representative cell lines) taken at 6 days are shown in Figure 4. Although several cells lines were shown to be resistant to morphological differentiation induced by RA, axonal outgrowth was evident in the majority of lines tested. SK-N-SH was an exception in that the cells changed from a mixed neuronal/flat cell morphology to a predominately flat cell phenotype with RA treatment.

3.5.2 [^{3}H]-thymidine Incorporation in Four NB Cell Lines

The effect of RA on the inhibition of DNA synthesis was analyzed in four cell lines by incorporation of [^{3}H]-thymidine after 1, 3 and 6 days. As shown in Figure 5, SMS-KAN was most sensitive to growth inhibition by RA, while IMR-5 showed a slight reduction in DNA synthesis. SK-N-MC was completely resistant to RA and SK-N-SH

TABLE 4

MORPHOLOGICAL CHANGES OBSERVED IN NB CELL LINES AFTER TREATMENT WITH RETINOIC ACID FOR 6 DAYS

CELL LINE	NEURITE OUTGROWTH	MORPHOLOGICAL ALTERATIONS
LA-N-1	1+ ---> 3+[a]	- extensive neurite outgrowth involving most cells
LA-N-2	1+ ---> 2+	- limited neurite outgrowth
SMS-KAN	0 ---> 4+	- cells spread and flatten - numerous short neurite extensions from individual cells
SMS-KCNR	0 ---> 3+	- same as SMS-KAN
IMR-5	1+ ---> 3+	- axonal extensions from cells at edges of large clumps
IMR-32	N.D.[b]	- neuronal morphology - no detectable changes
SK-N-SH	N.D.	- mixed neuronal/flat morphology to 100% flat cells
SK-N-MC	N.D.	- neuroblastic, round cells - no detectable changes
NMB/N7	N.D.	- same as IMR-32
CHP-126	1+ ---> 3+	- same as IMR-5

[a] extent of morphological differentiation: control cultures---> RA treated cultures. Cells were scored according to the degree of neurite outgrowth (processes at least twice as long as the soma diameter) and reported as percentage differentiated cells: 4+> 76%; 3+ = 51-75%; 2+ = 26-50%; 1+ = 5-25%; 0 = <5%.

[b] N.D. = none detected

Figure 4: Alterations in morphology in four NB cell lines treated with retinoic acid. Parallel cultures of each cell line were treated with 0.1 % ethanol (control) or 1 uM RA (in 0.1% ethanol) for six days.

(A) SMS-KAN control; (B) SMS-KAN RA treated; (C) IMR-5 control; (D) IMR-5 RA treated; (E) SK-N-SH control; (F) SK-N-SH RA treated; (G,H) SK-N-MC cells. (Phase contrast x 100)

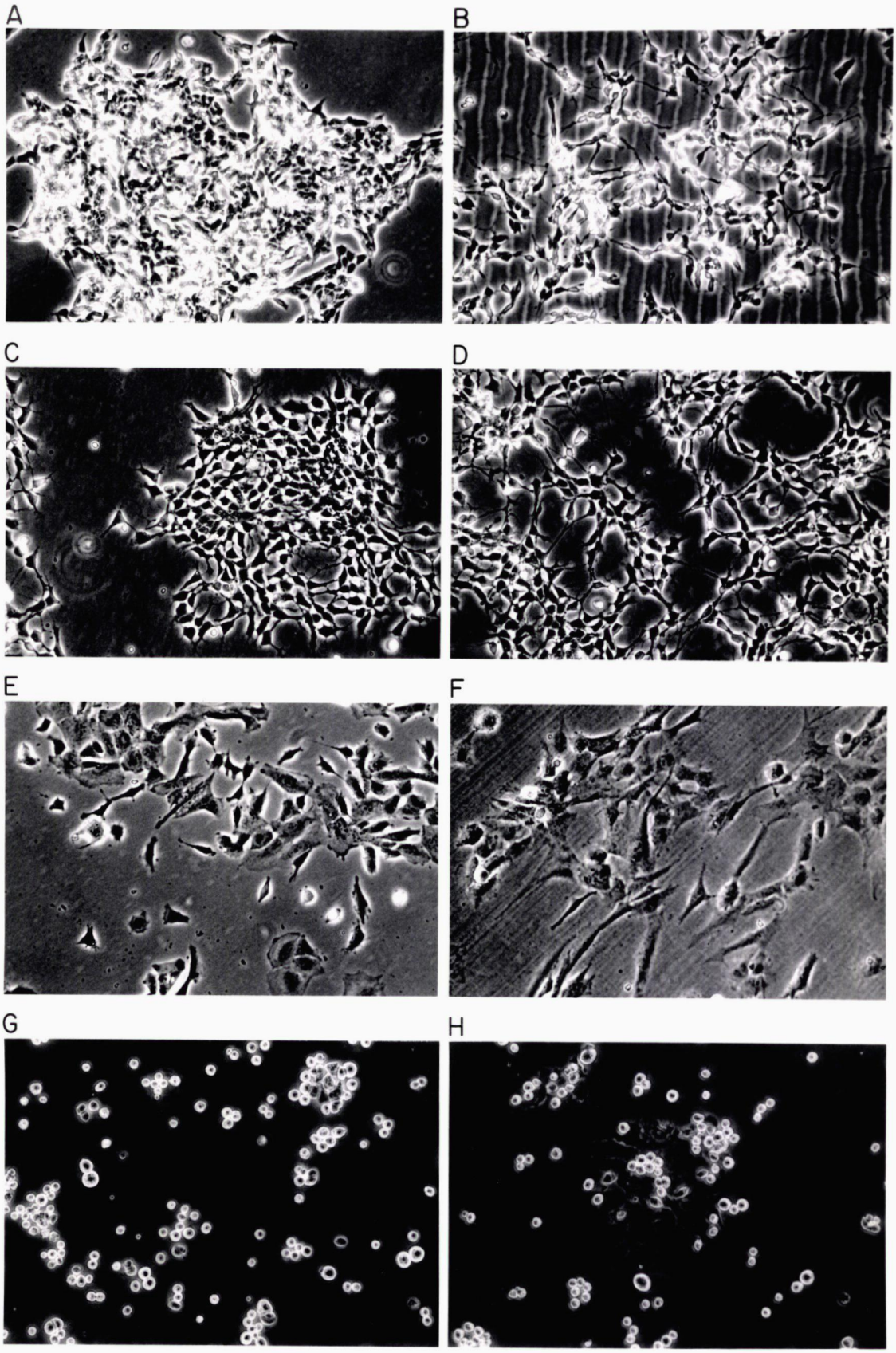
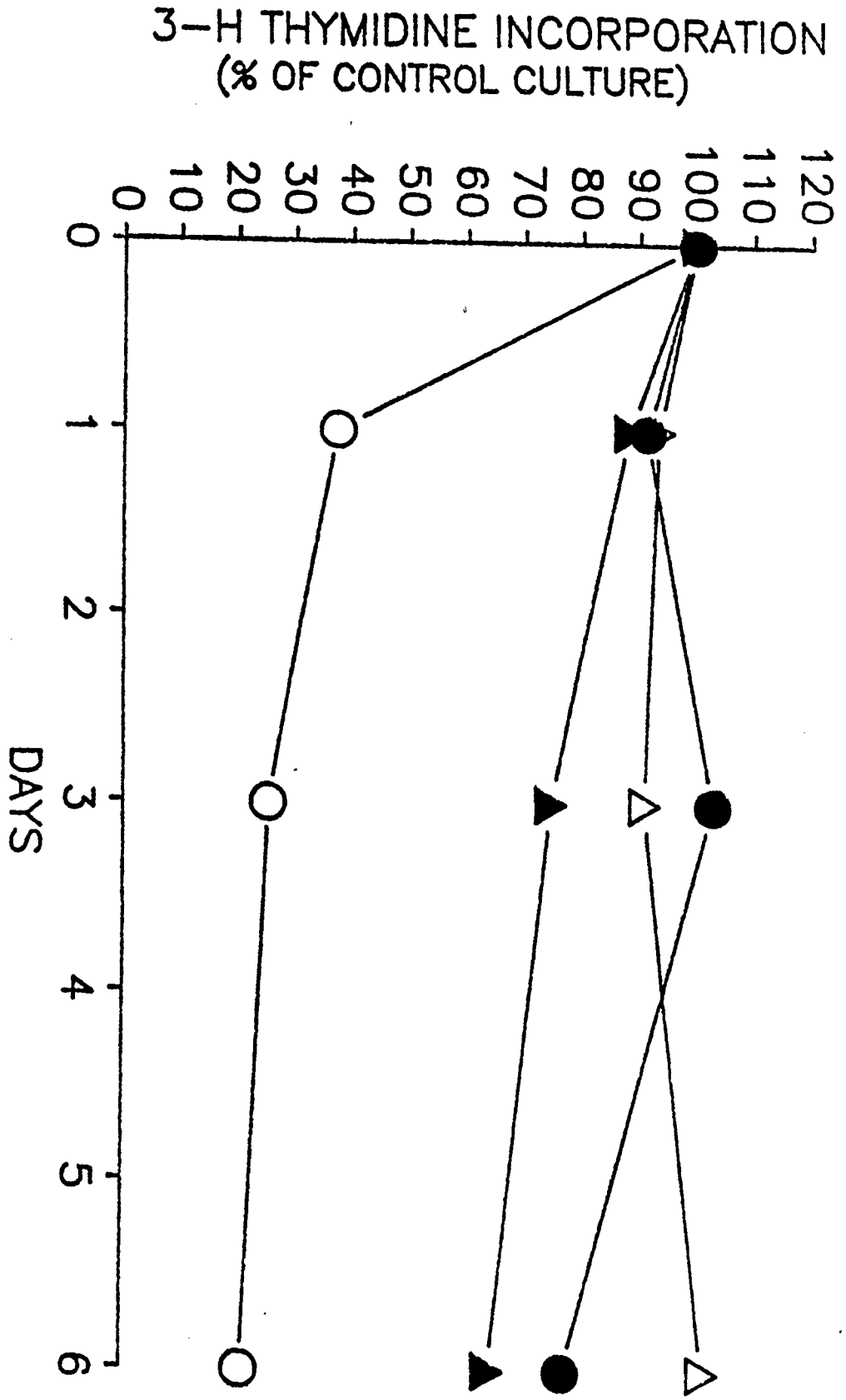

A
B
C
D
E
F
G
H

Figure 5: Time course experiment showing the effect of RA on DNA synthesis in four NB cell lines. [^{3}H]-thymidine incorporation was measured after 1, 3 and 6 days of treatment in ● SK-N-MC; Δ SK-N-SH; ▲ IMR-5; and O SMS-KAN. Values represent the average of quadruplicate cultures (SE < 10%).


3–H THYMIDINE INCORPORATION
(% OF CONTROL CULTURE)
0
10
20
30
40
50
60
70
80
90
100
110
120
DAYS
0
1
2
3
4
5
6

cells treated for 6 days actually showed a slight increase in DNA synthesis compared to untreated cells. These findings were corroborated by observations of cell density, suggesting that alterations in DNA synthesis were a reflection of cellular proliferation.

3.5.3 Fibrinolytic Enzyme Activity

In light of the variability in morphological response to retinoic acid of the different NB cell lines, it was of interest to compare these results to RA induced changes in PA and PAI activity of the same cell lines. Table 5 presents the results of zymographic analysis of the CM and cell lysates, indicating shifts in PA/PAI activity observed after 6 days of RA treatment. Representative zymographies (corresponding to cell lines shown in Figure 4) are presented in Figure 6 (supernatants) and Figure 7 (lysates).

As depicted in Panel A (Figures 6 and 7), the fibrinolytic enzyme profile of SMS-KAN cells was dramatically altered with RA treatment. A switch from secretion of UK and PAI in control cultures (Lanes a and b) to tPA in RA-treated cultures (Lanes c and d) was observed. An enhancement of cellular tPA was also observed in RA-induced cells compared to controls. IMR-5 cells (Panel B) exhibited a decrease of secreted UK and PAI and an apparent increase in cell-associated tPA activity. Overall, six of the NB cell lines exhibited an apparent increase in secreted and cell-associated tPA activity corresponding with morphological differentiation of the cells. In several of these cell lines, a decrease in UK secretion and UK- inhibitor complex formation accompanied the up-regulation of tPA (LA-N-1, IMR-5, SMS-KAN). In addition, these cell lines were generally most susceptible to growth inhibition by RA suggesting that PA activity, on a per flask basis, would represent a lower number of cells compared to control cultures.

TABLE 5

CHANGES IN PA/PAI ACTIVITY OF NB CELL LINES TREATED WITH RETINOIC ACID FOR 6 DAYS

CELL LINE	SECRETED				CELL-ASSOCIATED			
	HMW-UK	tPA	L.C.[a]	H.C.[b]	HMW-UK	tPA	L.C.	H.C.
LA-N-1	--[c]	++	-	nd[d]	NC[e]	+++	NC	nd
LA-N-2	NC	NC	NC	nd	nd	+++	nd	nd
SMS-KAN	---	+++	--	nd	NC	+++	NC	nd
SMS-KCNR	--	NC	nd	nd	NC	+++	nd	nd
IMR-5	---	NC	-	NC	NC	+++	nd	nd
IMR-32	NC	+	NC	NC	NC	NC	nd	nd
SK-N-SH	+++	nd	+++	+++	+++	nd	nd	+++
SK-N-MC	+	nd	+	nd	NC	nd	NC	nd
NMB/N7	-	NC	NC	-	-	nd	nd	nd
CHP-126	-	+	nd	nd	NC	+++	nd	nd

[a] L.C. - Low Molecular Weight Complex (68-75 kD)
[b] H.C. - High Molecular Weight Complex (90 kD)
[c] Equivalent volumes of CM or lysate from control and RA-treated cultures were assayed by SDS-PAGE and zymography. Results of zymographical analysis are tabulated according to the degree of change in size of the zone of lysis on the indicator plate observed in RA-treated samples compared to control samples. (-) and (+) indicate decrease and increase respectively.
[d] nd - none detected
[e] NC - no change

Figure 6: Fibrinolytic enzyme profile of four NB cell lines treated with retinoic acid. Equivalent volumes of sample from control (Lane a: CM; Lane b: CM incubated with LMW-UK) and RA treated (Lane c: CM; Lane d: CM incubated with LMW-UK) cultures were analyzed by SDS-PAGE and zymography. Lane e is a dialyzed human urine control (H Ur) in all panels except A where Lane e is a tPA standard. The cell lines correspond to those in Figure 5 where PANEL (A) SMS-KAN; (B) IMR-5; (C) SK-N-MC;(D) SK-N-SH.

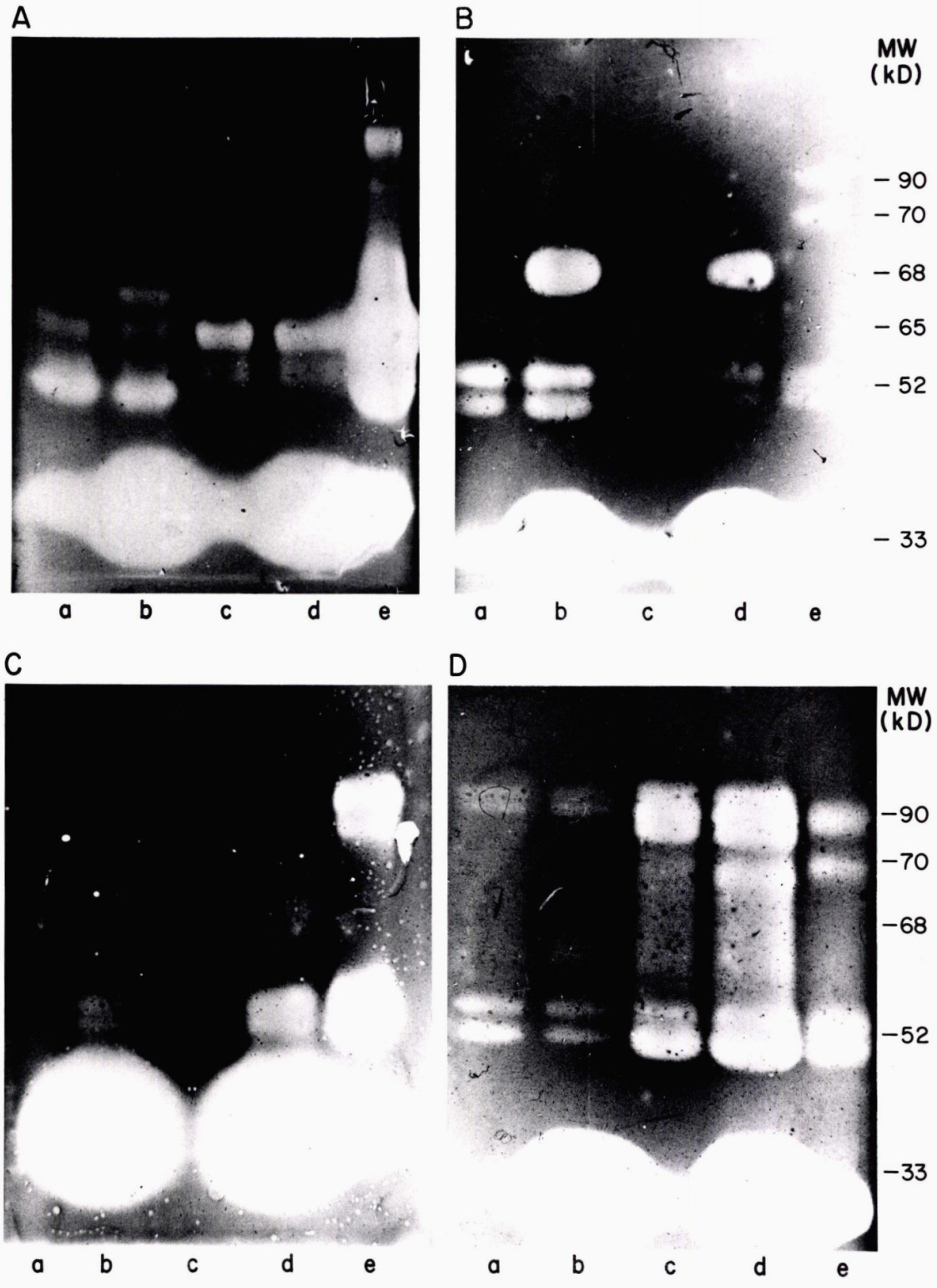
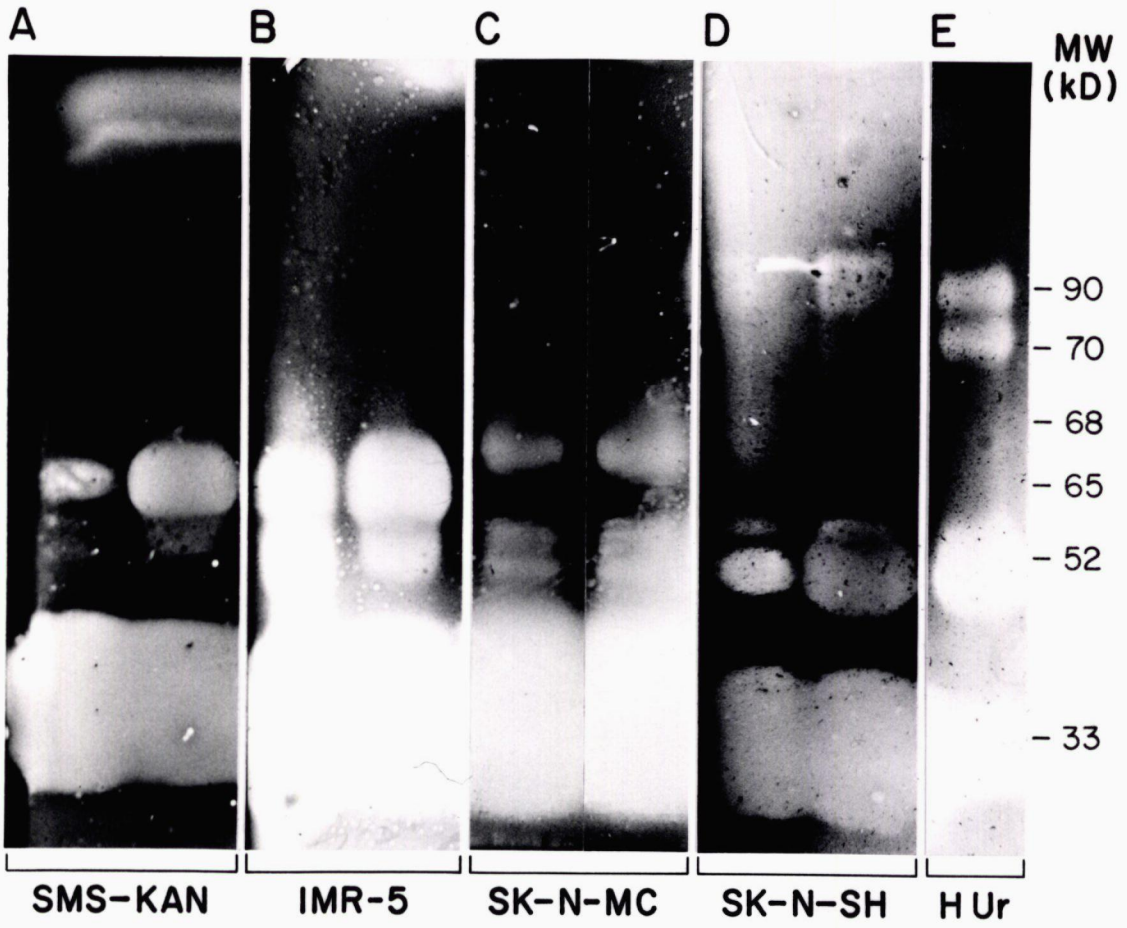

A
B
MW
(kD)
– 90
– 70
– 68
– 65
– 52
– 33
a b c d e
a b c d e
C
D
MW
(kD)
–90
–70
–68
–52
–33
a b c d e
a b c d e

Figure 7: Zymographic analysis of lysates from four NB cell lines treated with 1 uM retinoic acid for six days. Each panel shows PA activity of control (left lane) and RA treated (right lane) samples.

PANEL (A) SMS-KAN; (B) IMR-5; (C) SK-N-MC; (D)SK-N-SH; (E) dialyzed human urine (H Ur).


A
B
C
D
E
MW
(kD)
90
70
68
65
52
33
SMS-KAN
IMR-5
SK-N-MC
SK-N-SH
H Ur

In cell lines exhibiting no detectable morphological alterations on exposure to RA, no significant changes in fibrinolytic enzyme activity were observed. For instance, in SK-N-MC cells induced with RA, only a slight enhancement of UK and PAI was observed (Panel C). In one cell line, SK-N-SH, conversion to the flat cell phenotype correlated with an increase in cell-associated and secreted UK, as well as UK-inhibitor complex activity (Panel D).

3.6 Treatment of SMS-KAN with Differentiation Agents

3.6.1 Morphology

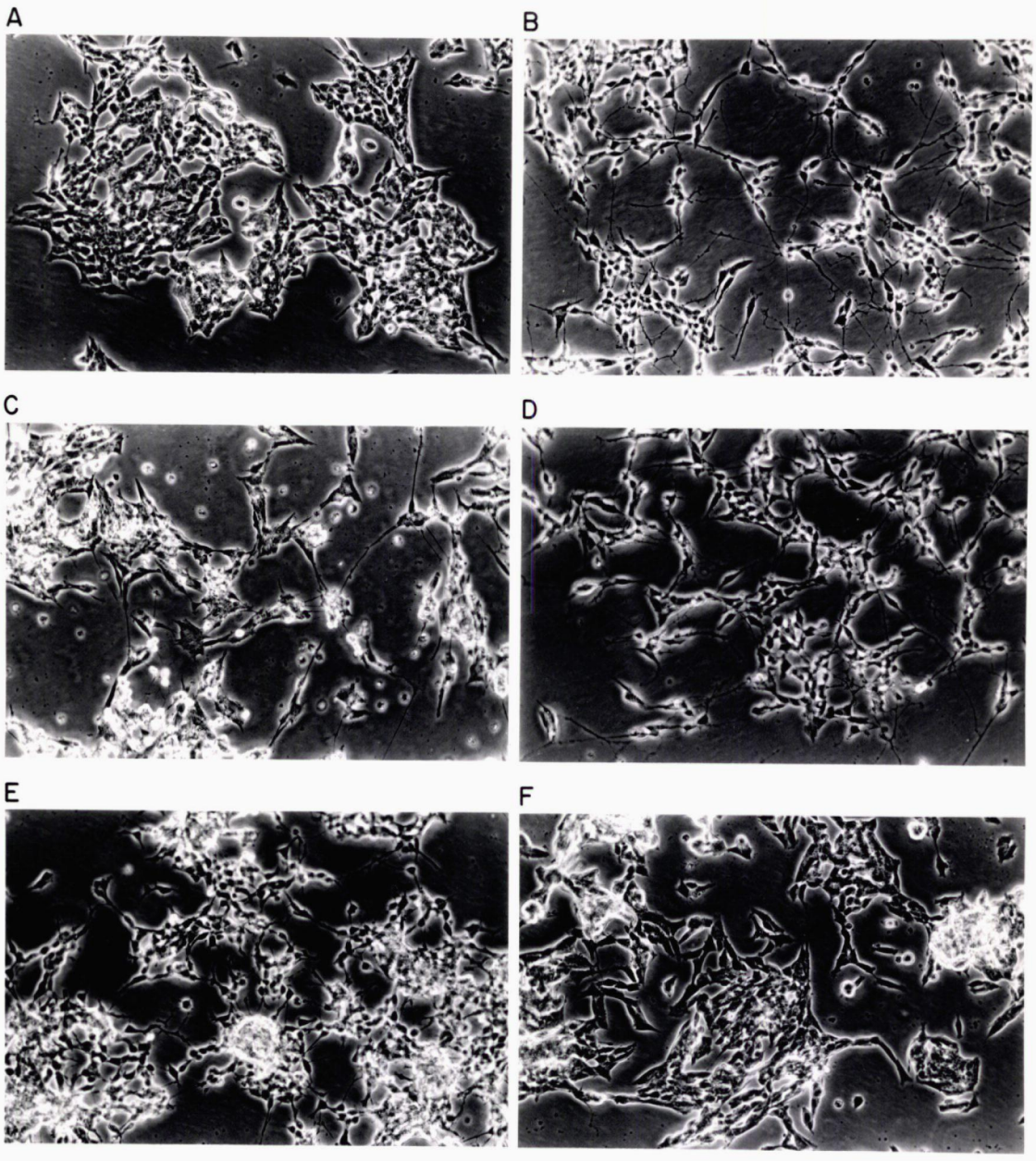
SMS-KAN cells have previously been utilized to investigate features of NB cells undergoing morphological differentiation. Table 6 and Figure 8 illustrate the changes in cellular morphology observed after treatment with the various agents for 6 days. Similar alterations were detected in all cases after 3 days, although the effects were less pronounced. RA induced the most dramatic changes, with extensive neurite outgrowth and spreading of cells (Figure 8, panel B). The effects of NaB were quite similar to those of RA (Figure 8, panel D), although some cell death was observed during the period of conditioning (approximately 10%). Dibutyryl cAMP caused axonal outgrowth, with flattening but no spreading of cells (Figure 8, panel C). 5-BrDU induced the formation of numerous short neurites although the time course of the response was considerably slower than that of the other agents (Figure 8, panel E). No neurite outgrowth was observed with PMA-treated cells although breaking apart of clumps and outward migration of cells from the clumps was apparent (Figure 8, panel F).

TABLE 6

MORPHOLOGY OF SMS-KAN CELLS TREATED WITH FIVE DIFFERENTIATING AGENTS

TREATMENT	CELL MORPHOLOGY
CONTROL (RPMI, 0.1% EtOH, or 0.1% DMSO)	-tear-drop shaped cells growing in clumps -very few neurite extensions -no detectable changes in morphology over six days
1 uM RA	-cells spread and flatten -extensive neurite outgrowth; short extensions from individual cells lengthening over course of treatment
1 mM dbcAMP	-limited neurite outgrowth -no spreading of cells
2 mM dbcAMP	-cells quite clumped with neurites extending from few cells on outer edge of clump -fewer neurites than with RA, but much longer
1 mM NaB	-identical to RA but slower time course (i.e. 6 day NaB like 4-5 day RA culture)
10 uM 5-BrDU	-similar to RA except cells more clumped -many neurites but much shorter
100 ng/ml PMA	-clumps of cells very 'rounded' -individual cells migrating away from clumps but no neurites observed

Figure 8: Morphological changes observed in SMS-KAN cells treated for six days with five differentiation agents. (A) untreated cells; (B) 1 uM RA; (C) 2 mM dbcAMP; (D) 1 mM NaB; (E) 10 uM 5-BrDU; (F) 100 ng/ml PMA. (phase contrast x 100)


A
B
C
D
E
F

3.6.2 [^{3}H]-thymidine Incorporation

Figure 9 demonstrates the effect of the differentiating agents on DNA synthesis in SMS-KAN cells. Compared to the appropriate controls, PMA had no detectable effect on [^{3}H]-thymidine incorporation by the cells. However, 2 mM dbcAMP was moderately effective in inhibiting cell proliferation (70% and 45% of control [^{3}H]-thymidine incorporation after 3 and 6 days of treatment respectively), while RA (5% of control), NaB (20% of control) and 5-BrDU (5% of control) were strong growth inhibitors .

3.6.3 Fibrinolytic Enzyme Activity

Initially, 1 uM RA was tested for its effect on PA and PAI activity; results of zymographic analysis of CM and lysates from cells treated for 3 days are presented in Figure 10. As described earlier, addition of exogenous LMW-UK induced the appearance of a band of activity with a molecular weight of 68 kD, suggesting the presence of a UK-binding protein with MW= 35 kD (Figure 10, lane a). After 3 days induction with RA, a switch in secretion from HUK to tPA occurred as well as a decrease in PAI synthesis (Figure 10, lane b). Cell-associated tPA also increased dramatically compared to controls (Figure 10, lanes d and c respectively). Since RA strongly inhibited the rate of cell growth (see above), RA samples loaded on a per flask basis clearly represented a significantly lower number of cells compared to ethanol controls.

The striking effects of RA on both morphological differentiation and fibrinolytic enzyme profile on these cells led us to test other agents reported to induce the differentiation of NB cells. Figure 11 illustrates the changes in PA and PAI activity in CM after 3 and 6 days treatment with 4 maturational agents (NaB is not included here but was very similar to RA- see Table 7). RA, dbcAMP and 5-BrDU caused an increase in secreted

Figure 9: Time course experiment showing the effect of five differentiation agents on DNA synthesis in SMS-KAN cells. [^{3}H]-thymidine incorporation was measured after 3 and 6 days of treatment with □ PMA; ▲ NaB; Δ 5-BrDU; ● dbcAMP; O RA. Values represent the average of quadruplicate cultures (SE < 10%).

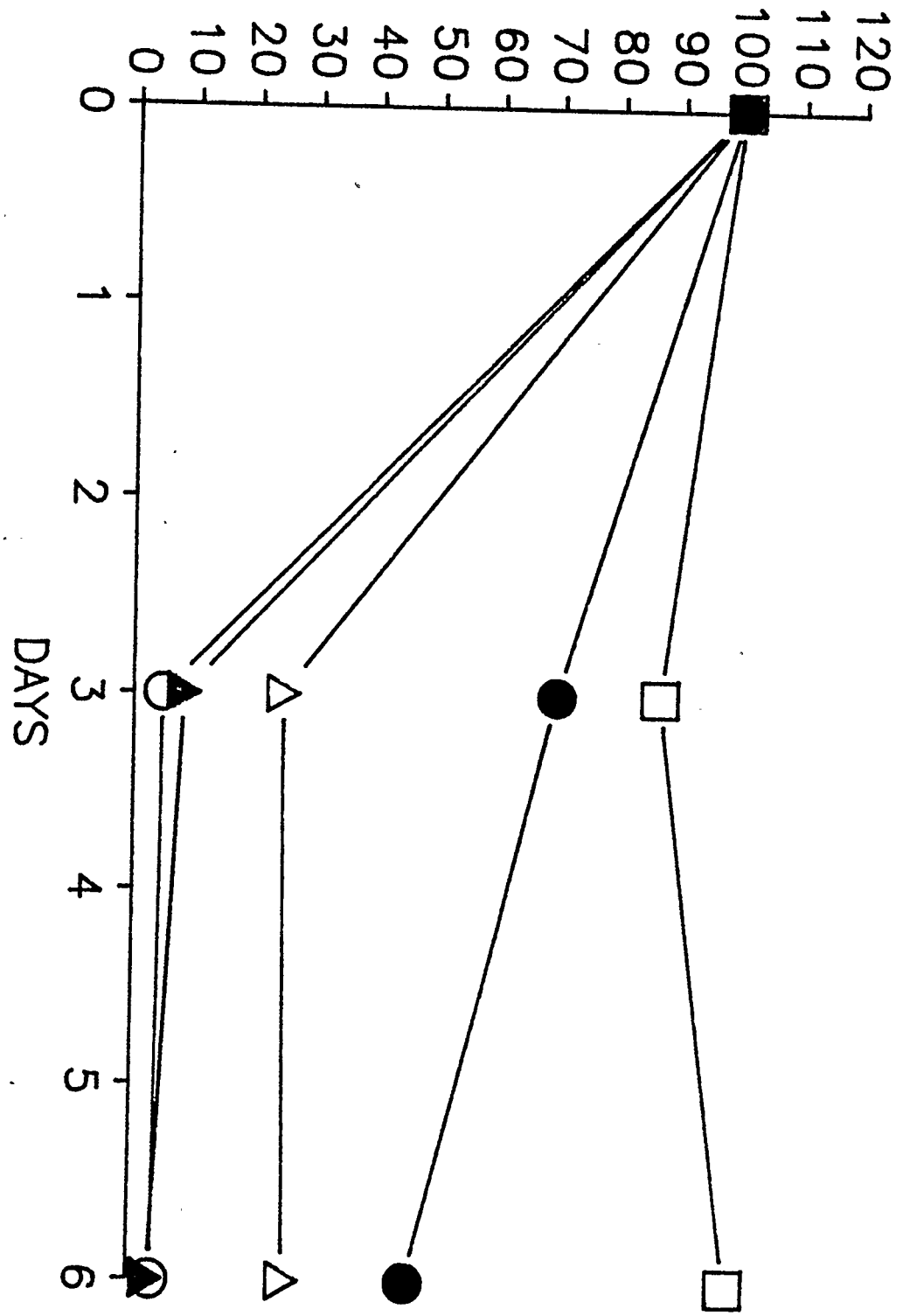

3–H THYMIDINE INCORPORATION
(% OF CONTROL CULTURE)
0
10
20
30
40
50
60
70
80
90
100
110
120
DAYS
0
1
2
3
4
5
6

Figure 10: Zymographic analysis of conditioned medium and lysates from SMS-KAN cells treated with 1 uM retinoic acid for three days. Equivalent volumes of samples from control and RA treated cultures were incubated with 1 unit LMW-UK prior to SDS-PAGE and zymography.

LANE (a) control cells- CM; (b) RA treated cells- CM; (c) control cells- lysate; (d) RA treated cells- lysate; (e) dialyzed human urine (H Ur).

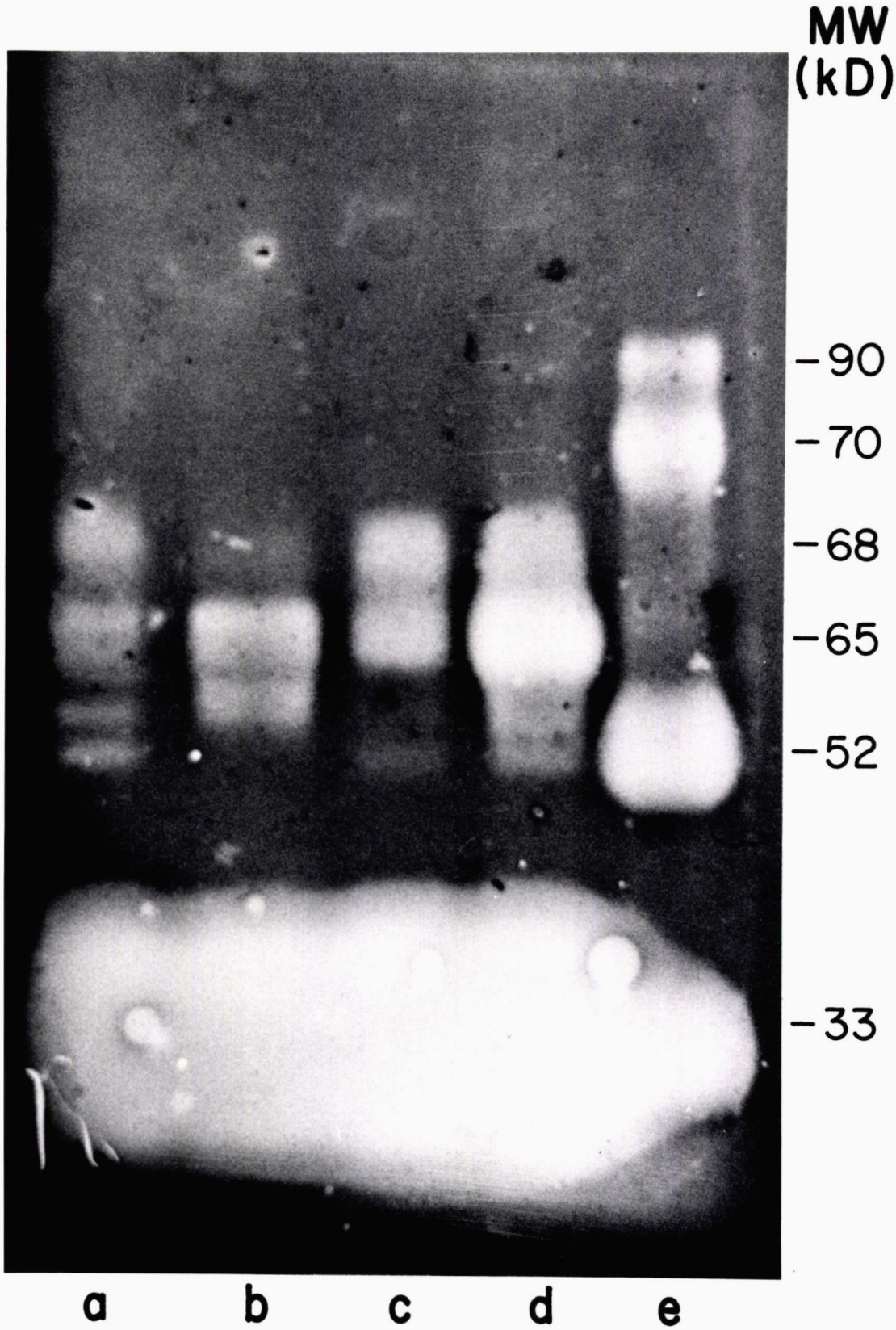
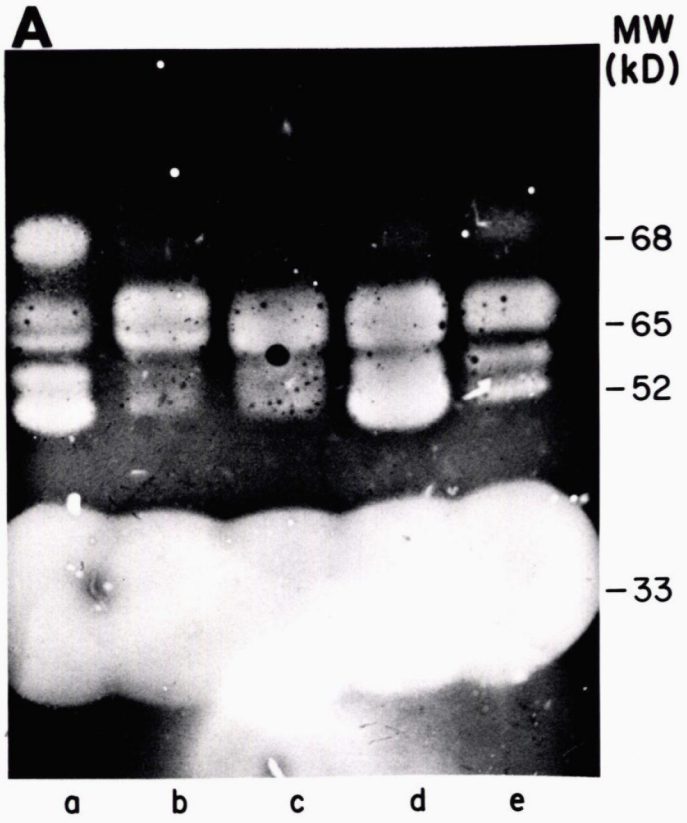
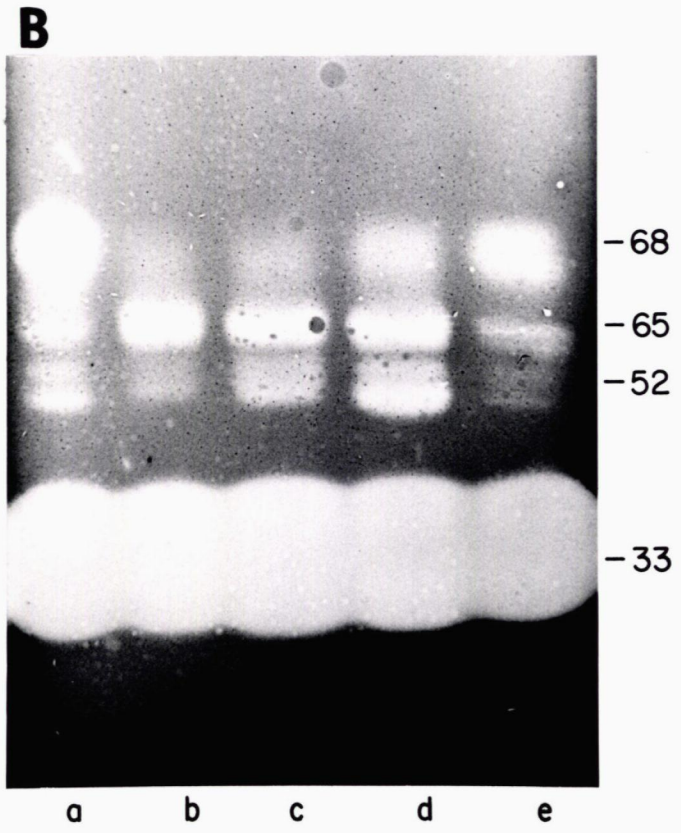

MW
(kD)
-90
-70
-68
-65
-52
-33
a
b
c
d
e

Figure 11: Alterations in fibrinolytic enzyme profile of CM from SMS-KAN cells induced with various differentiation agents. Cells were treated for 3 (PANEL A) or 6 (PANEL B) days.

Lane (a) control cells; (b) 1 uM RA; (c) 2 mM dbcAMP; (d) 10 uM 5-BrDU; (e) 100 ng/ml PMA.


A
MW
(kD)
68
65
52
33
a
b
c
d
e


B
68
65
52
33
a
b
c
d
e

tPA and a decrease in PAI (Figure 11, lanes b-d compared to a). PMA treated cells showed a similar profile to control cells (Figure 11, lane e). Table 7 summarizes alterations in the levels of fibrinolytic enzymes observed by zymography in CM and lysates with all treatments.

Fibrinolysis in gel assay results are presented in Table 8 and indicate quantitative differences in PA activity in morphologically differentiated versus control cultures. After 3 days of treatment, RA, dbcAMP and NaB caused an apparently higher increase in PA activity than 5-BrDU. However, endogenous PAI activity could affect this assay since samples with a lower PAI activity may show greater fibrinolysis. PMA had little effect on PA activity after 3 or 6 days of treatment.

3.6..4 Time Course Experiment of RA Treatment Over 6 Days

When examined daily for cellular morphology, RA treated cells exhibited a consistent increase in neurite outgrowth over a 6 day time period. To determine if changes in morphology correlated with alterations in PA and PAI activity, CM and lysates were prepared for 6 consecutive days and analyzed by zymography. As shown in Figure 12, the alterations described above for RA treatment (switch from HMW-UK to tPA and decrease in PAI secretion; increase in cell-associated tPA) were observed consistently over the 6 day period. In control samples (PANEL A), lanes a through e represent CM from proliferating cultures (days 2 to 6) whereas in RA treated samples (PANEL B), cell proliferation has been inhibited (see Figure 8)

TABLE 7

ALTERATIONs IN PA/PAI PROFILE OF SMS-KAN NB CELLS TREATED WITH VARIOUS DIFFERENTIATION AGENTS

	CONDITIONED MEDIUM [a]				LYSATE [a]			
	LMW-UK	HMW-UK	tPA	COMPLEX	LMW-UK	HMW-UK	tPA	COMPLEX
3 DAY								
CONTROL	+++	++	+	++	+	+/-	+	+
RA	++	+/-	+++	-	-	+	+++	+
dbcAMP	+	+	++	-	-	+	+++	+/-
5-BrDU	+	++	++	-	-	++	++	-
NaB	+	+	+++	-	-	+	+++	+/-
PMA	++	+	+	+/-	-	++	+	-
6 DAY								
CONTROL	-	++	+	+++	-	+/-	+	-
RA	-	+/-	+++	-	-	+	++	-
dbcAMP	-	+/-	++	+/-	-	+	++	-
5-BrDU	-	+++	++	+	-	+	++	-
NaB	-	+	+++	+/-	-	+/-	++	-
PMA	+	+/-	+/-	++	-	-	+	-

[a] Equivalent volumes of CM or lysate from each culture were assayed by SDS-PAGE and zymography. Results of zymcgraphic analysis are tabulated according to relative size of the zones of lysis on the indicator plate, where (-) indicates absence of PA activity and (+++) represents the highest level of PA activity detected.

TABLE 8

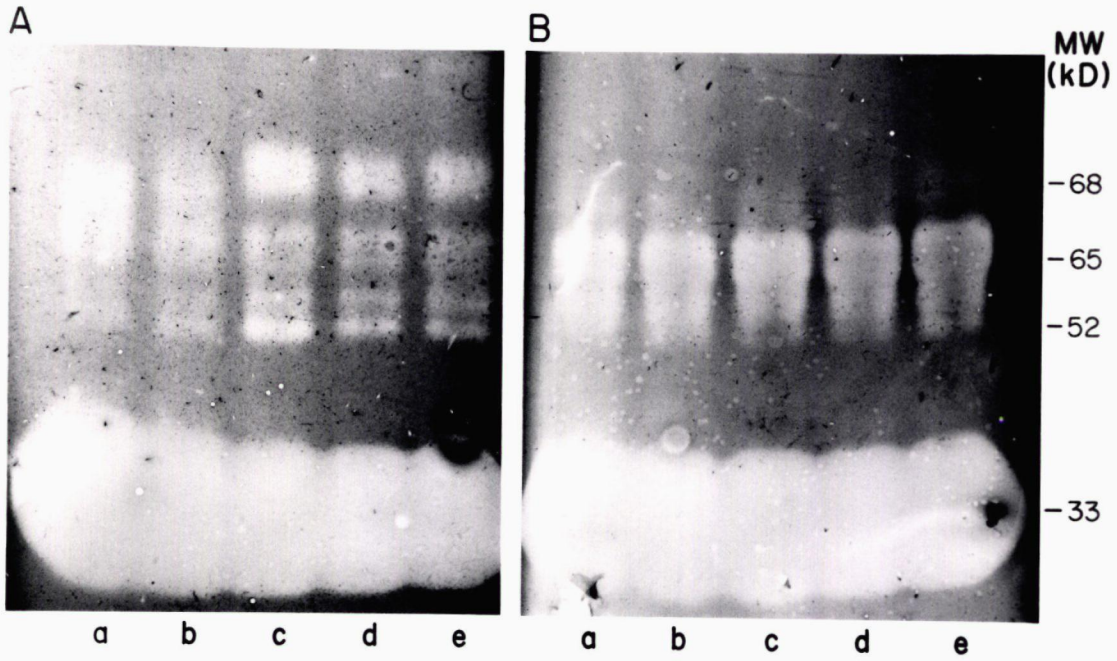
FIBRINOLYTIC ACTIVITY OF CM AND LYSATES FROM SMS-KAN CELLS INDUCED TO DIFFERENTIATE WITH FIVE AGENTS

	PA ACTIVITY (units/ 75 cm2 flask)[a]	
	CONDITIONED MEDIUM	LYSATE
3 DAY		
CONTROL	2.0[b]	3.0
RA	6.0	9.0
dbcAMP	4.5	4.5
5-BrDU	3.5	3.0
NaB	5.0	6.0
PMA	2.5	3.0
6 DAY		
CONTROL	2.0	3.0
RA	6.0	9.0
dbcAMP	4.5	6.0
5-BrDU	4.0	6.0
NaB	4.5	7.5
PMA	2.0	2.5

a CM and detergent lysates from control and differentiated SMS-KAN cultures were prepared as described in the Materials and Methods. 10 uL of each sample was applied to a fibrin plate and incubated at 37°C for 24 hours. The plates were stained and the diameter of the zone of lysis was measured. Serial dilutions of a LMW-UK standard were assayed simultaneously and used to calculate PA activity.

b Values represent the average of triplicate assays (SE < 5%).

Figure 12: Time course of PA/PAI profile of SMS-KAN cells treated with 1 uM retinoic acid for six consecutive days. CM from control (PANEL A) and RA treated (PANEL B) cultures were prepared every 24 hours for six days and analyzed by SDS-PAGE and zymography. Lanes (a)-(e) days 2-6.


A
B
MW
(kD)
68
65
52
33
a
b
c
d
e
a
b
c
d
e

4.0 DISCUSSION

Cultured neuroblastoma cells provide an excellent model to study both neoplastic and neuronal cell behavior. The availability of numerous cell lines from patients with advanced neuroblastoma has enabled detailed investigation of many features of this tumor in vitro. The spontaneous or agent-induced differentiation of cultured NB cells is of particular interest as it may reflect in vivo phenomena and aid in the development of therapeutic treatments for the disease.

Furthermore, NB cells possess many characteristics of normal neuronal cells and are often utilized to study aspects of neural cell development, differentiation and regeneration. NB cell lines, which are composed of primarily neuronal, glial or mixed phenotypes, can provide information about the behavior of different populations of neural crest-derived cells. Since primary cultures of human brain are obviously difficult to obtain, NB cell lines provide a unique source of human material for neural studies.

4.1 Fibrinolytic Activity of Human Neuroblastoma Cells

NB cell lines from rat, mouse and human sources secrete high levels of plasminogen activators (Becherer and Wachsman, 1980; Soreq and Miskin, 1981; Gilbert and Wachsman, 1982). Enhanced levels of PA activity have been associated with tumor invasion and metastases (Dano et al., 1985; Ramshaw et al., 1986) and thus may be an important aspect of the malignant behavior of NB.

In this study, all ten human NB cell lines were found to constitutively secrete urokinase (MW = 52 kD) and all except two, SK-N-SH and SK-N-MC, secreted tPA (MW = 67 kD). These latter two cell lines were derived from different patients and share no common features. However, there has been some suggestion that SK-N-MC is

not a true NB cell line as it lacks the chromosomal pattern characteristic of this tumor (a deletion in the short arm of chromosome 1) and is completely unresponsive to treatment with differentiation agents. Furthermore, SK-N-SH is unique in its morphological and biochemical response during differentiation and is reportedly unstable in long-term culture (Dr. P. Reynolds: personal communication).

A dramatic variability in quantity of PA secreted was observed amongst the cell lines. This finding is expected in light of the well-established heterogeneity of NB cell lines with respect to morphology, growth characteristics and many other biochemical parameters (Biedler et al., 1973; Biedler et al., 1978; Rettig et al., 1987; Sadee et al., 1987).

The presence of both UK and tPA has been reported in cultures of rat cerebellar tissue (Moonen et al., 1985). A considerably higher level of PA activity was detected in cultures of developing cerebellum (7 day old animals) compared to adults, suggesting a role for PAs in early establishment of the CNS. Both PAs have also been identified in the PNS (Alvarez-Buylla and Valinsky, 1985); Schwann cells are the major source of tPA and neurons produce UK (Baron-Van Evercooren et al., 1987). Since NB is a neural crest derived tumor, composed of a mixture of neuronal cells, Schwann-like 'flat' cells and melanocytes, it is not surprising that both UK and tPA are expressed. It is interesting to note, however, that SK-N-SH cells were not found to produce tPA in either the undifferentiated or differentiated state. Treatment of these cells with retinoic acid led to an unusually high proportion of Schwann-like/melanocytic cells which would be expected to express tPA. These observations emphasize the need to exercise caution when drawing direct comparisons between the biochemical activity of transformed cells and their normal counterparts.

No evidence has previously been presented for the presence of PAI molecules in neuroblastoma cells although molecules with PA inhibitory activity have been described for other neural-derived tumors (rat and human gliomas) and rat astrocyte cultures (Dano et al., 1986; Gloor et al., 1986; Gross et al., 1988; Rosenblatt et al., 1987). In this study, eight of ten human NB cell lines were demonstrated to constitutively secrete PA inhibitory molecules. Preliminary characterization of the NB-derived PAI(s) revealed two different molecular weight forms (approximately 35 and 40 kD) secreted by individual cell lines. Higher molecular weight species of PA (90 and 105 kD) observed in CM from some cell lines are believed to represent preformed complexes of PAI with endogenous HMW-UK (52 kD + PAI) and tPA (67 kD + PAI), respectively.

Four categories of cell-derived PA inhibitors have been described (PAI-1, PAI-2, PAI-3, and PN) which have molecular weights ranging from 47- 54 kD (see Table 3). The molecular weights of the PAIs secreted by NB cells are lower than those of the previously described molecules. This suggests that the NB-derived PAI molecules are a distinct group of PA inhibitors. Alternatively,the lower molecular weight species of PAI detected in most of the NB cell lines may represent post-translationally modified forms of these defined PAI molecules. However, there is other evidence apart from the molecular weight data to indicate that these NB cell lines are secreting serine protease inhibitors that form SDS-stable complexes with urokinase but are not identical to the other reported PAI molecules. This possibility is supported by the results of protease and heparin binding experiments of the PA inhibitors from two of the cell lines, SK-N-SH and SMS-KAN. The 40 kD PAI synthesized by SK-N-SH cells showed a high affinity for heparin-Sepharose (see Figure 3), suggesting that it may be a protease nexin-like molecule. However, this inhibitor bound only UK and trypsin but not plasmin or thrombin unlike PN which binds all four of these proteases (Scott et al., 1985). The

SMS-KAN derived PAI (35 kD) did not bind heparin and therefore could be PAI-1 or PAI-2. When tested for binding to iodinated proteases, this inhibitor also showed a high affinity for trypsin. Thus, it is apparent that both the SK-N-SH and the SMS-KAN PAI molecules have characteristics not typical of PN or PAI-1, PAI-2 and PAI-3. The unique substrate specificity of these NB inhibitors may indicate that they could functionally inhibit PAs as well as other enzymes with trypsin-like properties. In fact, a trypsin-like ectoenzyme which functions as a dynorphinase has been identified in NB cell membranes (Satoh et al., 1988) and this protein could potentially serve as a target for NB-derived inhibitors.

A PA inhibitor with characteristics similar to those described above for the SMS-KAN derived PAI has been identified in serum-free medium from a human glioblastoma cell line, U138 (Rehemtulla et al., in press). The U138 inhibitor was purified from bulk cell cultures and shown to have a high affinity for UK and trypsin, but not for heparin-Sepharose. Rao et al. (1988) characterized three distinct serine protease inhibitors in conditioned medium from a rat gliosarcoma: a PN-1 type inhibitor, a PAI-1 like molecule, and a third inhibitor which bound only UK and trypsin. These findings support the possibility that PA inhibitors different from those previously reported may be present in brain cells. Further characterization of these PA inhibitors with specific cDNA probes for PAI-1 and PAI-2 and an ELISA which is now available for PAI-1 will be necessary to resolve their identity.

4.2 Morphological Differentiation of Neuroblastoma Cells

Neuroblastoma cells may be induced to differentiate with numerous biological agents. In this study, all NB cell lines were treated with retinoic acid and examined daily for morphological changes. As indicated in Table 4 and Figure 4, responses to RA varied

dramatically amongst the cell lines. The extension of neurite-like processes, termed morphological differentiation, was observed in six of the ten cell lines. When [^{3}H]-thymidine incorporation was assayed during differentiation in two of these cell lines, an inhibition of DNA synthesis was observed and was most dramatic in the cell line exhibiting the most extensive morphological differentiation. Several cell lines were apparently resistant to the agent and showed little or no alteration in morphology or cell growth. SK-N-SH cells were unique in their response to RA; although DNA synthesis was not inhibited, morphological manifestations of the treatment were evident. The heterogeneity of responses to RA observed in NB cell lines may reflect a similar variation in sensitivity to this drug in vivo and thus be a consideration in clinical application of this treatment.

4.3 Differentiation-related Changes in PA Activity

Treatment of tumor cells with differentiation agents modulates fibrinolytic activity in a number of cell types (Ossowski and Belin, 1985; Nelson et al., 1987; Genton et al., 1987). Rodent NB cells differentiated with dibutyryl cAMP showed an enhancement of cellular and secreted PA activity (Laug et al., 1976; Soreq et al., 1983). Human NB cells exposed to a plasminogen-deficient medium exhibited extensive neurite outgrowth and a 5-fold increase in PA activity (Becherer and Wachsman, 1980). In agreement with these findings, we have observed an increase in total PA activity in differentiated NB cells compared to controls. Having characterized the PAs expressed by ten cell lines, we were able to further examine specific alterations in UK and tPA activity during this process.

Neurite outgrowth of the NB cells was found to correlate directly with the up-regulation of tPA synthesis. Urokinase activity was not similarly enhanced during differentiation; in fact, in some cell lines a reduction in UK secretion was observed.

Tissue and urokinase PA are products of different genes (Pennica et al., 1983) and thus might be expected to have separate functions and be subject to different regulatory controls. During the process of neuronal cell differentiation, this appears to be the case. Distinct physiological roles for UK and tPA in the nervous system may be due in part to different activation requirements and substrate specificity of each PA. This latter possibility has been suggested by Baron-Van Evercooren and coworkers (1987), who found that UK but not tPA is mitogenic for Schwann cells and astrocytes.

Tissue PA is best known for its role in the vasculature, where it aids in clot dissolution through a fibrin-dependent pathway. Since tPA requires the presence of fibrin for its activation, the presence or absence of fibrin is an important factor in interpreting changes in tPA activity. This study and others (described above), which have reported an increase in PA activity during differentiation, have been performed using fibrin-dependent assay systems. However, if fibrin is not present in the in vivo environment, the reduction in UK would indicate a net decrease in fibrinolytic activity during morphological differentiation. In fact, down-regulation of UK has been associated with more 'mature' or differentiated cells in a number of tumors including renal carcinoma (Ossowski and Belin, 1985; Nelson et al., 1987), colon carcinoma (Boyd et al., 1988) and human glioma (Gross et al., 1988).

Conversely, tPA may bind to specific receptors on neuronal cells enabling localization and possible activation of this protease by a fibrin-independent mechanism (Verrall and Seeds, 1987). In this case, an enhancement of tissue-type PA may be directly related to neurite process formation since PA release occurs predominantly at the growth cones of extending neurites in differentiating NB cells (Krystosek and Seeds, 1981a). Modulation of extracellular matrix components by tPA may also contribute to changes in cell adhesiveness and enable progression of nerve fibers.

To determine if these alterations in PA activity were unique to RA-induced differentiation, one cell line was treated with four maturational agents in addition to RA. Although the switch from secretion of UK to tPA was most evident in RA-treated cells (see Figure 10), both sodium butyrate and dibutyryl cAMP resulted in similar changes in the fibrinolytic enzyme profile. Treatment of cells with 5-BrDU caused an enhancement of tPA synthesis but little change in UK activity. As shown in Table 6 and Figure 8, all four of these agents caused differentiation of the cells as evidenced by neurite outgrowth and growth inhibition, although 5-BrDU induced less morphological changes. PMA had little detectable effect on the cells either biochemically or morphologically. Thus, it appears that alterations in fibrinolytic enzyme activity during differentiation, especially the up-regulation of tissue PA, are not RA-dependent but may be induced by other maturational agents.

4.4 PA Inhibitory Activity and Morphological Differentiation

The processes involved in migration and axonal outgrowth of neural cells during development and differentiation are complex, involving exogenous factors, neighboring cell adhesion molecules and interactions with the extracellular matrix. Serine protease inhibitory molecules also appear to play an important role in these processes.

Changes in levels of PAI relative to PA may be critical in interpreting the role of cellular fibrinolysis in differentiation. For instance, although an increase in PA was reported in PMA treated U-937 cells, a 50-fold excess of PAI-2 activity over urokinase indicated an overall decrease in fibrinolytic potential during cellular differentiation (Genton et al., 1987). In the present study, a decrease in PAI activity was observed in two of the cell lines which exhibited morphological differentiation when induced with RA. In one of these lines, SMS-KAN, treatment for three days with any of four agents

causing neurite outgrowth led to a corresponding reduction in both UK and PAI. In SK-N-SH cells, differentiation to a predominantly flat cell phenotype correlated with an up-regulation of both UK and PAI. Previous studies have demonstrated that PA and PAI can be regulated independently or co-ordinately within the same cell (reviewed in Hart and Rehemtulla, 1988). The findings presented here indicate that both UK and PAI are co-ordinately regulated during NB cell differentiation although tPA and PAI appear to be independently regulated.

The pattern of PAI expression observed during differentiation varied considerably amongst the cell lines (summarized in Table 2). As shown here, NB cell lines appear to be producing different PA inhibitors which would not be expected to respond during differentiation in an identical fashion.

The significance of the reduction of PAI secretion observed during morphological differentiation in some cell lines is unknown. Since the NB-derived PA inhibitors bound strongly to trypsin as well as PAs, we have raised the possibility that these inhibitors are unique and may functionally inhibit other enzymes such as trypsin-like dynorphinases in vivo. If this is the case, alterations in PAI activity during differentiation may be irrelevant with respect to overall fibrinolytic activity of the cell. Conversely, the binding of NB-derived PAIs to UK and/or tPA could influence physiological processes through specific PA inhibition.

As discussed earlier, the specificity of a protease inhibitor appears to be important in defining its role in neural processes. The more general serine protease inhibitor protease nexin is able to induce neurite outgrowth when added exogenously to neuroblastoma cells. It is presently unknown whether other cell-derived inhibitors such as PAI-1, PAI-2 or the NB-derived PAIs have similar effects to PN when added to NB cell cultures. In contrast to PAI molecules, protease nexin inhibits thrombin in addition to PAs. Since

the inhibition of thrombin is believed to be critical to morphological differentiation, the NB-derived PA inhibitors, which do not appear to bind thrombin, may not have this effect.

Furthermore, intracellular versus extracellular mechanisms underlying neurite outgrowth may differ. When a maturational agent such as retinoic acid is added to the cells, it acts through intracellular pathways which result in neurite extension. However, a differentiation agent acting intracellularly may induce changes in addition to the up-regulation of tPA such as the production of other proteases and/or inhibitors which may influence this process. In addition, the pattern of extracellular matrix components secreted by NB cells changes dramatically when the cells are treated with retinoic acid (Tsokos et al., 1987) and this factor may also affect the cellular morphology. In contrast, protease nexin inhibits extracellular proteolysis and may or may not act through the same intracellular mechanisms as differentiation agents.

4.5 Future Prospects

Clearly, the role of plasminogen activators and their potential inhibitors in the morphological differentiation of NB cells is a very complex issue. To further understand the mechanisms by which PAs influence this process, more extensive quantitation of PAs and PAIs would be useful. Fibrin-independent PA activity can be measured by ELISA using specific antibodies to UK and tPA, although these reagents measure both latent and active forms of the enzymes. Zymography, on the other hand, reflects active PA but may be misleading with respect to alterations in tPA since this assay is fibrin-dependent. Molecular probes for both PAs, as well as the PAI molecules, are becoming

commercially available. With the use of these probes, and more sensitive assays for levels of secreted and cell-associated PAs, more thorough studies of the regulation of these enzymes during the differentiation process could be performed.

We are presently in the process of attempting to obtain fresh neuroblastoma samples removed from patients during surgery. Since cell lines in long-term culture may have undergone some phenotypic alterations, the responses of fresh biopsied tumors to differentiation agents may be more indicative of in vivo processes. In fact, Sawaya and Highsmith (1988) have analyzed the PA activity and molecular weight pattern of biopsied human brain tumors, although neuroblastoma was not included in this study.

Urokinase secretion is apparently down-regulated during neurite outgrowth. It would be interesting to perform experiments where NB cells are differentiated in the presence of exogenous high molecular weight UK. If indeed depression of UK is relevant to the morphological differentiation event, this phenomenon may be inhibited if sufficient levels of UK are available to the cells. Similarly, the presence of anti-tPA antibodies in differentiating NB cell cultures should inhibit neurite outgrowth if tPA is essential to this process. Such experiments would require careful measurement of neurite outgrowth which would be facilitated by the use of time-lapse photography.

Since protease nexin derived from glial/glioma cells is able to induce morphological differentiation of NB cells, it possible that other cell-derived inhibitors may have similar effects. It would be interesting to assess morphological manifestations of NB cells treated with purified PAI-1, PAI-2, NB-derived PAI or the U138 glioblastoma-derived inhibitor. Such studies would help to define the specificity of inhibitors able to induce neurite outgrowth. Quantities of purified PAI-1 and U138 inhibitor, sufficient to perform such experiments, are currently being isolated in our laboratory.

Plating of NB cells on surfaces coated with various extracellular matrix components provides an alternate way of studying morphological differentiation. Measurement of alterations in PA and PAI occuring when cells are induced in this manner could be compared to effects of exogenous agents such as retinoic acid or PAIs. A further extension of these experiments would involve plating NB cells on different substrata, followed by agent-induced differentiation and measurement of PA/PAI activity. By comparing the fibrinolytic activity of cells plated on plastic, ECM components, and ECM in addition to a differentiation agent, changes in PA and PAI could be related to specific tumor cell-matrix interactions occuring during the differentiation process.

4.6 Implications

The involvement of serine proteases and their inhibitors in cellular interactions within the nervous system is well established (see Introduction). Characterization of these molecules will enable investigation of regulatory mechanisms at the biochemical and molecular level during different stages of neural development. Furthermore, dysregulation of fibrinolytic enzyme activity may lead to pathological sequelae in the nervous system. For example, during cerebellar development in the mouse mutant weaver, migration of granule neurons is abortive leading to premature death of the animal. Regulatory events involving PAs and their inhibitors may be important in this situation since PAs are released by granule neurons during migration and glial-derived protease nexin, a serine protease inhibitor, can interfere with this cellular migration (Monard, 1987).

Proteases and their inhibitors have been implicated in the process of regeneration which occurs following lesion in the PNS but not the CNS (Bignami et al., 1982). The presence of a Gd-NPF-like molecule has been reported after damage to the rat sciatic nerve (PNS) although this inhibitor is not found in optic nerve (CNS) which has been

similarly damaged (Patterson, 1985). It is possible that the existence and activity of molecules such as protease nexin in vivo may affect the potential for axonal regeneration.

Experiments aimed at understanding biochemical and molecular aspects of NB cell differentiation may be applicable therapeutically as well. Recently, differentiated neuroblastoma cells have been considered as a donor source for neural transplantation studies (Gash et al., 1986; Kordower et al., 1987). In these experiments, NB cells are treated prior to transplant so that they are no longer in a proliferative tumorous state and have taken on characteristics of normal neurons. Alterations in the proteolytic activity of NB cell lines in the differentiated state may be a factor in determining the ability of these cells to survive and function in the host.

Agents which induce the differentiation of NB cells in vitro are often considered for clinical use in the treatment of patients with advanced disease. The ability of protease inhibitory molecules to cause neurite outgrowth of cultured NB cells suggests that they may have therapeutic potential. Sawaya and coworkers (1986) have approached this issue by testing the effect of an antifibrinolytic agent (epsilon aminocaproic acid or EACA) on the growth of human glioblastomas transplanted subcutaneously in nude mice. Preliminary results from these experiments have indicated that EACA inhibits the growth of these experimentally induced brain tumors and prolongs survival of the animal.

As retinoic acid is being used in clinical trials for the treatment of NB (Dr. P. Reynolds; personal communication), the responsiveness or unresponsiveness of primary tumor to alterations in PA/PAI expression and/or neurite outgrowth could have predictive value in identifying those patients in which RA would have efficacy. In the case of acute myeloid leukemia (AML) a response to chemotherapy has been demonstrated in

patients whose AML cells secrete UK alone or with tPA but not in patients whose cells produce only tPA (Wilson and Francis, 1983). Thus, both qualitative and quantitative measures of plasminogen activators may have prognostic value in tumor therapy.

4.7 Summary and Conclusions

When treated with a maturational agent such as retinoic acid, neuroblastoma cell lines undergo biochemical and morphological alterations indicative of differentiation. One of the enzyme systems which appears to be affected during this process is the fibrinolytic cascade which is mediated largely by plasminogen activators and their inhibitors. We analyzed serum-free medium from ten human NB cell lines for PA and PAI activity using SDS-PAGE and zymography. All cell lines secreted the urokinase-type PA and eight also produced the tissue PA. The presence of molecules with PA inhibitory activity was also detected in most of the cell lines. Preliminary characterization of these PAI molecules revealed two apparently distinct forms which differed in molecular weight and heparin-binding affinity. The ability of NB-derived PA inhibitory molecules to bind strongly to trypsin in addition to PAs distinguishes them from previously described specific PAIs and protease nexin. Thus, NB cells appear to be secreting protease inhibitors of unique specificity that may function in the regulation of extracellular proteolysis in neural crest derived tissue.

During the differentiation process, cell lines which exhibited neurite outgrowth demonstrated an up-regulation of cellular and secreted tPA and, in some cases, a corresponding decrease in secreted UK and PAI. Several cell lines were apparently resistant to retinoic acid and showed little morphological or biochemical response. In one highly responsive cell line, treatment with other differentiation agents such as dibutyryl cyclic

AMP and sodium butyrate resulted in similar effects to those observed with RA. These findings indicated that the differentiation-related changes in morphology, growth and fibrinolytic activity were not specific to retinoic acid-mediated differentiation.

This study suggests that urokinase and tissue PA, both of which have been identified in several types of normal neural crest derived cells, may serve distinct and relevant functions during the process of neuronal differentiation. Furthermore, the pattern of fibrinolytic enzyme activity appears to change dramatically during agent induced differentiation in responsive neuroblastoma cell lines. This finding may have clinical implications as a diagnostic tool for responsiveness to therapeutic treatments of neuroblastoma.

REFERENCES

Adam, A. and Hochholzer, L. (1981) Ganglioneuroblastoma of the posterior mediastinum: A clinicopathologic review of 80 cases. Cancer, 47: 373-381.

Alvarez-Buylla, A. and Valinsky, J.E. (1985) Production of plasminogen activator in cultures of superior cervical ganglia and isolated Schwann cells. Proc. Natl. Acad. Sci. USA, 82: 3519-3523.

Amatruda, T.T., Sidell, N., Ranyard, J. and Koeffler, H.P. (1985) Retinoic acid treatment of human neuroblastoma cells is associated with decreased n-myc expression. Biochem. Biophys. Res. Comm., 126: 1189-1195.

Baker, J.B. and Gronke, R.S. (1986) Protease nexins and cellular regulation. Sem. in Thromb. and Haemostas., 12: 216-220.

Bakhit, C., Lewis, D., Billings, R. and Malfroy, B. (1987) Cellular catabolism of recombinant tissue-type plasminogen activator. Identification and characterization of a novel high affinity uptake system on rat hepatocytes. J. Biol. Chem., 262: 8716-8721.

Baron-Van Evercooren, A., Leprince, P., Rogister, B., Lefebvre, P.P., Delree, P., Selak, I. and Moonen, G. (1987) Plasminogen activators in developing peripheral nervous system, cellular origin and mitogenic effect. Dev. Brain Res., 36: 101-108.

Becherer, P.R. and Wachsman, J.T. (1980) Increased neurite development and plasminogen activator expression by exposure of human neuroblastoma cells to a plasminogen-deficient growth medium. J. Cell Phys., 104: 47-52.

Bergmann, B.L., Scott, R.W., Bajpai, A., Watts, S.. and Baker, J.B. (1986) Inhibition of tumor-cell-mediated extracellular matrix destruction by a fibroblast proteinase inhibitor, protease nexin 1. Proc. Natl. Acad. Sci. USA, 83: 996-1000.

Berthold, F. (1985) Current concepts on the biology of neuroblastoma. Blut, 50: 65-74.

Biedler, J.L., Helson, L. and Spengler, B.A. (1973) Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res., 33: 2643-2652.

Biedler, J.L., Roffler-Tarlov, S., Schachner, M. and Freedman, L.S. (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res., 38: 3751-3757.

Bignami, A., Cella, G. and Chi, N.H. (1982) Plasminogen activators in rat neural tissues during development and Wallerian degeneration. Acta. Neuropathol., 58: 224-228.

Blasi, F., Vassalli, J-D. and Dano, K. (1987) Urokinase-type plasminogen activator: Proenzyme, receptor and inhibitors. J. Cell Biol., 104: 801-804.

Boyd, D., Florent, G., Kim, P. and Brattain, M. (1988) Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res., 48: 3112-3116.

Breitman, T.R., Selonik, S.E. and Collins, S.J. (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA, 77: 2936-2940.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brodeur, G.M., Sekhon, G.S. and Goldstein, M.N. (1973) Chromosomal aberrations in human neuroblastomas. Cancer, 40: 2256-2263.

Carbonetto, S., Gruver, M.M. and Turner, D.C. (1983) Nerve fiber growth in culture on fibronectin, collagen and glycosaminoglycan substrates. J. Neurosci., 3: 2324-2335.

Cieplak, W. and Hart, D.A. (1985) Formation of stable complexes between urokinase and a human urinary component. Thromb. Haemostas., 52: 36-41.

D'Angio, G.J., Evans, A.E. and Koop, C.E. (1972) Special pattern of widespread neuroblastoma with a favorable prognosis. Lancet, 1: 1046-1049.

Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielson, L.S. and Skriver, L. (1985) Plasminogen activators, tissue degradation and cancer. Adv. in Cancer Res., 44: 139-266.

Dano, K,. Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1986) Urokinase-type plasminogen activators and modulation of its activity in cancer. Proc. 14th Intl. Cancer Cong., 3: 1112.

DeBruin, P.A.F., Griffioen, G., Verspaget, H.W., Verheijen, J.H., Dooijewaard, G., van den Ingh, H.F. and Lamers, B.H.W. (1988) Plasminogen activator profiles in neoplastic tissues of the human colon. Cancer Res., 48: 4520-4524.

DeBruin, P.A.F., Verspaget, H.W., Griffioen, G., Nap, M., Verheijen, J.H. and Lamers, B.H.W. (1987) Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis, 1: 57-62.

DeClerk, Y.A., Bomann, E.T., Spengler, B.A. and Biedler, J.L. (1987) Differential collagen biosynthesis by human neuroblastoma cell variants. Cancer Res., 47: 6505-6510.

Deutsch, D. and Mertz, E. (1970) Plasminogen: purification from human plasma by affinity chromatrography. Science, 170: 1095-1096.

Eaton, D.L. and Baker, J.B. (1983) Phorbol ester and mitogens stimulate human fibroblast secretions of plasmin-activatable plasminogen activator and protease nexin, an antiactivator/antiplasmin. J. Cell Biol., 97: 323.

Edgar, D., Trimple, R. and Thoenen, H. (1984) The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO, 3: 1463-1468.

Evans, A.E., Gerson, J. and Schnaufer, L. (1976) Spontaneous regression of neuroblastoma. Natl. Cancer Inst. Monogr., 44: 49-52.

Everson, T.C. (1964) Spontaneous regression of cancer. Ann. N.Y. Acad. Sci., 114: 721-735.

Festoff, B.W. (1985) Proteases, their inhibitors and the extracellular matrix: Factors in nerve-muscle development and maintenance. Adv. Exper. Med., 209: 25-39.

Gash, D.M., Notter, M.F.D., Okawara, S.H., Kraus, A.L. and Jones, P.J. (1986) Amitotic neuroblastoma cells used for neural implants in monkeys. Science, 233: 1420-1422.

Genton, C., Kriuthof, E.K.O. and Schleuning, W-D. (1987) Phorbol ester induces the biosynthesis of glycosylated and nonglycosylated plasminogen activator inhibitor 2 in high excess over urokinase-type plasminogen activator in human U-937 lymphoma cells. J. Cell Biol., 104: 705-712.

Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Identification of a receptor for the morphogen retinoic acid. Nature, 330: 624-628.

Gilbert, L.C. and Wachsman, J.T. (1982) Characterization and partial purification of the plasminogen activator from human neuroblastoma cell line, SK-N-SH- A comparison with human urokinase. Biochim. Biophys. Acta., 704: 450-460.

Gloor, S., Odink, K., Guenther, J., Nick, H. and Monard, D. (1986) A glia-derived neurite promoting factor with protease inhibitory activity belongs to the protease nexins. Cell, 47: 687-693.

Granelli-Piperno, A. and Reich, E. (1978) A study of proteases and protease inhibitor complexes in biological fluids. J. Exp. Med., 148: 223-234.

Gross, J.L., Behrens, D.L., Mullins, D.E., Kornblith, P.L. and Dexter, D.L. (1988) Plasminogen activator and inhibitor activity of human glioma cells and modulation by sodium butyrate. Cancer Res., 48: 291-296.

Gross, N., Beck, D., Favre, S. and Carrel, S. (1987) In vitro antigenic modulation of human neuroblastoma cells induced by IFN-gamma, retinoic acid and dibutyryl cyclic AMP. Int. J. Cancer, 39: 521-529.

Guenther, J., Nick, H. and Monard, D. (1985) A glia derived neurite promoting factor with protease inhibitory activity. EMBO, 4: 1963-1966.

Gurwitz, D. and Cunningham, D.D. (1988) Thrombin modulates and reverses neuroblastoma neurite outgrowth. Proc. Natl. Acad. Sci. USA, 85: 3440-3444.

Hart, D.A., Kramer, R. and Cieplak, W. (1984) Identification of subpopulations of human urinary plasminogen activators. Thromb. Haemostas., 51: 212-216.

Hart, D.A. and Rehemtulla, A. (1988) Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis and cell function. Comp. Biochem. Physiol., 90B: 691-708.

Hart, D.A., Rehemtulla, A. and Babins, E. (1986) Species differences in the detection of high molecular weight urinary plasminogen activators. Comp. Biochem. Physiol., 84B: 287-293.

Haussler, M.R., Donaldson, C.A., Kelly, M.A., Mangelsdorf, D.J., Bowden, G.T., Meinke, W.J., Meyskens, F.L. and Sidell, N. (1984) Identification and quantitation of intracellular retinol and retinoic acid binding proteins in cultured cells. Biochim. Biophys. Acta., 803: 54-62.

Haussler, M.R., Sidell, N., Kelly, M., Donaldson, C., Altman, A. and Mangelsdorf, D. (1983) Specific high-affinity binding and biologic action of retinoic acid in human neuroblastoma cell lines. Proc. Natl. Acad. Sci. USA, 80: 5525-5529.

Hawkins, R.L. and Seeds, N.W. (1986) Effect of proteases and their inhibitors on neurite outgrowth from neonatal mouse sensory ganglia in culture. Brain Res., 398: 63-70.

Heeb, M., Espana, F., Geiger, M., Collen, D., Stump, D.C. and Griffin, J.H. (1987) Immunological identity of heparin-dependent plasma and urinary protein C inhibitor and plasminogen activator inhibitor-3. J. Biol. Chem., 262: 15813-15816.

Helson, L., Helson, C., Peterson, R.I. and Das, S.K. (1976) A rationale for the treatment of metastatic neuroblastoma. J. Natl. Cancer Inst., 57: 727-729.

Imashuku, S., Sugana, T., Fujiwara, K., Todo, S., Ogita, S. and Goto, Y. (1982) Intraaortic prostaglandin E1 infusion in maturation of neuroblastoma. Experimentia, 38: 932-933.

Jetten, M.M., Anderson, K., Deas, M.A., Kagechika, H., Lotan, R., Rearick, J.I. and Shudo, K. (1987) New benzoic acid derivatives with retinoid activity: lack of direct correlation between biological activity and binding to cellular retinoic acid binding protein. Cancer Res., 47: 3523-3527.

Kalderon, N. (1982) Role of the plasmin-generating system in the developing nervous tissue: I. Proteolysis as a mitogenic signal for the glial cells. J. Neurosci. Res., 8: 509-519.

Knauer, D.J., Thompson, J.A. and Cunningham, D.D. (1983) Protease nexins: Cell-secreted proteins that mediate the binding, internalization, and degradation of regulatory serine proteases. J. Cell Phys., 117: 385-396.

Kordower, J.H., Notter, M.F.D. and Gash, D.M. (1987) Neuroblastoma cells in neural transplants: a neuroanatomical and behavioral analysis. Brain Res., 417: 85-98.

Kruithof, E.K.O., Gudinchet, A. and Bachmann, F. (1988) Plasminogen activator-1 and plasminogen activator-2 in various disease states. In press.

Kruithof, E.K.O., Schleuning, W.D. and Bachmann, F. (1985) Human tissue-type plasminogen activator. Production in continuous serum-free culture and rapid purification. Biochem. J., 226: 631-634.

Kruithof, E.K.O., Vassalli, J.D., Schleuning, W.D., Mattaliano, R.J. and Bachmann, F. (1986) Purification and characterization of a plasminogen activator inhibitor from the histocytic lymphoma cell line, U937. J. Biol. Chem., 261: 11207-11210.

Krystosek, A. and Seeds, N.W. (1981a) Plasminogen activator release at the neuronal growth cone. Science, 213: 1532-1533.

Krystosek, A. and Seeds, N.W. (1981b) Plasminogen activator secretion by granule neurons in cultures of developing cerebellum. Proc. Natl. Acad. Sci. USA, 78: 7810-7814.

Kumar, S., Steward, J.K., Waghe, M., Pearson, D., Edwards, D.C., Fenton, E.L. and Griffith, A.H. (1970) The administration of the nerve growth factor to children with widespread neuroblastoma. J. Pediatr. Surg., 5: 18-22.

Kyritsis, A.P., Fletcher, R.T. and Chader, G.J. (1986) Laminin: An initiating role in retinoblastoma cell attachment and differentiation. In Vitro Cell. Dev. Biol., 22: 418-422.

Lammle, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lammle, B. and Griffin, J.H. (1985) Formation of the fibrin clot: the balance of procoagulant and inhibitory factors. Clin. Haematol., 14: 281-342.

Lander, A.D., Fujii, D.K. and Reichardt, L.F. (1985) Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. Proc. Natl. Acad. Sci. USA, 82: 2183-2187.

Larsson, L.I., Skriver, L., Nielson, L.S., Grondahl-Hansen, J., Kristensen, P. and Dano, K. (1984) Distribution of urokinase-type plasminogen activator immunoreactivity in the mouse. J. Cell Biol., 98: 894-903.

Laug, W.E., Jones, P.A., Nye, C.A. and Benedict, W.F. (1976) The effect of cyclic AMP and prostaglandins on the fibrinolytic activity of mouse neuroblastoma cells. Biochem. Biophys. Res. Comm., 68: 114-119.

Lee, L.S. and Weinstein, I.B. (1978) Epidermal growth factor, like phorbol esters, induces plasminogen activator in HeLa cells. Nature, 274: 696-697.

Lindner, J., Guenther, J., Nick, H., Zinser, G., Antonicek, H., Schachner, M. and Monard, D. (1986) Modulation of granule cell migration by a glia-derived protein. Proc. Natl. Acad. Sci. USA, 83: 4568-4571.

Littauer, U.Z., Zutra, A., Rybak, S. and Ginzburg, I. (1985) The expression of tubulin and various enzyme activities during neuroblastoma differentiation. In: A. Evans, G.J.D'Anjou and R.C.Seeger (eds.), Adv. in Neuroblastoma Res., pp. 193-208, New York: Alan R. Liss. Inc.

Lotan, R. and Lotan, D. (1980) Stimulation of melanogenesis in a human melanoma cell line by retinoids. Cancer Res., 40: 3345-3350.

Luckenbill-Edds, L. and Kleinman, H.K. (1988) Effect of laminin and cytoskeletal agents on neurite formation by NG108-15 cells. J. Neurosci. Res., 19: 219-229.

Ludwig, K.W., Lowey, B. and Niles, R.M. (1980) Retinoic acid increases cyclic AMP dependent protein kinase activity in murine melanoma cells. J. Biol. Chem., 255: 5999-6002.

Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E. and Varon, S. (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol., 97: 1882-1890.

Mattsson, M.E.K., Ruusala, A. and Pahlman, S. (1984) Changes in inducibility of ornithine decarboxylase activity in differentiating human neuroblastoma cells. Exp. Cell Res., 155: 105-112.

Mehta, J., Mehta, P., Lawson, D. and Saldeen, T. (1987) Plasma tissue plasminogen activator inhibitor levels in coronary artery disease: correlation with age and serum triglyceride concentrations. J. Amer. Coll. Cardiol., 9: 263-266.

Mimura, J. and Luskatoff, D.J. (1987) The extracellular matrix of cultured bovine aortic endothelial cell contains functionally active type-1 plasminogen activator inhibitor. Blood, 70: 721-728.

Monard, D. (1985) Neuronal cell behavior: modulation by protease inhibitors derived from non-neuronal cells. Cell Biol. Intl. Reports, 9: 297-305.

Monard, D. (1987) Commentary- Role of protease inhibition in cellular migration and neuritic growth. Biochem. Pharmacol., 36: 1389-1392.

Monard, D., Niday, E., Limat, A. and Solomon, F. (1983) Inhibition of protease activity can lead to neurite extension in neuroblastoma cells. Prog. Brain Res., 58: 359-364.

Monard, D., Solomon, F., Rentsch, M. and Gysin, R. (1973) Glia-induced morphological differentiation in neuroblastoma cells. Proc. Natl. Acad. Sci. USA, 70: 1894-1897.

Moonen, G., Grau-Wagemans, M.P.G. and Selak, I. (1982) Plasminogen activator-plasmin system and neuronal migration. Nature, 298: 753-755.

Moonen, G., Grau-Wagemans, M.P.G., Selak, I., LeFebvre, P., Rogister, B., Vassalli, J.D. and Belin, D. (1985) Plasminogen activator is a mitogen for astrocytes in the developing cerebellum. Dev. Brain Res., 20: 41-48.

Nelson, N.A., Cieplak, W., Dacul, S.C. and Prager, M.D. (1987) Modulation of secreted plasminogen activator activity of human renal carcinoma cells by dimethylsulfoxide, butyrate and retinoate. Cancer Lett., 34: 305-316.

Ossowski, L. and Belin, D. (1985) Effect of dimethylsulfoxide on human carcinoma cells, inhibition of plasminogen activator synthesis, change in cell morphology, and alteration of response to cholera toxin. Mol. Cell Biol., 5: 3552-3559.

Ossowski, L. and Reich, E. (1983) Antibodies to plasminogen activator inhibit human tumor metastasis. Cell, 35: 611-619.

Pahlman, S., Ruusala, A., Abrahamsson, L., Mattsson, M.E.K. and Esscher, T. (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Diff., 14: 135-144.

Patterson, P.H. (1985) On the role of proteases, their inhibitors and the extracellular matrix in promoting neurite outgrowth. J. Physiol., 80: 207-211.

Pennica, D., Holmes, W., Kohr, W., Harkins, R.N., Vehar, G., Ward, C., Bennett, W., Yelverton, E., Seebing, P., Heyneker, H., Goedder, H. and Collen, D. (1983) Cloning and expression of human tissue type plasminogen activator cDNA. Nature, 301: 214-221.

Plet, A., Gerbaud, P., Anderson, W.B. and Brion, E. (1985) Changes in the activity and subcellular distribution of cyclic-AMP-dependent protein kinases with retinoic acid induced differentiation of embryonal carcinoma cells. Diff., 30: 159-164.

Prager, M.D., Nelson, N.G., Cieplak, W. and Dacus, S.C. (1986) Characterization of plasminogen-activator from two human renal carcinoma cell lines and effect of differentiation inducing agents. Proc. Amer. Assoc. Cancer Res., 27: 3-8.

Prashad, N., Lotan, D. and Lotan, R. (1987) Differential effects of dibutyryl cyclic adenosine monophosphate and retinoic acid on the growth, differentiation, and cyclic adenosine monophosphate-binding protein of murine neuroblastoma cells. Cancer Res., 47: 2417-2424.

Ramshaw, I.A., Badenoch-Jones, P., Grant, A., Maxted, M. and Claudianos, C. (1986) Enhanced plasminogen activator production by highly metastatic variant cell lines of a rat mammary adenocarcinoma. Invas. Metastas., 6: 133-144.

Rao, J.S., Tennant, K.D. and Festoff, B.W. (1988) Characterization of plasminogen activator inhibitors produced by rat brain tumor cells in vitro. FASEB J., 2: A1737.

Rehemtulla, A., Gates, J. and Hart, D. (1987) The induction of an inhibitor of plasminogen activators in mouse cells which binds to both human and mouse enzymes. Comp. Biochem. Physiol., 88B: 277-283.

Rehemtulla, A., Murphy, P., Dobson, M. and Hart, D.A. (1988) Purification and characterization of a plasminogen activator inhibitor from the human astrocytoma, U138. Biochem. Cell Biology, in press.

Rettig, W.J., Spengler, B.A., Chesa, P.G., Old, L.J. and Biedler, J.L. (1987) Coordinate changes in neuronal phenotype and surface antigen expression in human neuroblastoma cell variants. Cancer Res., 47: 1383-1389.

Reynolds, C.P., Biedler, J.L., Spengler, B.A., Reynolds, D.A., Ross, R.A., Frenkel, E.P. and Smith, R.G. (1986) Characterization of human neuroblastoma cell lines established before and after therapy. J. Natl. Cancer Inst., 76: 375-387.

Reynolds, C.P. and Maples, J. (1985) Modulation of cell surface antigens accompanies morphological differentiation of human neuroblastoma cell lines, In: A. Evans, G.J.D'Anjou and R.C.Seeger (eds.), Adv. in Neuroblastoma Res., pp. 3-37, New York: Alan R. Liss.Inc.

Robson, J.A. and Sidell, N. (1985) Ultrastructural features of a human neuroblastoma cell line treated with retinoic acid. Neurosci., 14: 1149-1162.

Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J. and Furcht, L.T. (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol., 98: 212-220.

Rosenblatt, D.E., Cotman, C.W., Nieto-Sampedro, M., Rowe, J.W. and Knauer, D.J. (1987) Identification of a protease inhibitor produced by astrocytes that is structurally and functionally homologous to human protease nexin-I. Brain Res., 415: 40-48.

Sadee, W., Yu, V.C., Richards, M.L., Pries, P.N., Schwab, M.R., Brodsky, F.M. and Biedler, J.L. (1987) Expression of neurotransmitter receptors and myc protooncogenes in subclones of a human neuroblastoma cell line. Cancer Res., 47: 5207-5212.

Saksela, O., Hovi, T. and Vaheri, A. (1985) Urokinase-type plasminogen activator and its inhibitor secreted by cultured human monocyte-macrophages, J. Cell Phys., 122: 125-132.

Sappino, A.P., Busso, N., Belin, D. and Vassalli, J.D. (1987) Increase in urokinase-type plasminogen activator gene expression in human lung and breast carcinomas. Cancer Res., 47:4043-4046.

Satoh, M., Yokosawa, H. and Ishii, S. (1988) Membrane-bound trypsin-like enzyme functioning in degradation of dynorphin in neuroblastoma cells. Purification and characterization. J. Biochem., 103: 493-498.

Sawaya, R. and Highsmith, R. (1988) Plamsinogen activator activity and molecular weight patterns in human brain tumors. J. Neurosurg., 68: 73-79.

Sawaya, R., Mandybur, T., Ormsby, I. and Tew, J.M. (1986) Antifibrinolytic therapy of experimentally grown malignant brain tumors. J. Neurosurg., 64: 263-268.

Schlesinger, H.R., Rorke, L., Jamieson, R. and Hummler, K. (1981) Neuronal properties of neuroectodermal tumors in vitro. Cancer Res., 41: 2573-2575.

Schurch-Rathgeb, Y. and Monard, D. (1978) Brain development influences the appearance of glial factor-like activity in rat brain primary cultures. Science, 273: 308-309.

Scott, R.W. and Baker, J.B. (1983) Purification of protease nexin. J. Biol. Chem., 258: 10439-10440.

Scott, C.F., Carrell, R.W., Glaser, C.B., Kueppers, F., Lewis, J.H. and Colman, R.W. (1986) Alpha 1-antitrypsin-Pittsburgh. A potent inhibitor of human plasma factor XIa, kallikrein and factor XIIf. J. Clin. Invest., 77: 631-634.

Scott, R.W., Bergman, B.L., Bajpai, A., Hersh, R.T., Rodriguez, H., Jones, B.N., Barreda, C., Watts, S. and Baker, J.B. (1985) Protease nexin: Properties and a modified purification procedure. J. Biol. Chem., 260: 7029-7034.

Seeger, R.C., Rayner, S.A., Banerjee, A., Chung, H., Laug, W.E., Neustein, H.B. and Benedict, W.F. (1977) Morphology, growth, chromosomal pattern and fibrinolytic activity of two new human neuroblastoma cell lines. Cancer Res., 37: 1364-1371.

Selak, I., Rogister, B., Lefebvre, P.P., Leprince, P. and Moonen, G. (1986) Plasminogen activators in brain development. Adv.in the Biosci., 61:193-201.

Sidell, N. (1982) Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. J. Natl. Cancer Inst., 68: 589-596.

Sidell, N., Altman, A., Haussler, M.R. and Seeger, R.C. (1983) Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. Exp. Cell Res., 148: 21-30.

Sidell, N. and Horn, R. (1985) Properties of human neuroblastoma cells following induction by retinoic acid, In: A. Evans, G.J.D'Anjou and R.C. Seeger (eds.), Adv. in Neuroblastoma Res., pp. 39-53, New York: Alan R.Liss Inc.

Sidell, N., Lucas, C.A. and Kreutzberg, G.W. (1984) Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. Exp. Cell Res., 155: 305-308.

Sidell, N., Sarafian, T., Kelly, M., Tsuchida, T. and Haussler, M. (1986) Retinoic acid-induced differentiation of human neuroblastoma: a cell variant system showing two distint responses. Exp. Cell Biol., 54: 287-300.

Sommer, J., Gloor, S.M., Rovelli, G.F., Hofsteenge, J., Nick, G., Meier, R. and Monard, D. (1987) cDNA sequence coding for a rat glia-derived nexin and its homology to members of the serpin superfamily. Biochem., 26: 6407-6410.

Soreq, H. and Miskin, R. (1981) Plasminogen activator in the rodent brain. Brain Res., 216: 361-374.

Soreq, H., Miskin, R., Zutra, A. and Littauer, U.Z. (1983) Modulation in the levels and localization of plasminogen activator in differentiating neuroblastoma cells. Dev. Brain Res., 7: 257-269.

Sprengers, E.D. and Kluft, C. (1987) Plasminogen activator inhibitors. Blood, 69: 381-387.

Sprengers, E.D., Princen, H.M.G., Kooistra, T. and Van Hinsbergh, V.W.M. (1985) Inhibition of plasminogen activators by conditioned medium of human hepatocytes and hepatoma cell line HepG2. J. Lab. Clin. Med., 105: 751-761.

Stone, S., Nick, H., Hofsteenge, J. and Monard, D. (1987) Glia-derived neurite-promoting factor is a slow-binding inhibitor of trypsin, thrombin and urokinase. Arch. Biochem. Biophys., 252: 237-244.

Stump, D.C., Thienpont, M. and Collen, D. (1986) Purification and characterization of a novel inhibitor of urokinase from human urine. Quantitation and preliminary characterization in plasma. J. Biol. Chem., 261, 12759-12764.

Sugimoto, T., Sawada, T., Matsumura, T., Yoshihiro, H., Kemshead, J.T., Suzuke, Y., Okada, M., Tagaya, O. and Hino, T. (1987) Morphological differentiation of human neuroblastoma cell lines by a new synthetic polyprenoic acid (E5166). Cancer Res., 47: 5433-5438.

Thiele, C.J., Reynolds, C.P. and Israel, M.A. (1985) Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature, 313: 404-406.

Tissot, J.D., Hauert, J. and Bachmann, F. (1984) Characterization of plasminogen activators from normal human breast and colon and from breast and colon carcinomas. Int. J. Cancer., 34: 295-302.

Triche, T.J. and Askin, F.B. (1983) Neuroblastoma and the differential diagnosis of small, round blue-cell tumors. Human Pathol., 14: 569-595.

Tsokos, M., Ross, R.A. and Triche, T.J. (1985) Neuronal, Schwannian and melanocytic differentiation of human neuroblastoma cells in vitro, In: A. Evans, G.J.D'Anjou and R.C. Seeger (eds.), Adv. in Neuroblastoma Res., pp. 55-68, New York: Alan R. Liss Inc.

Tsokos, M., Scarpa, S., Ross, R.A. and Triche, T.J. (1987) Differentiation of human neuroblastoma recapitulates neural crest development. Am. J. Pathol., 128: 484-496.

Tumilowicz, J.J., Nichols, W.W., Cholon and J.J., Greene, A.E. (1970) Definition of a continuous human cell line derived from neuroblastoma. Cancer Res., 30: 2110-2118.

Unsicker, K., Vey, J., Hofmann, H-D., Muller, T.H. and Wilson, A.J. (1984) C6 glioma cell-conditioned medium induces neurite outgrowth and survival of rat chromaffin in vitro: Comparison with the effects of nerve growth factor. Proc. Natl. Acad. Sci. USA, 81: 2242-2246.

VanMourik, J.A., Lawrence, D.A. and Luskutoff, D. (1984) Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J. Biol. Chem., 259: 14914-14921.

Varani, J., Lovett, E.J., McCoy, J.P., Shibata, S., Maddox, D.E., Goldstein, I.J. and Wicha, M.S. (1983) Differential expression of a laminin-like substance by high and low metastatic tumor cells. Amer. J. Pathol., 111: 27-34.

Varani, J., McKeever, L., Fligiel, S.E.G. and Sitrin, R.G. (1987) Plasminogen activator production by human tumor cells: effect on tumor cell-extracellular matrix interactions. Int. J. Cancer, 40: 772-777.

Varon, S., Rudge, J.S., Davis, G.E., Skaper, S.D. and Manthorpe, M. (1986) Astroglia production of neuronotrophic and neurite-promoting agents. In Advances in the Biosciences, 61:203-211.

Vassalli, J.D., Vaccino, D. and Belin, D. (1985) A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell Biol., 100: 86-92.

Verrall, S. and Seeds, N.W. (1987) Binding of tissue plasminogen activator to cerebellar neurons in culture. Abstr. for Amer. Soc. Cell Biol., J. Cell Biol., 105: 1801.

Wilson, E.L. and Francis, G.E. (1983) Differentiation-linked secretion of urokinase and tissue plasminogen activator by normal human hemopoietic cells. J. Exp. Med., 165: 1609-1623.

Wohlwend, A., Belin, D. and Vassalli, J.D. (1987) Plasminogen activator specific inhibitors in mouse macrophages: in vivo and in vitro modulation of their synthesis and secretion. J. Immunol., 139: 1278-1284.