

AN ABSTRACT OF THE THESIS OF

Michele L. Solem for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on July 22, 1992.

Title: Studies of Genes Expressed in the Brain and Regulated by Transforming Growth Factor β .

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Abstract approved: _____

David W. Barnes

Human S-protein is a serum glycoprotein that binds the activated complement complex, mediates coagulation, and also functions as a cell adhesion protein through interactions with its integrin receptor. A cDNA clone for human S-protein was isolated from a lambda gt11 cDNA library of mRNA from HepG2 hepatocellular carcinoma cell line. The cDNA clone in lambda was subcloned into pUC18 for Southern and Northern blot experiments. Hybridization with ^{32}P -radiolabeled human S-protein cDNA revealed a single copy gene encoding S-protein in the human and mouse genome. A 1.6 Kb mRNA for S-protein was detected in mRNA from mouse liver and brain. S-protein is known to be regulated by transforming growth factor β , although the mouse brain-derived glial cells, SFME, are not

induced by the growth factor to express S-protein mRNA.

Transforming growth factor β promotes the differentiation of serum-free mouse embryo cells (SFME) into astroblasts that express the astrocyte-specific marker glial fibrillary acidic protein (GFAP). In order to find other genes induced by TGF β , a cDNA library made from mRNA of SFME grown in the presence of TGF β for 48 hours was differentially screened using cDNA probes made from mRNA of SFME grown with and without TGF β . After screening approximately 10,000 clones, 14 clones were detected by the TGF β (+) probe but not by the TGF β (-) probe. Sequencing of one clone revealed that it was a full length copy of cystatin C cDNA.

Other growth factors do not induce cystatin C, including nerve growth factor, basic fibroblast growth factor and platelet-derived growth factor. Hydrocortisone at 10^{-7} M does induce cystatin C in SFME at comparable levels to TGF β . When transcription is inhibited for 8 hours, cystatin C mRNA is stable in the presence of TGF β compared to its observable decay when TGF β is not present. Also, a 4 Kb RNA species is detected by Northern blot experiments and is induced by TGF β in SFME. This message may be the primary cystatin C transcript, which may suggest regulation of cystatin C in the nucleus.

Studies of Genes Expressed in the Brain
and Regulated by Transforming Growth Factor β

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STUDIES OF GENES EXPRESSED IN THE BRAIN
AND REGULATED BY TRANSFORMING GROWTH FACTOR β

CHAPTER I

Introduction and Literature Review

As we witness cells mingling through the microscope, we are challenged to understand how these tiny units of life communicate. Through genetics, we have begun to map the intricate journey of the cell cycle that is traversed by cells during their replication. The regulation of the cell cycle has been elaborately described (Hartwell and Weinert, 1989; Murray and Kirshner, 1989; Nurse, 1990), and this process involves both an intrinsic genetic programming and essential extrinsic factors that govern cells making up the organism. Growth regulators are cues for intercommunication; they coordinate cell growth and activities during embryonic development and throughout life.

Cells grown in culture have similar growth requirements as cells growing in vivo (Sato, 1979). Nutrients provide fuel for the cell. Growth regulators control cell growth and differentiation. A substratum is often required for the cells to grow; some examples of cell adhesive proteins include fibronectin, laminin, and collagen. Cell attachment proteins anchor cells to allow internal organization and to permit cell-cell interactions.

Cells have been traditionally derived and cultured in the presence of serum, but serum is a complex substance. There is often variability in the components that make up serum. This makes for problems in reproducibility when serum is used in experiments. Serum contains a mix of peptide growth factors and hormones that may interfere with determining specific growth regulator's effects on cell activities (Temin et al., 1972; Brooks, 1975). Also, there are growth inhibitors in serum that interfere with cell growth (Rawson et al., 1991).

Mouse embryo cells derived and maintained in vitro in serum-supplemented media will at first go through a number of cell divisions and then enter a growth crisis. Only genomically altered cells will continue to grow; those that are tetraploid or heteroploid, and with severe chromosomal aberrations. The normal karyotype of the cells will be lost. Swiss, BALB and NIH lines (3T3, 3T6, 3T12) as well as C3H 10T1/2, which are commonly used in studies of carcinogenesis and growth control, are all derived this way, and all exhibit major chromosomal abnormalities. However, mouse embryo cells derived in a defined, serum-free media remain karyotypically normal and do not undergo a growth crisis (Loo et al., 1987). Serum-free cells have been used to study specific growth factor's effects on their activities.

Serum-free cell culture allows us to define and selective manipulate of the cells' environment. Although serum-free cell culture remains a largely empirical science, some

guidelines for growing various types of cells have emerged (Barnes and Sato, 1980a). Cells in vivo are exposed to a complex array of nutrients, growth factors and extracellular matrices. If the cell is to survive in culture, the medium must provide an adequate environment. Serum-free media contains all the essential ingredients that cells require. Besides the proper balance of salts, buffers and amino acids, all cells require hormones and growth factors. For example, insulin is required by most cells for survival in serum-free medium. The concentration of insulin required by serum-free cells is often much higher than physiological levels. This suggests that insulin at these higher concentrations may mimic the effects of other growth factors.

Binding proteins, including transferrin and albumin, are also required by most cells and may serve to provide essential nutrients and minerals and to bind up and remove toxins. An essential growth factor for the survival of many ectodermal and neuroectodermal cell types is epidermal growth factor (EGF); EGF stimulates the proliferation of many cells and is required by most normal cells growing in culture. Interestingly, often the transformation of cells obviates the need for specific growth factors, like EGF. For example, mouse serum-free derived cells (SFME) when transformed by ras oncogenes lose the requirement for EGF (Shirahata et al., 1990).

Most cells require a substratum, and this can be provided

using specific attachment factors, such as fibronectin, cold-insoluble globulin or serum spreading factor. The use of the polymer, polylysine, can also promote cell attachment, presumably by its highly positive charge.

Peptide growth factors are growth regulators that bind to cell surface receptors and stimulate cell proliferation and/or differentiation (Deuel, 1987, Cross and Dexter, 1991). They regulate a variety of responses in the cell. There is a multitude of signaling pathways to the nucleus that varies between growth factors, and the same growth factor may signal many different pathways in the same cell. All peptide growth factors are known to have their own cell surface receptor. Commonly, the receptor responses to binding of the growth factor by signaling a tyrosine kinase activity; the EGF, PDGF and CSF receptors are tyrosine kinases (Yarden and Ullrich, 1988). Growth factors also activate other kinases, including protein kinase C, and G proteins.

Many oncogenes are related to growth factors or growth factor receptors (Varmus, 1987); for example the *sis* oncogene shares homology with platelet-derived growth factor, the *erbB* and *neu* oncogenes are related to the EGF receptor. Much research has focused on growth factors' control of cell growth, and how oncogenes abrogate this control (Cantley et al., 1991). The finely regulated communications by peptide growth factors can be disrupted at several points. Tyrosine

kinase activity of the growth factor receptor can be altered, creating a continuous and unregulated cell signaling event. Alterations in protein kinases (e.g. RAF), phosphorylases and phosphatases also may jeopardize internal order and communication with outside the cell. The checkpoints that regulate the cell cycle events of normal cells may be lost in transformed cells.

Serum-free mouse embryos cells (SFME) transformed with the Ha-ras, N-ras and neu oncogenes are capable of growing in the absence of epidermal growth factor, which is essential for the growth of untransformed SFME (Shirahata et al., 1990; Rawson et al., 1990a). Serum free mouse embryo cells have been used in our laboratory to identify new oncogenes, taking advantage of this unique property of the cell line.

Serum Free Derived Mouse Embryo Cells

The cell-line, known as serum-free mouse embryo cells (SFME), was isolated from a sixteen day old Balb/c mouse embryo, and has been continuously passaged in serum free medium without loss of its normal karyotype (Loo et al., 1987). These cells have a doubling time of approximately 20 hours, and a fibroblast-like morphology that changes with the culture conditions. For instance, in the presence of TGF β , SFME become elongated (Fig. 1.1), and stick tightly to the tissue culture flask, presumably do to the increased synthesis

of extracellular matrix proteins. The recipe used for growing SFME is a 1:1 mixture of Ham's F12 /DMEM medium in the presence of insulin, transferrin, epidermal growth factor, high density lipoprotein (HDL), and selenium in a fibronectin precoated flask to provide cell attachment (Loo et al., 1989). The cells require both insulin and EGF for growth. Transferrin provides iron and may regulate other responses, selenium serves as an antioxidant, HDL provides the cells with essential lipids, and fibronectin promotes cell attachment. After 20 hours of EGF removal, the cells will enter a programmed cell death, which is irreversible but can be halted by inhibitors of protein synthesis and transcription (Rawson et al., 1990). Serum-free mouse embryo cells are reversibly growth-inhibited in the presence of 10% fetal calf serum (Rawson et al., 1991). Recently it was determined (Sakai et al., 1990) that in the presence of transforming growth factor β (TGF β) or 10% calf serum, SFME will express the astrocyte-specific differentiation marker glial fibrillary acidic protein (GFAP). This is an intermediate filament found only in mature astrocytes. The expression of GFAP by SFME demonstrates the differentiation-promoting effects of TGF β on these astroblast precursors. Interestingly, the effects are reversible upon removal of the growth factor. A major part of this thesis will discuss TGF β induction and regulation of specific genes in SFME.

Astrocytes and the Brain

Astrocytes are a type of glial cell. They are the most abundant cells found in the brain, and are noted for their stellate appearance (Fedoroff and Vernadakis, 1986). They are distinguished from other cells by their cell-specific expression of a 49,000 M.W. intermediate filament, glial fibrillary acidic protein. Astrocytes provide many functions in the brain. They are responsible for the regulation of the brain's extracellular environment, and monitor the concentrations of excitatory ions and neurotransmitters released by neurons into the extracellular space. Astrocytes provide nutrients and growth factors for neurons and glial cells. Some types of astrocytes regulate what passes into the brain at the blood brain barrier. There, they form a close junction with endothelial cells lining the capillaries.

There are many types of astrocytes; the radial glial cells are a type of astrocyte that provide a scaffold for the migration of neurons and other brain cells from their site of origin in the developing neuroectoderm, to their final destination in the mature central nervous system. Some astrocytes are phagocytes and can remove the debris from degenerated neurons and from inflammatory activities of microglia. Astrocytes are immunocompetent cells, and can phagocytose and express foreign antigens and the major histocompatibility class II antigen at their surface. They release cytokines, like the interleukins, which stimulates the

activities of T helper cells that migrate into the brain. Some astrocytes can depolarize and may be the recipient of "synaptic" neuronal transmissions, although the consequences, if any, are not yet understood (Muller et al., 1992).

The brain is an intricate web of network; spun into its pathways lies the primal program of instinct interwoven with the powers of creativity and imagination. Many other cells in the brain besides neurons coordinate activities (Ramon y Cajal, 1909), these include the astrocytes, the microglia which are phagocytic cells, the oligodendrocytes that insulate the neuronal axons and ependymal cells.

The development of glial cells has been closely examined in vivo and in vitro, and in each case the progression towards glial differentiation is the same (Lillien and Raff, 1990). The neuroectoderm gives rise to the neuroblasts and glioblasts. The neuroblast proceeds the glioblast in its maturation. The first type of glial cell to appear is the type I astrocyte, detected by its expression of GFAP in mouse embryos at day 16. The oligodendrocytes are next to appear, typically during the first day postnatally. Type II astrocytes arise from the same cell precursor, and will be next to differentiate at the beginning of the second week following birth. The direction of oligodendrocyte-astrocyte (O-2A) progenitor cells differentiation has been elaborately examined using the rat optic nerve as a model system for CNS glial cell development (Raff et al., 1983, Raff, 1989). It

appears that the type I astrocytes provide signals, including the release of a 23,000 M.W. protein known as ciliary neurotrophic factor, that stimulate differentiation of the O-2A progenitor cells. Ciliary neurotrophic factor and other glial cell regulatory molecules associated with the extracellular matrix are believed to mediate their effects on O-2A progenitor differentiation simultaneously.

The extracellular matrix can influence the behavior of vertebrate neural cells by promoting their adhesion and neurite outgrowth (Neugebauer et al., 1988). Neurite outgrowth requires calcium dependent cell adhesion molecules and the integrin extracellular matrix receptors. The integrins will bind to specific cell attachment proteins, including fibronectin, vitronectin and laminin (Rauvala et al., 1989). Laminin, which is the most effective of the extracellular matrix proteins in promoting neurite outgrowth of retinal neurons, is expressed on the surface of cultured astrocytes. Vitronectin and its $\alpha V \beta 3$ integrin receptor have recently been detected in the brains of Alzheimer's victims (Akiyama et al., 1991). Proteases and protease inhibitors are also involved in neurite outgrowth, and may be finely regulated by localized glia to coax the growth of neurons during development and regeneration. Some proteases and protease inhibitors that are known neurite-regulating proteins include protease nexins, thrombin and plasminogen activator (Knauer and Cunningham, 1984). Interestingly, the cell-

secreted glia-derived protease inhibitor nexin complexed with thrombin have been found to specifically interact with the cell adhesion molecule, vitronectin (Rovelli et al., 1990).

S-Protein: Alias serum-spreading factor, vitronectin

S-protein is a 75,000 M.W. serum glycoprotein produced at high concentrations by the liver. It has also been detected in the brain (Akiyama et al., 1991). This protein has many functions; it is involved in blood coagulation and will bind and protect thrombin from inactivation by antithrombin III (Podack et al., 1986). S-protein is also known as serum spreading factor, and was purified from serum as a cell attachment factor in culture (Barnes et al., 1980b). It is also known as vitronectin since it can bind to glass (Hayman et al., 1983). S-protein can bind other proteins including the nexins (Rovelli et al., 1990), plasminogen activator inhibitor (Declerck et al., 1988), and members of the complement family, the cascade of cell lysis proteins (Podack et al., 1977). When bound to nascent complement components, it may protect bystander cells. Recently it has been determined that TGF β will enhance the expression of S-protein in human HepG2 hepatoma cells (Koli et al., 1991). TGF β also regulates the expression of other cell attachment proteins and their receptors, including fibronectin, collagen, and the integrins. One of integrin receptors binds S-protein at an arg-gly-asp sequence at the amino terminal region of the

protein (Jenne and Stanley, 1985).

The ability of S-protein to bind glial-derived nexin-thrombin complex may potentiate the neurite-promoting activity of the serine protease inhibitor (Rovelli et al., 1990). S-protein and its receptor have also been detected in the brains of Alzheimer's victims, possibly indicating inflammatory responses within these diseased brains (Akiyama et al., 1991).

The neurodegenerative disease, Alzheimer's disease affects seniors, typically over the age of 65 years (Yamaguchi et al., 1988). This disease is characterized by diffuse plaques and internal neurotangles found associated is the marker protein β amyloid. β amyloid deposits are due to aberrations in the processing of its precursor, amyloid precursor protein (APP). The exact function of the precursor is not fully known (Selkoe, 1990). Some possibilities include a role in blood hemostasis, cell attachment, and gene activation (Deryk Loo, personal communication). Two of the three alternatively spliced transcripts contain the Kunitz serine protease inhibitor. β amyloid is toxic to neurons and other cells at high concentrations (Yankner et al., 1989; Yankner et al., 1990) and it possibly is growth stimulatory at low concentrations. Both neurons and glial cells (Frederickson, 1992) may be involved in the deposition of the β amyloid plaques and tangles. Genetic linkage analysis has found that the autosomal dominant condition known as hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D) shows a

single point mutation in the β amyloid protein (Levy et al., 1990). Similarly, the Icelandic version of HCHWA has a single base point mutation in the gene coding for cystatin C (Cohen et al., 1983; Grubb et al., 1984). These genetic aberrations are believed to be primarily responsible for the onset of these diseases. Victims of HCHWA-I typically begin showing symptoms, including cerebral hemorrhage and cerebral amyloid deposits, in their fifth decade of life.

Prologue to the Cystatin C Research

We have found that TGF β strongly induces the protease inhibitor cystatin C in proastroblasts, as well as inducing their differentiation into astroblasts that express the developmental marker glial fibrillary acidic protein. The induction of cystatin C in vivo may be both a developmental and an inflammatory response in the brain. It may be involved in the regulation of peptide neuromodulators released into the extracellular space in the brain.

Cystatin C is a secreted cysteine protease inhibitor of the family of proteases that include papain and the cathepsins B, H, and L (Rawlings and Barrett, 1990). The cathepsins are lysosomal proteases, and cathepsins B and D have been associated with amyloid deposits in Alzheimer's brains (Cataldo and Nixon, 1990). Cystatin C is a competitive inhibitor of these proteases, with a K_i value ranging between 10^{-7} and 10^{-11} M. Cystatin is produced by many types of cells

and tissues, and is found at high concentrations in body fluids, including serum, semen, urine, and the cerebral spinal fluid of the central nervous system (Abrahamson et al., 1990).

There are three classes of cysteine proteinase inhibitors. Class I and II are low molecular weight compounds (approx. 12,000 to 17,000 M.W.) and account for most of the cysteine protease inhibitory activity found in the body. Some of these include the cystatins A, B, C, and S and the stefins. Type III protease inhibitors include the kininogens, high molecular weight proteins (approx. 78,000 M.W.) that contain three cysteine protease inhibitory domains and the sequence for bradykinin at their carboxyl termini. The kininogens are found predominantly in the blood, functioning as both protease inhibitors and as regulators of inflammation. Interestingly, they are induced during aging (Sierra et al., 1989).

Thus far, cystatin C cDNA has been cloned in human (Abrahamson et al., 1987), rat (Cole et al., 1989) and mouse (Solem et al., 1990). All contain an amino terminal signal sequence between 17-23 amino acids in length; the mature protein consists of 12 amino acids. There are two disulfide bonds and a putative asparagine-linked glycosylation site. Cystatin C has been found to be growth stimulatory for 3T3 mouse fibroblasts (Sun, 1989), and also for certain kidney cells (Tavera et al., 1992). It is a major secretory product of human alveolar macrophages (Chapman et al., 199). In these cells, it is down regulated during activation responses

and is reduced in the macrophages of smokers. Cystatin C is produced to high concentration by the choroid plexus of the brain, the region responsible for the bulk of the cerebral spinal fluid. Cystatin C is produced by astrocytes (Zucker-Franklin et al., 1987). This secreted protein is strikingly found associated with the filaments and bundles of fibrils in the cytoplasm and nucleus. The presence of cystatin C gives evidence that astrocytes have some properties of monocytes, possibly they play a role in the development of some forms of amyloidosis. There may be a developmentally-related expression of cystatin C, since astrocyte precursors do not express the gene at comparable levels (Solem et al., 1990). Cystatin C expression has been studied in the developing brain and compared with the expression of transthyretin and the β protein (β amyloid). It is detected at low levels in the embryo, and reaches its highest concentration directly after birth, remaining at this level throughout adulthood. It's increased presence has been correlated with the development of the choroid plexus (Thomas et al., 1989).

The gene for the human cystatin C has been cloned and sequenced (Abrahamson et al., 1990). Cystatin C gene contains three exons and two introns that span 4 kilobases. The gene is expressed in all tissues examined, including kidney, liver, pancreas, intestines, stomach, antrum, lung and placenta. The apparent non-tissue specific expression of the gene is believed to be because it has housekeeping features in the

regulatory 5' flanking region (i.e., lacks typical CAAT-box, is notably GC-rich, and contains binding sites for transcription factor Sp1).

Little is known about the regulation of cystatin C expression although it is a highly expressed and common protein. Other class II cystatins are tissue-specific; an example is cystatin S which is mainly found in the submandibular glands and is induced in response to β -adrenergic agonists such as isoproterenol (Shaw et al., 199). In this thesis I will discuss what appears to be an mRNA stability regulation of cystatin C in mouse astroblasts by the peptide growth factor TGF β .

An Introduction to Transforming Growth Factor β

Transforming growth factor β plays multiple roles in cell growth and differentiation of many types of cells (Massague, 1990). In particular, the development of mesodermal tissue seems to be affected by the growth factor (Heine et al., 1987). Recently, TGF β has been attributed to a variety of responses in the brain, with astrocytes being predominantly influenced (Saad et al., 1991; Wahl et al., 1991).

There are five known sub-types of TGF β (Rizzino, 1988). The degree of homology ranges from 64% (TGF β_1 vs TGF β_4) to 82% (TGF β_2 vs TGF β_4). TGF β_1 is a prototype; a disulfide-linked dimer of two identical chains of 112 amino acids. TGF β is synthesized as the C-terminal domain of a 390 amino acid

precursor. The receptor for TGF β is found on every cell type, and does not contain a tyrosine kinase moiety like many peptide growth factor receptors.

TGF β homologues include the activins, inhibins and the Mullerian inhibitory substance, all very important factors that modulate gonadal activities and embryonic development. Biological activities of TGF β are diverse and cell-specific (Sporn et al., 1987). TGF β regulates the proliferation of many cells, including the growth arrest of certain lung epithelial cells and keratinocytes. TGF β also exerts a strong antiproliferative action on B lymphocytes, T lymphocytes, and thymocytes. The mechanism of growth inhibition by TGF β may involve the suppression of retinoblastoma protein phosphorylation. TGF β is a mitogen for certain cell types, and it stimulates proliferation of certain fibroblasts and osteoblasts. SFME cells do not show a growth rate response to TGF β .

TGF β induces the expression of many genes that are often involved with the production of the extracellular matrix. TGF β induces the genes for fibronectin and collagen in several fibroblast cell lines. TGF β also regulates the expression of vitronectin, proteoglycans, and other matrix proteins. The receptors for some of these matrix proteins are also regulated by TGF β . TGF β regulates the synthesis of several different proteases and protease inhibitors that affect matrix stability and turnover. TGF β induces the protease inhibitors

plasminogen activator inhibitor, TIMP, and cystatin C and down regulates the synthesis of collagenase, plasminogen activator and transin/stromelysin. TGF β also modulates cell adhesion by upregulating the synthesis of LFA-1, a cell-cell adhesion receptor which binds to the intercellular adhesion molecule ICAM-1 on the surface of lymphoid cells.

Transforming growth factor β promotes the differentiation of SFME cell proastroblasts, inducing the gene for the astrocyte-specific differentiation marker glial fibrillary acidic protein. TGF β also induces in astrocytes the genes for nerve growth factor (Lindholm et al., 1990), NCAM and L1 cell adhesion molecules (Saad et al., 1991) and cystatin C. The regulation of certain genes induced by TGF β has been explored (Massague, 1990). TGF β increases the mRNA for collagen, fibronectin and thrombospondin both in the presence and absence of mRNA stability changes, depending on cell density (Penttinen et al., 1988). TGF β suppresses c-myc expression by inhibiting the initiation of transcription of the gene. Inhibition of this growth related gene by TGF β appears to be through its action on the retinoblastoma protein (Laiho et al., 1990). In this report, we discuss mRNA stability regulation of cystatin C by the growth factor.

mRNA Stability: a Level of Gene Regulation

There are many points where the expression of a gene can

be controlled. Regulation of transcription is common, and this can be coordinated at initiation, during elongation, or at termination. Splicing and further processing of the nuclear RNA may be regulated, as well as transport of the mature nuclear mRNA from the nucleus. In the cytoplasm, mRNA may be regulated by changes in stability. Events during translation may determine the fate of a gene's expression. Some examples of mRNA stability regulation will be discussed, since we find regulation of cystatin C mRNA stability by TGF β in serum-free mouse embryo cells.

Messenger RNA decay is now recognized as a major control point in the regulation of gene expression. (Brawerman, 1989) Certain regions of the mRNA may influence its stability. Often it is the 5' and 3' untranslated portions of the message that are involved. Cellular factors (specific ribonucleases or other cellular proteins) typically are associated with regulation at these target regions.

The mRNAs encoding ferritin and transferrin have specific sequences in their 5' and 3' untranslated regions (UTRs) that form a stem-loop structure to which the iron responsive element (IRE) interacts and is regulated by iron (Mullner et al., 1989; Leibold and Munro, 1988). In the absence of iron, IRE will interact with these loops, and in the case of ferritin, translation will be blocked. IRE also binds to the 3' end of transferrin and stabilizes the mRNA.

The 3'-untranslated region of certain short-lived mRNAs,

including c-myc, c-fos, and certain lymphokines contains multiple copies of AU-rich motifs that are responsible for the instability of these messages. Introducing several repeats of the consensus sequence UAUUUAU to the 3'UTR of globin mRNA dramatically reduces its stability (Shaw and Kamen, 1986).

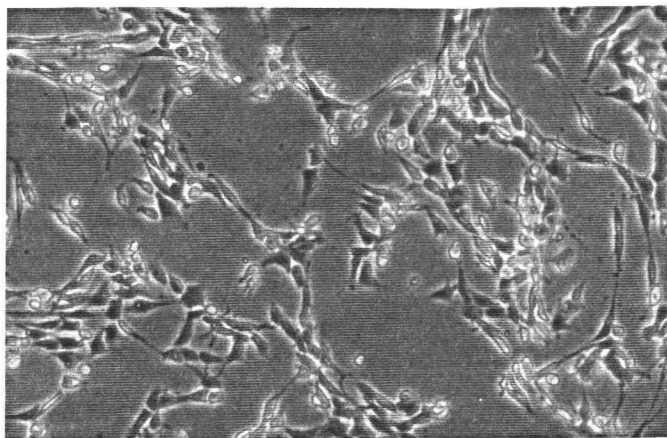
Regions at both the 5' and 3' ends of several histones' mRNA are necessary for regulating stability (Ross and Kobs, 1986; Morris et al., 1986). A destabilization of histones' message takes place when DNA synthesis is complete after S phase. There is also evidence that there is post-transcriptional regulation of the primary transcript in the nucleus.

A very profound effect on mRNA stability is seen in regulation of the vitellogenin mRNA by estrogen (Blume et al., 1987). In *Xenopus* liver, vitellogenin mRNA has a half life of 5 hours when estrogen is present, and 160 hours if estrogen is removed from the culture medium. The 5' and 3'UTR of the message seems to be required for this regulation (Nielsen and Shapiro, 1990).

In this thesis, I report studies that examine the regulation of cystatin C mRNA stability by TGF β . We have found that TGF β regulates the cytoplasmic stability of cystatin C mRNA, and also I believe the primary transcript can be detected in the total RNA of SFME grown in the presence of TGF β . Although the detection of primary transcript RNA by Northern blot analysis is not common, there are reports that

clearly identify unprocessed RNA, particularly when genes are strongly induced (Ferrari et al., 1992).

(A)



(B)

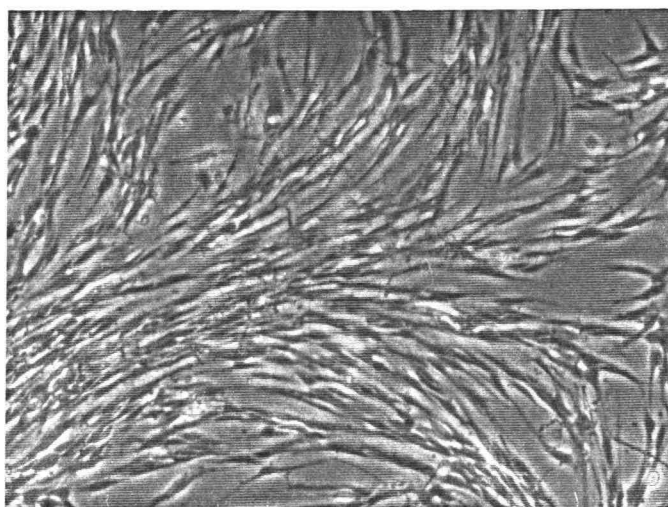


Fig. 1.1: Morphology of SFME grown with and without TGF β .
(A) SFME grown under serum-free conditions (F12/DMEM medium containing high density lipoprotein (10 $\mu\text{g/ml}$), insulin (10 $\mu\text{g/ml}$), transferrin (25 $\mu\text{g/ml}$), epidermal growth factor (50 ng/ml) in dishes precoated with fibronectin (10 $\mu\text{g/ml}$). (B) TGF β (2 ng/ml) added to the plates for 24h.

CHAPTER II

Human and mouse S-protein mRNA detected in Northern blot
experiments and evidence for the gene encoding S-protein
in mammals by Southern blot analysis

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SUMMARY

Human S-protein is a serum glycoprotein that binds and inhibits the activated complement complex, mediates coagulation through interaction with antithrombin III and plasminogen activator inhibitor I, and also functions as a cell adhesion protein through interactions with extracellular matrix and cell plasma membranes. A full length cDNA clone for human S-protein was isolated from a lambda gt11 cDNA library of mRNA from HepG2 hepatocellular carcinoma cell line using mixed oligonucleotide sequences predicted from the amino-terminal amino acid sequence of human S-protein. The cDNA clone in lambda was subcloned into pUC18 for Southern and Northern blot experiments. Hybridization with radiolabeled human S-protein cDNA revealed a single copy gene encoding S-protein in human and mouse genomic DNA. A 1.7 Kb mRNA for S-protein was detected in RNA from human liver and from the PLC/PRF5 human hepatoma cell line. No S-protein mRNA was detected in mRNA from human lung, placenta, or leukocytes or in total RNA from cultured human embryonal rhabdomyosarcoma (RD cell line) or cultured human fibroblasts from embryonic lung (IMR9 cell line) and neonatal foreskin. A 1.6 Kb mRNA for S-protein was detected in mRNA from mouse liver and brain. No S-protein mRNA was detected in mRNA from mouse skeletal muscle, kidney, heart or testis.

INTRODUCTION

Human S-protein is a serum glycoprotein that was first identified as an inhibitor of the formation of the complement membrane attack complex, preventing complement-dependent cell lysis (Podack et al., 1977, 1978; Podack and Muller-Eberhard, 1979). S-protein functions additionally in the terminal stages of the coagulation system by binding and protecting thrombin from inactivation by antithrombin III (Podack et al., 1986), and also binds heparin (Barnes et al., 1985) and plasminogen activator inhibitor I (Declerck et al., 1988). The protein exists in serum as a 75,000 molecular weight molecule and a 65,000 molecular weight molecule that arises from the 75,000 molecular weight form by proteolytic cleavage. In addition thrombin-catalyzed cleavage of human S-protein yields a single 57,000 molecular weight protein (Silnutzer et al., 1984).

Determination of the base sequence for the gene encoding human S-protein revealed that this protein was identical to a cell adhesion molecule previously isolated independently in several laboratories and termed spreading factor or epibolin (Jenne and Stanley, 1985; Know et al., 1979; Barnes et al., 1980b; Stenn et al., 1981; Barnes et al., 1983; Barnes and Silnutzer, 1983; Fuquay et al., 1986). The name vitronectin has been suggested for this protein (Hayman et al., 1983).

Cell membrane receptors for the protein bind to an arg-gly-asg sequence located at position 45-47 from the amino terminal of S-protein (Suzuki et al., 1985). Previous work from Jenne and Stanley (1987) indicates that human S-protein shares homology and exon-intron arrangement with genes for hemopexin, transin and interstitial collagenase.

Human S-protein is synthesized by liver in vivo and by HepG2 human hepatoma cells in culture (Suzuki et al., 1983; Jenne and Stanley, 1987; Barnes et al., 1985), and monoclonal antibodies to human S-protein detect the molecule associated with several cell types, including human platelets (Barnes et al., 1983) and cultured human fibroblasts (Hayman et al., 1983). The protein also has been reported to be present in muscle, kidney, lung and placenta (Hayman et al., 1983). It has been suggested that S-protein may be produced by fibroblastic cells (Hayman et al., 1983), leading to deposition of the protein in multiple localizations in vivo. However, we can detect the protein produced in vitro only in cultures of liver-derived cells (Barnes et al., 1985). For this reason we examined the pattern of synthesis of S-protein mRNA in various tissues. In addition, we were interested in the relative evolutionary conservation of the S-protein gene in various species, because monoclonal and polyclonal antibody to human S-protein recognized only the human and monkey proteins (Barnes et al., 1983; Barnes et al., 1985), suggesting possible unique features in this protein among

primates.

In this paper we report the results of Northern blot experiments probing human and mouse mRNA from several different types of cell lines and tissues. Although mRNA for the protein was detected in liver and liver-derived cell lines, no mRNA for the protein was detected in cultured fibroblasts or in RNA from kidney, placenta or muscle, suggesting that the S-protein detected in these cases (Hayman et al., 1985) originated from other sources. In addition, the genomic DNA of human, mouse, dog, rat, monkey, rabbit and cow were found to contain a single copy gene for S-protein in a sufficiently related form to be detected by Southern blot analysis.

METHODS

Embryonal lung human fibroblast (IMR9), human embryonal rhabdomyosarcoma (RD) and human hepatoma (PLC/PRF5) cell lines were obtained from the American Type Culture Collection, Rockville, Md. Human liver, lung, placental and leukocyte mRNA and mouse liver, brain, skeletal muscle, heart, kidney and testis mRNA and a nylon DNA blot containing 8 μ g each of human, monkey, mouse, rat, dog, cow, rabbit, chicken and yeast genomic DNA digested with Eco RI were purchased from Clonetech, Palo Alto, Ca. A nylon DNA blot containing 8.0 μ g each of human placental genomic DNA or Balb/c mouse genomic DNA cut with 5 different restriction enzymes (Eco RI, Hind III, Bam HI, Pst I and Bgl II) was also purchased from Clonetech.

α - and γ -³²P-dATP and α -³²P-dCTP were purchased from New England Nuclear, Boston Mass. An oligonucleotide-primed extension labeling kit was purchased from Boehringer Mannheim. Bovine serum and fetal bovine serum were purchased from Gibco Laboratories. F12 nutrient mixture and DMEM were purchased from Gibco Laboratories, Grand Island, N.Y. Insulin and transferrin were purchased from Sigma Chemical Company, St Louis, Mo. A synthetic degenerative oligonucleotide probe complementary to codons 5 through 12 of the base sequence of human S-protein was prepared on an Applied Biosystems

synthesizer and purified at the OSU Gene Research Program.

Isolation of human S-protein cDNA clone from a gt11 cDNA library of HepG2 mRNA

A human hepatoma cDNA library was screened for human S-protein sequences by in situ hybridization (Davis et al., 1986) using a degenerative oligonucleotide probe derived from the amino-terminal sequence of human S-protein (Jenne and Stanley, 1985; Suzuki et al., 1985; Barnes et al., 1983c). The lambda gt11 vectors contained greater than 1 million inserts ranging in size from 600 to 4000 bases. Approximately ten thousand plaques were plated and screened with radiolabeled probe. The oligonucleotide probe was end-labeled using T4 polynucleotide kinase in the presence of γ -³²P dATP (specific activity > 10⁶ cpm/ μ g).

Hybridization was conducted at 55°C for 16 hours in the presence of 2 X SSC, 0.2% polyvinyl pyrrolidone (PVP), 10 X Denhardt's solution, sonicated salmon sperm DNA (150 μ g/ml), yeast RNA (1.7 mg/ml) and 10⁶ cpm/ml radiolabeled probe. Blots were washed after hybridization with 2 X SSC at 42°C and twice at 35°C for 30 minutes each (Davis et al., 1986). After the tertiary screening of positive hybridizing colonies, single plaque-purified representations of human S-protein cDNA-lambda gt11 clones were obtained.

Subcloning of human S-protein cDNA and the 5'-end 564-base fragment of human S-protein into pUC18

The human S-protein cDNA clone was subcloned into the Eco RI site in the polylinker region of pUC18 (Davis et al., 1986). The 5'-end 564-base fragment of human S-protein cDNA was subcloned into the Eco RI and Kpn I sites in the polylinker region of pUC18. Since digesting the Kpn I generates three fragments that are of similar size, the human S-protein cDNA was first digested with Cla I, the 5'-end fragment was then isolated and digested with Kpn I.

Competent DH5 α cells (lacZ -) were transfected with the human S-protein-pUC18 clone (HSP-pUC18) and the 5'-end of human S-protein-pUC18 (HSP564-pUC18). Successful ligation of insert into pUC18 disrupted the plasmid's ability to cleave β -galactosidic bonds, which resulted in loss of blue colony formation on X-gal agar plates (Davis et al., 1986).

Isolation of cytoplasmic RNA and preparation of RNA Blots

Cytoplasmic RNA was isolated from cell lines as described (Rosenthal et al., 1986). Cells were grown in the presence of 10% calf serum or 10% fetal bovine serum. Isolated RNA samples were analyzed by electrophoresis through a 1.2% formaldehyde/agarose gel. 2.5 μ g of poly A-selected mRNA or 20 μ g total RNA were run on a 1.2% formaldehyde/agarose gel, and transferred to nitrocellulose by standard methods (Southern, 1975).

DNA and RNA blot hybridization

Hybridization of DNA and RNA blots were performed by standard methods (Southern, 1975). Nylon blots containing genomic DNA were prehybridized for 4 hours in the presence of 6 X SSC, 10 X Denhardt's solution, 100 $\mu\text{g/ml}$ sonicated salmon sperm DNA and 0.5% SDS at 65°C. Nitrocellulose blots containing poly A-selected mRNA or total RNA were prehybridized for 4 hours in the presence of 50.0% deionized formamide, 50 mM sodium phosphate (pH 6.5), 0.8 M NaCl, 1.0 mM EDTA, 0.1% SDS, 10 X Denhardt's solution, 250 $\mu\text{g/ml}$ sonicated salmon sperm DNA and 500 $\mu\text{g/ml}$ yeast RNA at 57°C. The cDNA inserts of HSP-pUC18 and HSP564-pUC18 were radiolabeled (specific activities $> 10^6$ cpm/ μg) by oligonucleotide-primed extension in the presence of α - ^{32}P -dATP and α - ^{32}P -dCTP, and were purified on a Sephadex G50 column.

The DNA blots were hybridized for 16-20 hours at 65°C with 1.5×10^6 cpm/ml radiolabeled HSP-pUC18 cDNA insert and fresh hybridization buffer. The RNA blots were hybridized for 16-20 hours at 57°C with 2.0×10^6 cpm/ml radiolabeled HSP564-pUC18 cDNA insert and fresh hybridization buffer. DNA blots were washed at 65°C with 2 X SSC for 15 minutes, and then washed with 2 X SSC and 0.1% SDS for 30 minutes. Stringent washes were then done at 65°C with either 0.5 X SSC and 0.1% SDS, 0.25 X SSC and 0.1% SDS or 0.1 X SSC and 0.1% SDS as indicated in the figure legends.

RNA blots were washed twice at room temperature with 1 X

SSC, 0.1% SDS for 3 minutes, and then washed twice at 65°C with either 0.5 X SSC, 0.1% SDS or 0.1 X SSC, 0.1% SDS. The blots were wrapped in plastic and exposed to film overnight or longer at -80°C in an X-ray film cassette containing an intensifying screen.

RESULTS

Southern blot analysis

A human HepG2 cDNA library was screened with a radiolabeled oligonucleotide probe containing the nucleotide sequence derived from the amino-terminal portion of human S-protein (Jenne and Stanley, 1985; Hayman et al., 1983; Barnes et al., 1983c). Three full length human S-protein cDNA-lambda clones were purified separately after the tertiary screening. The cDNA insert of one of these clones was subcloned into the Eco RI site of pUC18 (HSP-pUC18), and the 5'-end 564 fragment of the human S-protein cDNA was subcloned into the Eco RI and Kpn I site of pUC18.

Figure 2.1 shows the ethidium bromide-stained restriction fragments from HSP-pUC18 after digesting with Eco RI alone or in combination with either Kpn I, Cla I or Acc I. The restriction fragment sizes are identical to those predicted from the reported sequence of human S-protein cDNA (Jenne and Stanley, 1985). Digesting with Eco RI alone generated the 2.7 Kbp pUC18 vector and the 1.54 Kbp human S-protein cDNA insert. Digesting with Eco RI and Kpn I produced the 2.7 Kbp plasmid and 3 fragments of approximately 410 bp, 560 bp and 570 bp. Digesting with Eco RI and Cla I produced the 2.7 Kbp plasmid and two fragments of approximately 600 and 940 bp. Digesting with Eco RI and Acc I produced the 2.7 Kbp plasmid and 2

fragments of 740 bp and 800 bp. Digesting with Eco RI and either Hind III, Sma I, Pst I or Bam HI produced the 2.7 Kbp plasmid and the full 1.54 Kbp human S-protein cDNA insert (not shown).

Southern hybridization of radiolabeled probe with human placental genomic DNA identified single bands that hybridized under stringent conditions in each of the lanes containing DNA digested with 5 different restriction enzymes (Fig 2.2a). The following bands were detected: Eco RI-digested DNA, 20-21 Kbp band; Hind III-digested DNA, 17-18 Kbp band; Bam HI-digested DNA, 9.0-9.5 Kbp band; Pst I-digested DNA, 2.3-2.5 Kbp band; Bgl II-digested DNA, 13-14 Kbp band. The sizes of the bands produced from digesting with Eco RI, Hind III, Pst I and Bam HI are the same as those previously reported for human genomic DNA from peripheral blood lymphocytes (Jenne and Stanley, 1987).

Southern blot analyses of mouse genomic DNA digested with Eco RI, Hind II or Bam HI identified single bands of sizes 7.0-7.5 Kbp, 5.5-6.0 Kbp and 3.5-4.0 Kbp respectively (Fig. 2.2b). Since digesting with each of the three restriction endonucleases yielded a single band no greater than 7.5 Kbp in size, it is likely that mouse S-protein is a single copy gene. Jenne and Stanley have previously made a similar conclusion based on similar analysis (Jenne and Stanley, 1987).

A DNA blot containing the Eco RI-digested genomic DNA of human, monkey, rat, mouse, dog, cow, rabbit, chicken and yeast

was hybridized with radiolabeled human S-protein cDNA (Fig. 2.3). After washing at low stringency (2 X SSC, 0.1% SDS) and exposing the blot to film overnight, single bands were detected in DNA from rat (7.0-7.5 Kbp), mouse (5.5-6.0 Kbp), dog (18-19 Kbp), cow (5.5-6.0 Kbp) and rabbit (9.5-10 Kbp). These bands were still present after washing at high stringency (0.25 X SSC, 0.1% SDS) (Fig 2.3). At low stringency, high background hybridization was observed in human and monkey Eco RI-digested DNA. At high stringency (0.1 X SSC, 0.1% SDS) single bands in lanes containing the monkey (5.5-6.0 Kbp) and human (20-21 Kbp) DNA were detected.

Northern blot analysis

Northern blots analyzing total RNA or mRNA of human and mouse tissues and cultured cells were hybridized with radiolabeled HSP564 cDNA. A single 1.7 Kb S-protein mRNA was detected in human liver mRNA after an overnight exposure (Fig. 2.4); no S-protein mRNA was detected in human lung, placental and leukocyte mRNA after a two week exposure. A 1.7 Kb mRNA for S-protein in human liver has previously been reported (Jenne and Stanley, 1987). A single 1.6 Kb S-protein mRNA was detected in mouse liver mRNA after an overnight exposure and a faint S-protein mRNA band was detected in mouse brain after 5 days exposure (Fig 2.5). No S-protein mRNA was detected in RNA from mouse skeletal muscle, heart, kidney and testis mRNA after several weeks exposure.

Human S-protein mRNA was detected in RNA from the human hepatoma cell-line PLC/PRF5 after an overnight exposure (Fig. 2.6a). No human S-protein mRNA was detected in total RNA from cultured RD rhabdomyosarcoma, foreskin or IMR9 fibroblasts after 6 days. After the final exposures, the nitrocellulose blots were stripped for 30 minutes at 80°C in a 1.0% glycerol solution and reprobed with β -actin to examine the integrity of the mRNA. In all cases, there was little or no degradation detected in the samples. Figure 2.6b shows the autoradiographs of human foreskin, PLC/PRF5, RD and IMR9 total RNA probed with β -actin after two days exposure.

DISCUSSION

Human S-protein is a multidomain serum glycoprotein with diverse functions. The first 44 amino acids of its amino-terminus contains the entire somatomedin B peptide (Jenne and Stanley, 1985; Suzuki et al., 1985; Barnes et al., 1983c); directly following is the cell-binding site that permits cell attachment and spreading (Hayman et al., 1985). The carboxy-half of S-protein contains complement-binding and heparin-binding domains (Jenne and Stanley, 1985). The human S-protein gene contains eight exons and 7 introns in an arrangement and sequence related to hemopexin (Jenne and Stanley, 1987).

In this paper we report that the gene for S-protein is present in other mammals, including monkey, rat, mouse, dog, cow and rabbit. The S-protein gene was not detected in chicken or yeast genomic DNA, but this does not exclude the possibility that the analogous protein shares homology with mammalian S-protein in some conserved regions. Immunological cross-reactivity between human and chicken hemopexins has been observed (Chen et al., 1988).

Human S-protein is synthesized by the liver and is found in serum at a concentration of 100-400 μ g/ml (Suzuki et al., 1985; Jenne and Stanley, 1987; Barnes et al., 1985; Shaffer et al., 1984). Hayman et al. (1983) reported that S-protein

could be detected in cultured fibroblasts as well as in muscle, kidney, lung and placental tissue. However, our Northern hybridization analyses suggest that cultured fibroblasts do not produce mRNA for human S-protein at a level detectable after several days exposure. Additionally, no message was detected in RNA from muscle, kidney or placenta. These results suggest that matrix-bound S-protein identified by immunological or other methods may derive from the circulation in vivo or from serum in vitro, possibly by interaction of S-protein with matrix components through binding sites on the molecule for other matrix proteins or through interaction with plasminogen activator inhibitor, which is secreted by many cell types. Our results are in agreement with our previously reported failure to detect S-protein in cultured fibroblasts, kidney or muscle-derived cells (Barnes et al., 1985).

Both human and mouse liver produce S-protein mRNA detectable after an overnight exposure; a low level of S-protein mRNA also was detected in mouse brain mRNA. The cell type that produces S-protein mRNA in mouse brain is unknown. We could detect no immunological reactivity to spreading factor in a human neuroblastoma line (Barnes et al., 1985). Fibronectin and possibly other adhesion proteins have been detected in cultured astrocytes (Price et al., 1985), and glial cells may be the source of brain S-protein. However, we were unable to detect S-protein mRNA in one astrocyte cell

line tested. Further work will be necessary to determine the cell type synthesizing S-protein in the brain.

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ADDITIONAL COMMENTS

Although we detected S-protein in mouse brain mRNA, we were unable to detect the message in SFME proastroblasts. Recently, it has been demonstrated that TGF β increases the S-protein mRNA in HepG2 cells. TGF β did not induce S-protein in SFME that we could detect by Northern analysis. The distribution and regulation of S-protein in the brain remains to be determined. Recently, S-protein and its receptor was detected at high levels in the brains of Alzheimer's disease victims. It is suggested that this protein may play a role in inflammatory responses associated with the disease. S-protein and its receptor have been recently detected in the astroglial-derived tumor, glioblastoma multiforme, which may potentiate glioblastoma cell invasion of normal brain (Gladson and Cheresch, 1991). The mouse S-protein cDNA has recently been isolated and used as a probe for S-protein in the mRNA of fourteen different mouse tissues, including liver, lung and brain (Seiffert et al., 1991). S-protein mRNA was only detected in the liver. Based on a dose response analysis, the authors reported that the other tissues examined contain at least 40-fold less S-protein than the liver. Hayman et al. (1983) reported the detection of vitronectin (S-protein) by immunofluorescence in the human diploid fibroblast cell line, IMR90. However, we could not detect the mRNA for S-protein in

the IMR90 cell line. Barnes and Reing (1985) also could not detect the protein in IMR90 cells using enzyme-linked immunoassay techniques. Enzyme-linked immunoassay is a very sensitive method for detecting a protein. Since each enzyme molecule acts catalytically to generate many thousands of molecules of product, this technique allow even tiny amounts of antigen to be detected. I have examined the sensitivity of Northern blot experiments in our laboratory using 3^2P -radiolabeled probe (specific activity $> 10^8$ cpm/ μg), and I estimate that I should be able to detect femtogram amounts of a single mRNA type after several days exposure. I believe the sensitivity of Northern blot methods is comparable to immunofluorescent detection of the corresponding protein. Using monoclonal antibodies directed towards human S-protein, Hayman et al. (1983) could not detect the bovine S-protein present in the serum used to culture the IMR90 cell line. However, they detected what appeared to be a fibrillar-like pattern of S-protein associated with the cell surface of IMR90 cells and surrounding extracellular matrix. It may be that bovine S-protein's interaction with the extracellular matrix may change it's conformation such that the monoclonal antibodies directed to human S-protein can recognize this bovine serum-derived protein. The authors did not report whether other cell line, such as epithelial-derived cell lines, were negative as controls. We can detect the gene for S-protein in cow using human S-protein cDNA as probe, and

there should be conserved regions in certain functional areas of both proteins, like the cell attachment site, that a monoclonal antibody could recognize. If, in fact, the monoclonal antibodies used by Hayman et al. (1983) were not detecting S-protein derived from the serum, it may be that the particular batch of serum used in their experiments contained a unique factor not present in our system that was recognized by the monoclonal antibodies, or was responsible for the expression of S-protein in the fibroblast cells that they examined.

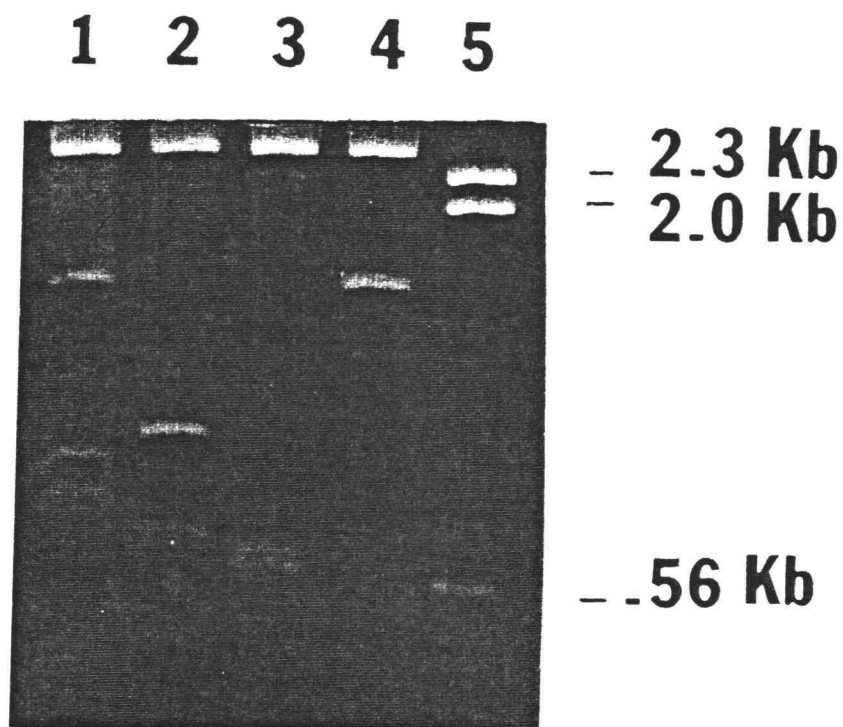
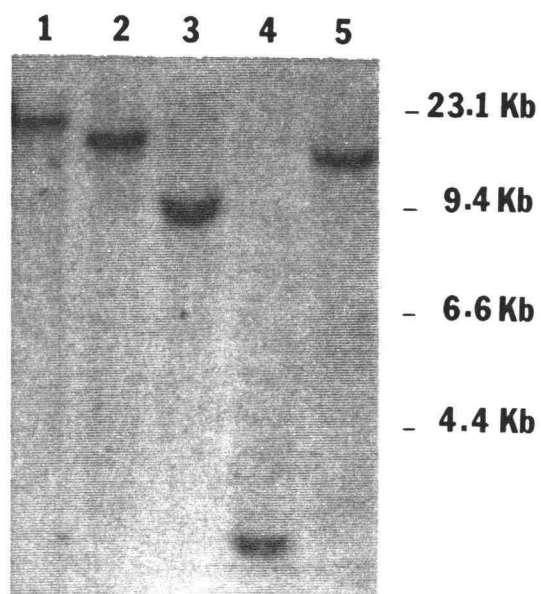
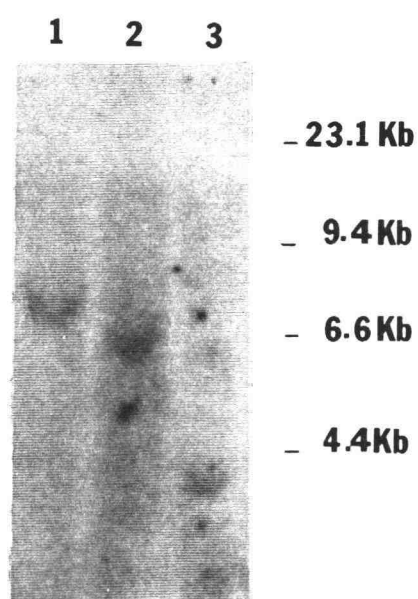


Fig. 2.1: Characterization of human S-protein cDNA by restriction enzyme digestion analysis. Human S-protein cDNA-pUC18 fractionated on an 0.8% agarose gel and stained with ethidium bromide after digesting with 4 different restriction enzymes. Lane 1, Eco RI and Acc I double digested HSP-pUC18; lane 2, Eco RI and Cla I double digested HSP-pUC18; lane 3, Eco RI and Kpn I double digested HSP-pUC18; lane 4, Eco RI digested HSP-pUC18; lane 5, Hind III-digested lambda DNA (size markers).

Fig. 2.2: Southern blot analysis of human and mouse genomic DNA probed for the S-protein gene. (A) An autoradiograph of a Southern blot containing human placental genomic DNA digested with the indicated enzymes. The blot was hybridized with radiolabeled human S-protein cDNA (HSP-pUC18 cDNA) for 2 hours and then washed under stringent conditions (0.1 X SSC, 0.1% SDS, 65°C). Lanes 1-5 contain Eco RI, Hind III, Bam HI, Pst I and Bgl II-digested genomic DNA, respectively. (B) An autoradiograph of Balb/c mouse genomic DNA probed with radiolabeled human S-protein cDNA and washed under stringent conditions (0.5 X SSC, 0.1% SDS, 65°C). Lanes 1, 2 and 3 contain Eco RI, Hind III and Bam HI-digested genomic DNA respectively. The positions of lambda DNA (Hind III-digested) molecular weight markers are shown.

A**B**

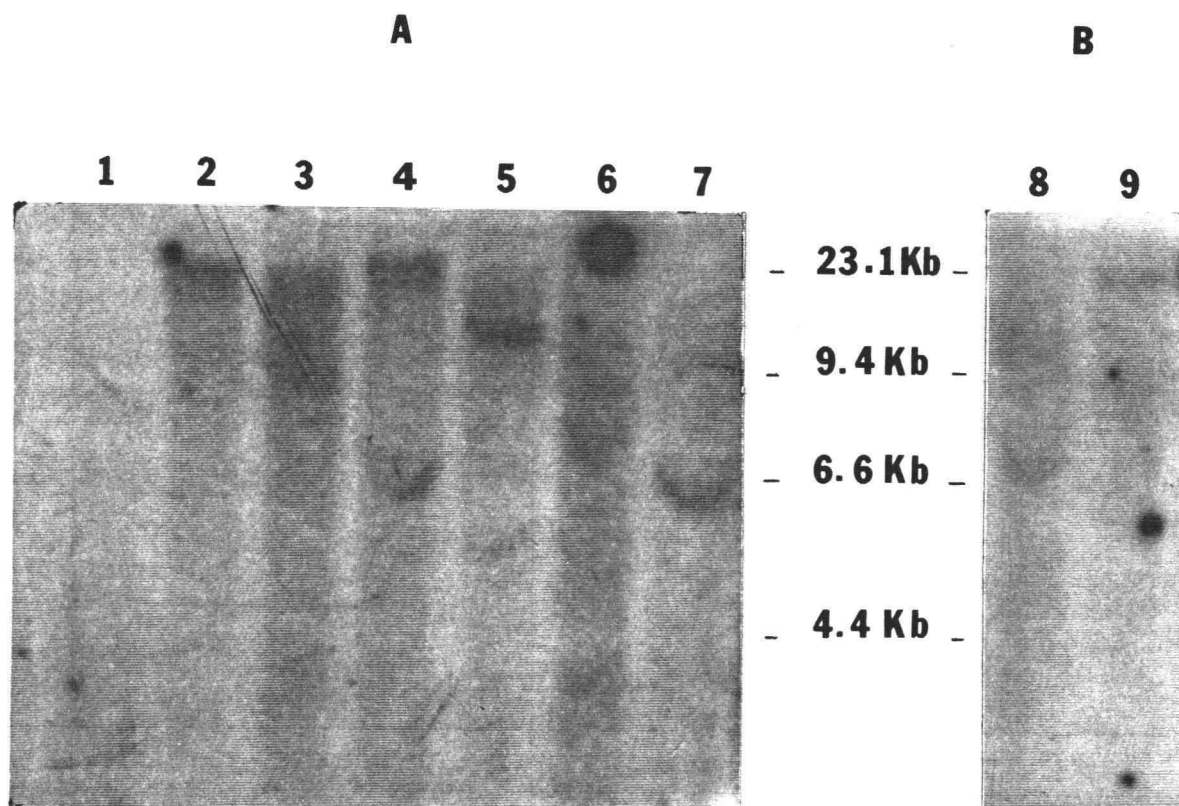


Fig. 2.3: The gene for S-protein detected in genomic DNA of seven mammals. Radiolabeled human S-protein cDNA (HSP-pUC18 cDNA) was used to probe the genomic DNA of yeast (1), chicken (2), rabbit (3), cow (4), dog (5), rat (6), mouse (7), monkey (8) and human (9) digested with Eco RI and blotted onto nylon. The hybridized blot was washed with 0.25 X SSC, 0.1% SDS at 65°C.

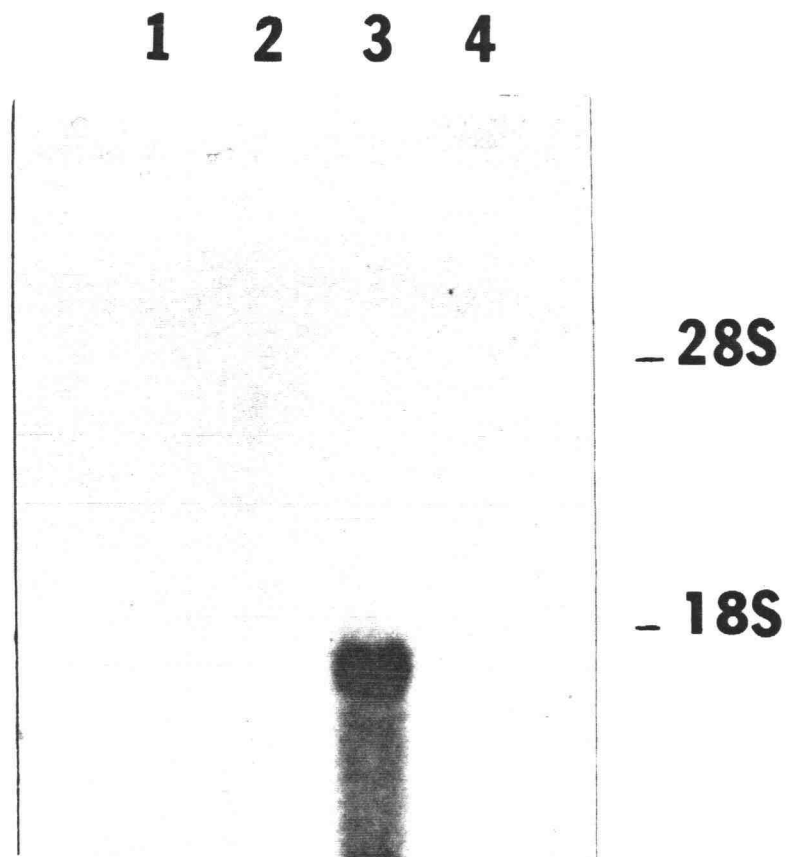


Fig. 2.4: Northern blot analysis of the mRNA from several human tissues probed for human S-protein. Autoradiograph of a Northern blot containing 2.5 μ g human leukocyte (1), placental (2), liver (3) and lung (4) poly A mRNA probed with radiolabeled human S-protein (5'-end 564-base fragment) cDNA. After hybridization, the blot was washed with 0.5 X SSC, 0.1% SDS at 65°C and exposed to film. After an overnight exposure, a single band of 1.7 Kb was detected in the human liver mRNA lane. 28S ribosomal RNA is 5.1 Kb and 18S ribosomal RNA is 2.0 Kb.

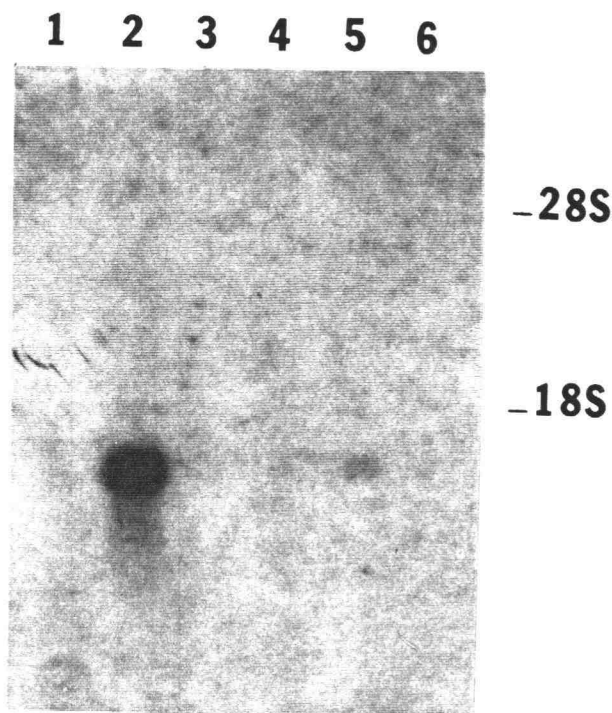


Fig. 2.5: Northern blot analysis of the mRNA from several mouse tissues probed for S-protein. Autoradiograph of a Northern blot containing 2.5 μ g mouse testis (1), liver (2), kidney (3), heart (4), brain (5) and skeletal muscle (6) poly A mRNA after probing with radiolabeled human S-protein (5'-end 564-base fragment). After hybridization, blots were washed at high stringency (0.5 X SSC, 0.1% SDS at 65°C) and then exposed to film. The autoradiograph was developed after exposure for 14 days. A single band of 1.6 Kb was detected in the mouse liver (strong band) and mouse brain (faint band) mRNA.

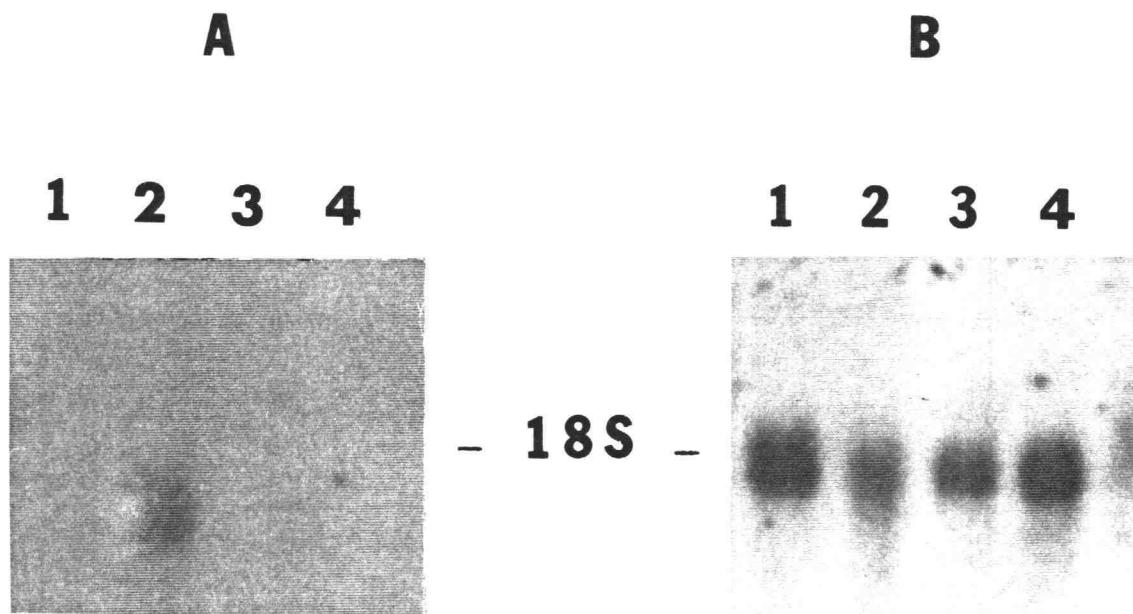


Fig. 2.6: Northern blot analysis of the mRNA from several human cell-lines probed for human S-protein and β -actin. Autoradiograph of a Northern blot containing 2 μ g total RNA from human foreskin (1), PLC/PRF5 (2), RD (3) and IMR9 (4) cell lines probed with radiolabeled human (5'-end 564-base fragment) (A) and β -actin (B). After hybridization, blots were washed at high stringency (0.1 X SSC, 0.1% SDS at 65°C) and then exposed to film. After an overnight exposure, a single band of 1.7 Kb for human S-protein mRNA was detected.

CHAPTER III

Transforming Growth Factor β Regulates Cystatin C in Serum-Free Mouse Embryo (SFME) Cells

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SUMMARY

Differential screening of cDNA library derived from mRNA of TGF β -treated serum-free mouse embryo (astrocyte precursor) cells isolated a strongly TGF β -regulated mRNA that codes for cystatin C, a cysteine protease inhibitor. Increase in cystatin C mRNA level was observed within four hours after treatment with picomolar concentrations of TGF β . The increase was reversible upon removal of TGF β and was not prevented by cycloheximide. These results suggest that cystatin C expression may represent a developmentally regulated differentiated function of astrocytes, and also suggest that cystatin C expression may be involved in the response of brain cells to platelet release of TGF β after trauma or injury.

INTRODUCTION

The Balb/c serum-free mouse embryo (SFME) cell line is cultured in basal nutrient medium supplemented with insulin, transferrin, epidermal growth factor, high density lipoprotein and fibronectin (Loo et al., 1987; Loo et al., 1989a; Loo et al., 1989b; Loo et al., 1990; Shirahata et al., 1990; Rawson et al., 1990; Sakai et al., 1990). Unlike conventional mouse embryo cell lines derived in serum-containing medium, these cells do not exhibit growth crisis or gross chromosomal aberrations (Loo et al., 1987; Loo et al., 1989). Proliferation of SFME cells is reversibly inhibited by serum (Loo et al., 1987; Loo et al., 1990; Shirahata et al., 1990; sakai et al., 1990), and treatment of SFME cells with serum or transforming growth factor β (TGF β) leads to the appearance of glial fibrillary acidic protein, a specific marker for astrocytes (Sakai et al., 1990; De Vellis et al., 1986). TGF β is a platelet-derived serum peptide growth factor that affects proliferation and differentiation of a variety of cell types (Sporn and Roberts, 1988; Rizzino, 1988).

We constructed a cDNA library from mRNA isolated from SFME cells after treatment with TGF β and selected by differential screening clones representing TGF β -regulated mRNA. A clone markedly regulated by TGF β in SFME cultures

was identified as cystatin C, a member of a family of cysteine protease inhibitors that also includes the prohormone kininogens (Barrett, 1987; Rawlings and Barrett, 1990). Cystatin C is a major central nervous system-derived component of cerebrospinal fluid and is the predominant constituent of amyloid fibrils in brains of patients with a form of hereditary cerebral hemorrhage (Ghiso et al., 1986; Cole et al., 1989; Maruyama et al., 1990). The protein also modulates leukocyte chemotaxis and phagocytosis-associated respiratory burst and has been suggested to be a regulator in inflammatory processes (Leung-Tack et al., 1990a; Leung-Tack et al., 1990b).

METHODS

Procedures for culture of Balb/c SFME cells have been described in detail in Loo et al. (1987), Loo et al. (1989a), Loo et al. (1989b). RNA isolation was done as follows. Cells growing in plastic dishes were washed twice with ice-cold PBS. They were then gently scraped off the dish (in 1-2 ml of PBS) with a rubber policeman, and pelleted. Cells were suspended in 140 mM NaCl/1.5 mM MgCl₂/10 mM Tris HCl, pH 7.6/200 µg/ml heparin (Sigma Chemical Company, MO) and lysed by adding 0.5% Nonidet P-40 (Sigma Chemical Company, MO). After brief mixing the lysate was centrifuged at 400 x g for 1 min. 10% SDS and 10 mM EDTA was added to the supernatant followed by extraction twice each with phenol, phenol/chloroform (1:1), and chloroform/isoamyl alcohol (24:1). The RNA was precipitated by adding 2.5 vol of 95% ethanol and 0.3 M sodium acetate (pH 6.0). Residual DNA was removed with RNase-free DNase I in the presence of vanadyl riboside complex (both from Bethesda Research Laboratories, MD).

Preparation of a TGF β-inducible cDNA library

A cDNA library in bacteriophage lambda gt11 was prepared using a cDNA synthesis kit (Pharmacia). The library was prepared from mRNA of SFME grown in serum-free F12/DMEM medium containing HDL (10 µg/ml), insulin (10 µg/ml), transferrin (25

$\mu\text{g/ml}$) and EGF (50 ng/ml) in dishes precoated with bovine fibronectin (10 $\mu\text{g/ml}$) supplemented additionally with 10 ng/ml TGF β for 48 hours. The mRNA was purified from approximately 1.0 mg of total RNA using an oligo-dT column (BRL). 3.0 μg of this mRNA was used in the synthesis of cDNA in the presence of α - ^{32}P -dCTP. First-strand cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase and oligo d(T)12-18 as primer. Second-strand synthesis involved a modification of the procedure of Gubler and Hoffman (1983). A portion of this cDNA was run on an agarose gel along with molecular weight markers and exposed to film; the major portion of the cDNA ranged between 0.5 to 2.0 Kbp, although some higher molecular weight cDNA up to 5.0 Kbp was observed. The cDNA was linked to EcoRI adaptors containing internal Not I sites, and a portion was ligated to gt11 lambda arms (Promega) overnight. The library was packaged into Promega Packagene. The titer of the library was determined by transfecting an overnight culture of Y1090 bacteria grown in the presence of 10 mM MgSO_4 and 0.2% maltose with several different amounts of packaged library. The library was found to contain over 1 million independent clones and approximately 70% of the individual plaques contained cDNA. The library was plated on LB-agarose 150 cm plates to approximately 500 plaques per plate using the bacteria host Y1090. Duplicate nitrocellulose filter lifts were taken from each plate, and the cDNA was denatured in 0.2 M NaOH, 1.5 M NaCl for 5 min.,

neutralized for 5 min. in 0.4 M Tris HCl (pH 7.4), 2 X SSC and washed for 5 min. in 2 X SSC. The filters were dried for one hour and then baked at 80°C for an additional 2 hours. Radiolabeled single-stranded cDNA probes were synthesized using as template 1.0 µg mRNA from SFME cells cultured in serum-free medium with or without 10 ng/ml TGF β for 48 hours. The reaction included 100 mM Tris HCl (pH 8.6), 125 mM KCl, 10 mM MgCl₂, 2.5 mM DTT, 1 mM dATP, dGTP, dTTP, 1 µg oligo(dT), 1 unit RNasin and 100 µCi α-³²P-dCTP (3000 Ci/mmol). The mixture was incubated at 42°C for 2 hours, the mRNA was hydrolyzed with 25 µl 0.15 M NaOH at 65°C for 1 hour, and the radiolabeled cDNA was purified on a Sephadex G50 column. Specific activities ranged between 1 to 3 x 10⁸ cpm/ug mRNA for both probes. Differential screening of duplicate nitrocellulose filters was performed using 2-3 x 10⁷ cpm of the radiolabeled probes in hybridization buffer containing 10% dextran sulfate, 40% formamide, 4 X SSC, 10 X Denhardt's solution, 0.2 M Tris HCl (pH 7.4), 1.0 µg/ml poly (dA) (Pharmacia) and 200 µg/ml sonicated salmon sperm DNA. Filters were first prehybridized in hybridization buffer for 4 hours, and then hybridized at 42° C for 3 days. Plaques that were detected by the TGF β-induced cDNA probe and not by the serum-free cDNA probe were selected and twice rescreened.

Subcloning of Mouse Cystatin C

One of 14 positive tertiary screened differential plaques was

used to transfect an overnight culture of Y1090 bacteria. Bacteriophage DNA was purified from the lysed plates using LambdaSorb Phage Absorbent provided by Promega. The cDNA insert was subcloned into the EcoRI site of the polylinker region of pUC18 and competent DH5 α (Clonetech) cells were transfected with a portion of the ligation mixture. Successful insert ligation into pUC18 resulted in the loss of blue colony formation on X-gal agar plates with the appearance of larger, white colonies. Single white colonies were selected and grown in an overnight culture in LB broth (ampicillin, 100 μ g/ml). Plasmid DNA was purified by alkali hydrolysis of the bacteria and subsequent CsCl gradient centrifugation. The cDNA insert was determined to be approximately 660 bp by agarose gel electrophoresis analysis.

Sequencing of Mouse Cystatin C

The CsCl-purified plasmid preparation was used as a template for sequencing the cDNA insert in both the forward and reverse directions using the Sequenase method of the United States Biochemical Corporation for dideoxy-double stranded DNA sequencing. The reactions were conducted in the presence of α -³⁵S-dCTP (800 Ci/mmol), and were run on a 6.0 % polyacrylamide gel. The gel was fixed, baked and exposed to film for 18-72 hours at -88°C using an intensifying screen cassette. Sequences in the middle portion of the cDNA were determined by preparing an exonuclease-digested clone that had

lost the initial 150 bases using the Erase-a-Base system of Promega. Also, a confirmation of the full sequence was provided by the Center for Gene Research at the Oregon State University using an automated laser sequencer. Cystatin C was quantified as described (Lah et al., 1989), using as standard chicken egg white cystatin (Sigma), (see Appendix for details).

RESULTS

Differential plaque screening of approximately 10^4 cDNA clones of mRNA from SFME cells cultures for 48 hours in serum-free medium with TGF β identified 14 bacteriophage clones that hybridized more strongly through tertiary screens to probe derived from TGF β treated cells than to probe derived from cells not exposed to TGF β (Fig. 3.1). The cDNA insert (660 bp) from one bacteriophage clone was recloned into pUC18. Radioactively labeled insert from this clone was used to screen the other positive clones, and all were found to cross-hybridize to this insert, suggesting that the mRNA represented by this clone was strongly regulated by TGF β .

Northern blots confirmed that the mRNA recognized by the clone was markedly increased in SFME cells after exposure to TGF β (Fig. 3.2a). Message size was approximately 0.8 Kb. Increased message level was detected after a 4 hour incubation of SFME cells with TGF β (Fig. 3.2a). The increase in message was reversible upon removal of TGF β from the culture medium. No increase in the level of this message was seen in an identical experiment using Balb/c 3T3 and 10T1/2 mouse cells (Fig. 3.3). TGF β at 1.0 ng/ml produced a near maximal response in SFME cells (Fig. 3.4), and exposure to nerve growth factor (10 ng/ml), platelet-derived growth factor (10

ng/ml) or fibroblast growth factor (10 ng/ml) for 24 hours did not lead to increase in the message levels (Fig 3.5).

Figure 3.6 represents a Northern analysis of SFME grown in the presence and absence of TGF β when protein synthesis is inhibited. Although cystatin C mRNA is still induced by TGF β in the presence of cycloheximide, GFAP induction is abolished. This may indicate differential regulation of the two genes by TGF β .

Determination of the nucleotide sequence of the cDNA clone of the TGF β -regulated mRNA identified 3' and 5' untranslated regions, translation start and stop codons, consensus initiation sequence, signal sequence and polyadenylation signal (Fig. 3.7). The cDNA sequence for the mouse cystatin C showed 88% homology to rat cystatin C and 73% homology to human cystatin C (Cole et al., 1989; Abrahamson et al., 1987). Assay of culture medium from TGF β -treated SFME cells confirmed that the growth factor regulated the appearance of the secreted protein (Fig. 3.8). Northern blot hybridization analysis of mRNA from various mouse tissues with the cDNA as probe confirmed high levels of a 0.8 Kb message expressed in brain, and also in heart and lung (Fig. 3.9). Differentiated astrocytes in primary culture recently have been reported to produce cystatin C (Zucker-Franklin et al., 1987), although no regulation was explored in this system.

DISCUSSION

In other cell culture systems TGF β suppresses synthesis of proteases and stimulates synthesis of inhibitors of serine or metalloprotease (Denhardt et al., 1986; Edwards et al., 1987; Lund et al., 1987; Kerr et al., 1988). Our work shows that in SFME cells TGF β can stimulate the appearance of a member of another class of protease inhibitors. TGF β also increases production of extracellular matrix in some cell types, and regulation of synthesis of proteases and inhibitors may stabilize the increased deposition of extracellular matrix components in the central nervous system (De Vellis et al., 1986), and part of the function of TGF β regulation of cystatin C in astrocyte precursors may be to assist in the protection of extracellular matrix.

Cystatin C also exerts effects on the immune response (Leung-Tack et al., 1990a; Leung-Tack et al., 1990b), and increased production of cystatin due to TGF β released by platelets in response to injury or trauma may additionally protect tissue in the area of a wound from cathepsin released by damaged cells. In addition, cystatin C and other protease inhibitors, as well as the cystatin C target protein cathepsin, have been implicated in the development of amyloid deposits in brain associated with Alzheimer disease or

cerebral hemorrhage (Ghiso et al., 1986; Cole et al., 1989; Maruyama et al., 1990; Meier et al., 1989; Cataldo et al., 1990; Abraham et al., 1988; Wagner et al., 1989). Data presented here regarding regulation of cystatin C, taken with this previous work, suggests a possible role for TGF β in the etiology of some amyloid-associated brain diseases.

ACKNOWLEDGEMENTS

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Differential Screening of a TGF β -inducible
cDNA Library

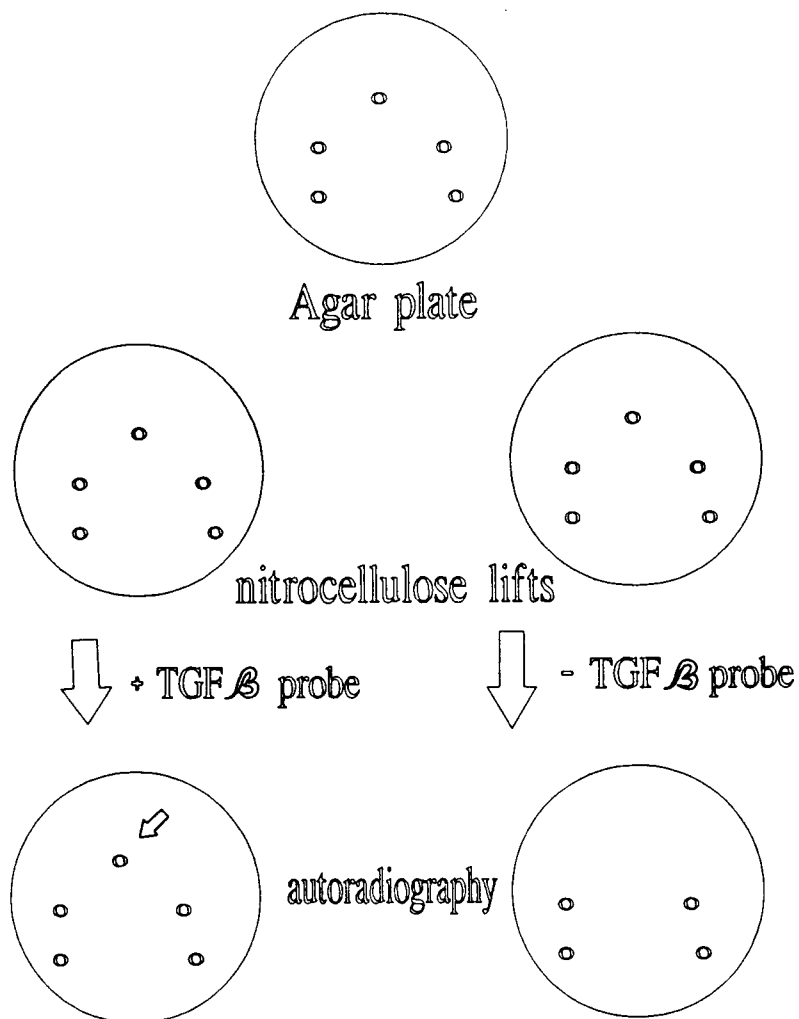


Fig. 3.1: Protocol for differential screening of a TGF β -inducible cDNA library

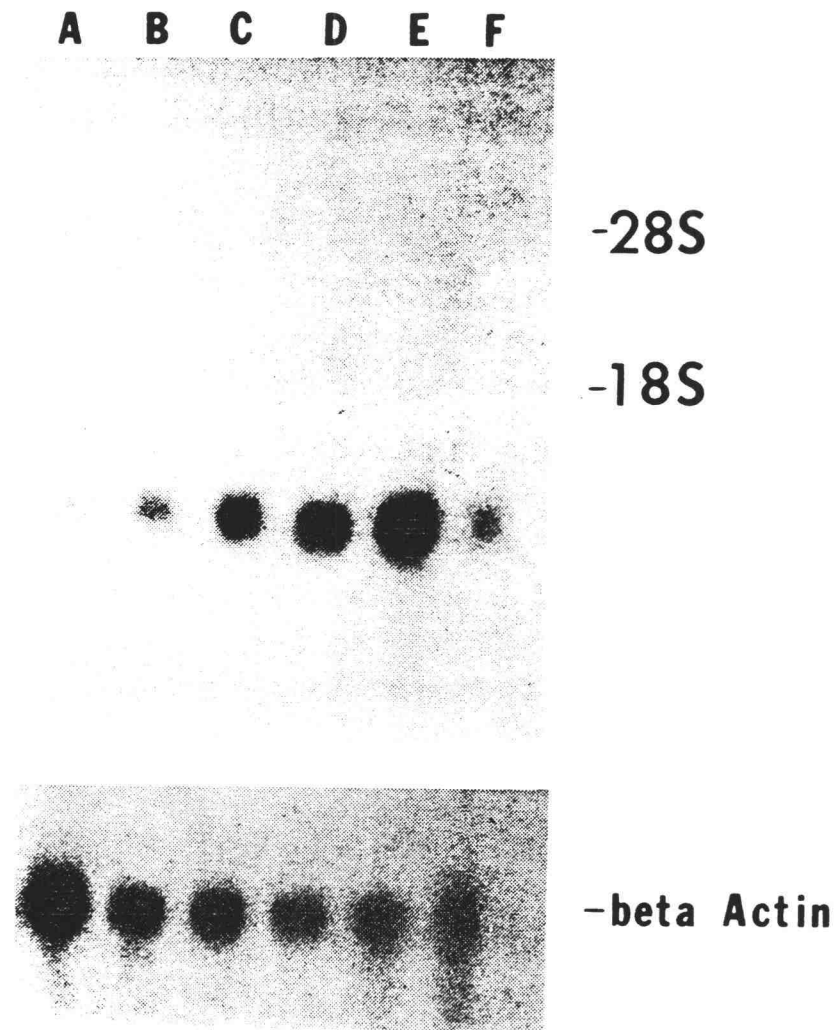


Fig. 3.2: Northern experiment examining the time course of cystatin C induction by TGF β and reprobbed with β -actin. SFME cells were treated with TGF β (10 ng/ml), 20 μ g cytoplasmic RNA was isolated and Northern hybridization blot analysis carried out using as probe a cDNA clone identified as representing a TGF β -regulated mRNA by differential screening of a library derived from TGF β -treated SFME mRNA. Top: (A), no TGF β , 48 hour incubation; (B), TGF β , 4 hours; (C), TGF β , 16 hours; (D) TGF β , 24 hours; (E) TGF β , 48 hours; (F), TGF β 24 hour, followed by 24 hour incubation without TGF β . Bottom: reprobbed with β -actin.

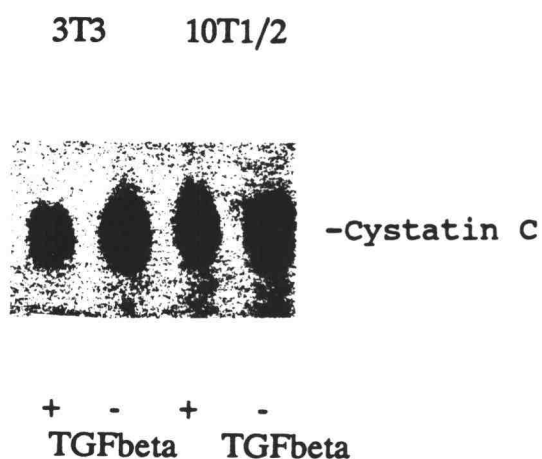


Fig. 3.3: NIH 3T3 and 10T1/2 cells are not induced by TGF β for induction of cystatin C mRNA. NIH 3T3 and 10T1/2 cells were grown in serum-free medium for 48 hours. TGF β (10 ng/ml) was added for an additional 48 hours to half of the cells.

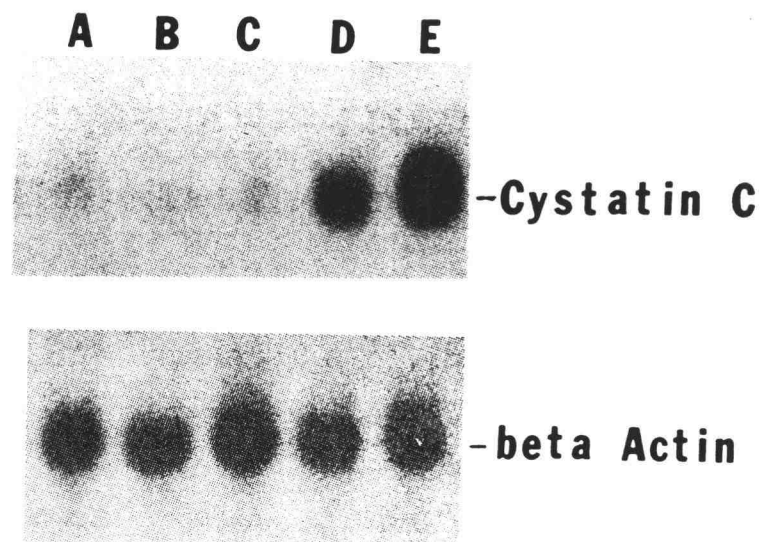


Fig. 3.4: Northern experiment examining the dose response for cystatin C induction by TGF β . SFME cells were treated for 24 hours with or without TGF β and 20 μ g cytoplasmic RNA analyzed as described in Fig. 8. Top: (A), no TGF β ; (B), 10 pg/ml TGF β ; 100 pg/ml TGF β ; (D), 1 ng/ml TGF β ; (E), 10 ng/ml TGF β . Bottom: reprobed with β -actin.

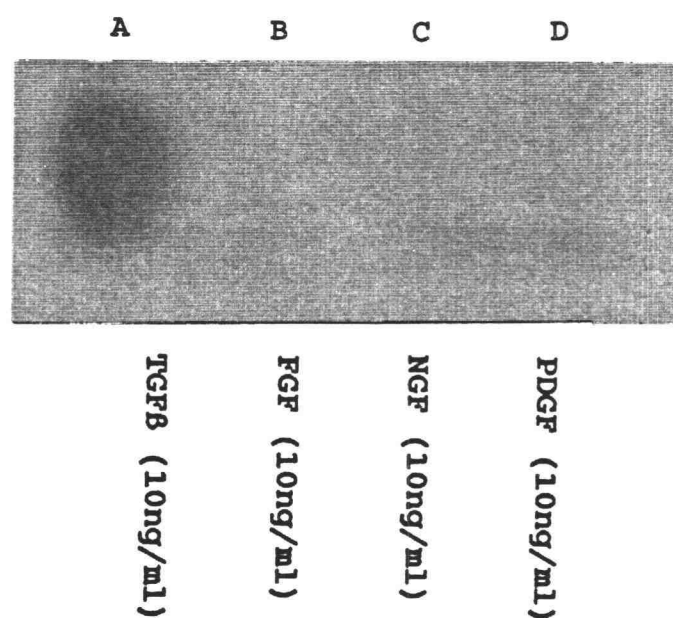


Fig. 3.5: Other growth factors do not induce cystatin C in SFME. Cells were grown serum-free in the presence of the following growth factors for 24 hours. (A), TGF β (10 ng/ml); (B), bFGF (10 ng/ml); (C), NGF (10 ng/ml); (D), PDGF (10 ng/ml).

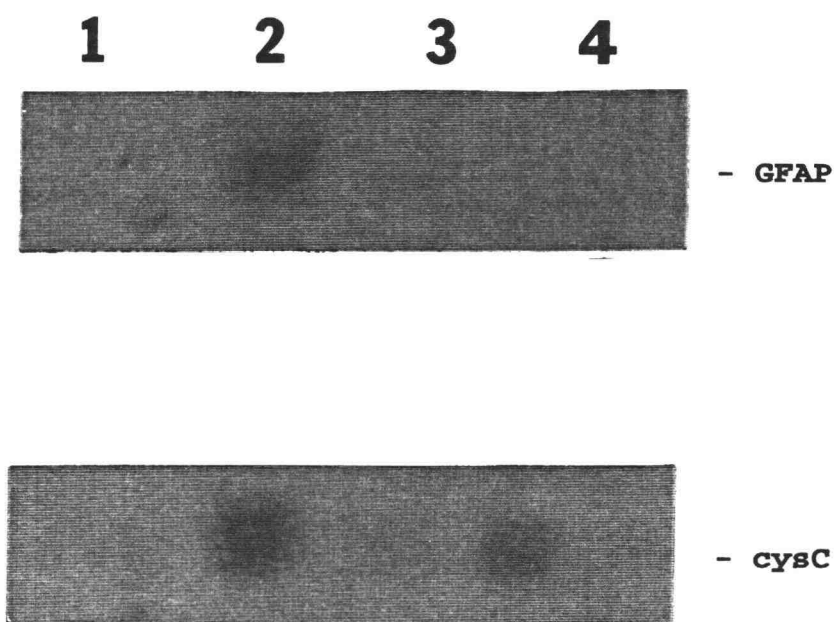


Fig. 3.6: Cystatin C mRNA induction by TGF β is not abolished by cycloheximide treatment. SFME cells were treated with TGF β (10 ng/ml) with or without cycloheximide (10 μ g/ml), and cytoplasmic RNA analyzed as described in Fig. 3.2. (1), no TGF β or cycloheximide; (2), TGF β , 8 hours; (3) cycloheximide, 8 hours; (4), cycloheximide and TGF β , 8 hours. Northern blot probed with α - 32 P-radiolabeled GFAP cDNA and cystatin C cDNA.

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TTTATCCCTTTGTCTAGCCAACC ATG GCC AGC CCG CTG CGC TCC TTG CTG TTC CTG CTG
Met Ala Ser Pro Leu Arg Ser Leu Leu Phe Leu Leu

GCC GTC CTG GGC GTG GCC TGG GCG GCG ACC CCA AAA CAA GGC CCG CGA ATG TTG
Ala Val Leu Gly Val Ala Trp Ala Ala Thr Pro Lys Gln Gly Pro Arg Met Leu
-1 +1
10

GGA GCC CCG GAG GAG GCA GAT GCC AAT GAG GAA GGC GTG CGG CGA GCG TTG GAC
Gly Ala Pro Glu Glu Ala Asp Ala Asn Glu Glu Gly Val Arg Arg Ala Leu Asp
20

TTC GCT GTG AGC GAG TAC AAC AAG GGC AGC AAC GAT GCG TAC CAC AGC CGC GCC
Phe Ala Val Ser Glu Tyr Asn Lys Gly Ser Asn Asp Ala Tyr His Ser Arg Ala
30 40

ATA CAG GTG GTG AGA GCT CGT AAG CAG CTC GTG GCT GGA GTG AAC TAT TTT TTT
Ile Gln Val Val Arg Ala Arg Lys Gln Leu Val Ala Gly Val Asn Tyr Phe Phe
50 60

GAT GTG GAG ATG GGC CGA ACT ACA TGT ACC AAG TCC CAG ACA AAT TTG ACT GAC
Asp Val Glu Met Gly Arg Thr Thr Cys Thr Lys Ser Gln Thr Asn Leu Thr Asp
70 80

TGT CCT TTC CAT GAC CAG CCC CAT CTG ATG AGG AAG GCA CTC TGC TCC TTC CAG
Cys Pro Phe His Asp Gln Pro His Leu Met Arg Lys Ala Leu Cys Ser Phe Gln
90 100

ATC TAC AGC GTG CCC TGG AAA GGC ACA CAC TCC CTG ACA AAA TTC AGC TGC AAA
Ile Tyr Ser Val Pro Trp Lys Gly Thr His Ser Leu Thr Lys Phe Ser Cys Lys
110

AAT GCC TAAGGGCTGAGTCTAGAAGGATCATGCAGATTGTTTCCTTACTTGTGCTCCTTCCCTATAGTGT
Asn Ala
120

TTCATCTCGCAGAAGGGTGCTCCGGCTCTGGAGGGCACCGCCAGTGTGTTTGCACCAGGAGACAGTAAAGGA
GCTGCTGCAGGCAGGTTCTGCACATCTGAACAGCTGTCCCTGGCTCCACTCTTCTTGCAGTACCTATCATG
CCTTGCTCAATTAAAAAGCGGCCGCGAA

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Fig. 3.7: The complete nucleotide sequence of mouse cystatin C cDNA and the deduced amino acid sequence. The putative signal peptide appears before the amino-terminal alanine, residue 1. The polyadenylation signal is underlined. A consensus initiation sequence (ACCATGG) appears at the translation start site.

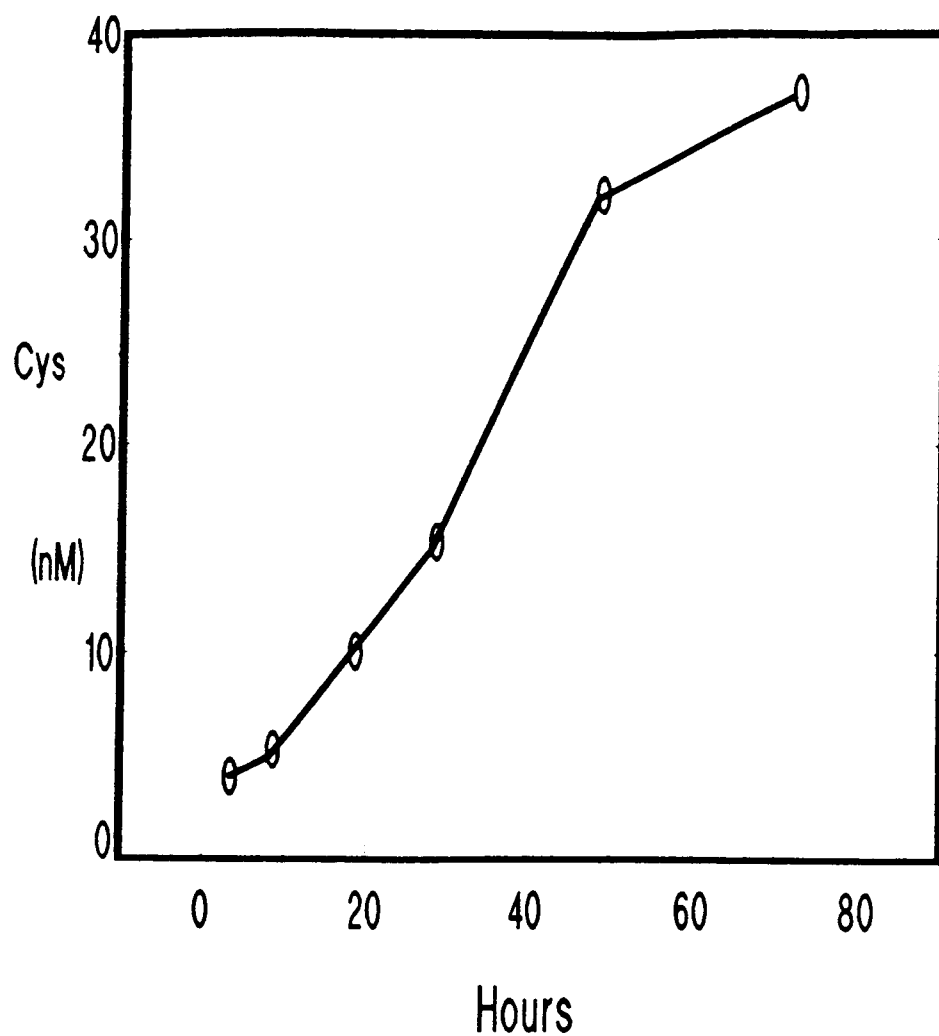


Fig. 3.8: Cystatin C in culture medium from TGF β -treated SFME cells. Medium from cells treated for the indicated times with TGF β (10 ng/ml) was assayed for cystatin C by a quantitative assay of inhibition of papain-mediated proteolysis, using as standard chicken egg white cystatin C.

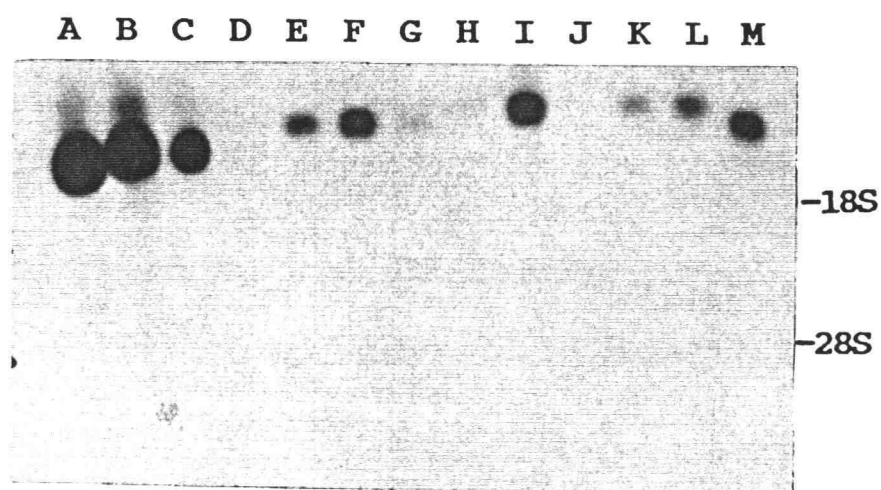


Fig. 3.9: Northern analysis of cystatin C expression in various mouse tissues. (A) 20 μ g total RNA of SFME with TGF β (48 hours, 2 ng/ml) (B) 20 μ g total RNA of SFME with 10 % calf serum, 24 h, (C) 5 μ g mRNA of SFME with 10% calf serum, 24 h. 2 μ g of poly A-containing RNA from (D) spleen, (E) testis, (F) spleen, (G) skeletal muscle, (H) pancreas, (I) lung, (J) liver, (K) kidney, (L) heart, (M) brain.

CHAPTER IV

Gene Regulation of Cystatin C

SUMMARY

Cystatin C is induced to high levels in a serum-free derived proastroblast cell-line known as SFME, in response to transforming growth factor β . We were interested in examining the regulation of the cystatin C gene with TGF β . By inhibiting RNA polymerase II transcription by α -amanitin, cystatin C mRNA decay was measured in the presence and absence of the growth factor. It appears that cystatin C mRNA is stabilized by TGF β compared to its observed decay when TGF β is removed from the culture medium. The cystatin C cDNA was cloned into a mammalian expression vector containing the bacterial β -galactosidase gene as a reporter under the control of the Rous sarcoma viral promoter. We were unable to regulate the constitutive expression of the lacZcysC transgene by TGF β , based on histochemical analysis. However, by extended Northern blot exposures, we have detected a 4 Kb message that hybridizes specifically to the cystatin C probe

that is regulated by TGF β . This message possibly represents the primary nuclear cystatin C transcript, and this suggests that cystatin C is regulated in the nucleus. Cystatin C mRNA is also induced by hydrocortisone in SFME grown under serum-free conditions, although GFAP is not induced. DIA (differentiation inhibitory activity), which induces GFAP in SFME at comparable concentrations as TGF β , does not induce cystatin C mRNA.

INTRODUCTION

Transforming growth factor β is responsible for multiple and cell-specific effects on the growth and differentiation of many types of cells and tissues (Massague, 1990). Recently, our laboratory (Sakai et al., 1990) has found that the serum-free derived mouse embryo cell-line, known as SFME, will be induced by TGF β to differentiate into astroblasts that express the astrocyte-specific marker protein, glial fibrillary acidic protein (GFAP). Also, TGF β strongly induces the cysteine protease inhibitor, cystatin C, in SFME (Solem et al., 1990).

A common feature of TGF β is the regulation of genes that are a part of the extracellular matrix and its construction (Rizzino, 1988). These include the genes for fibronectin and collagen (Raghow et al., 1987), and also certain proteases and protease inhibitors, including plasminogen activator and its inhibitor, metalloprotease and TIMP (tissue-specific inhibitor of metalloprotease) are regulated by TGF β (Edwards et al., 1987). The regulation of some of these genes is post-transcriptional; often, mRNA stability is affected by TGF β (Raghow et al., 1987; Penttinen et al., 1988). We were interested in determining if TGF β regulates cystatin C in SFME by stabilizing messenger RNA. By inhibiting

transcription with α -amanitin, we observe cystatin C mRNA is stabilized by TGF β , but it is destabilized when TGF β is removed from the culture medium.

Often, sequences within the mRNA of certain genes have been associated with stability-regulation. Such is the case for the transferrin receptor mRNA which contains sequences known as iron response element (IRE) in the 3' untranslated region (Mullner et al., 1989). Also, the cell-cycle regulated expression of certain histone genes is due to the presence of untranslatable mRNA sequences controlling regulated destabilization (Morris et al., 1986; Ross and Kobs, 1986). We were interested in determining if cystatin C mRNA contains sequences that confer stability-regulation by TGF β . We have constructed a mammalian expression vector containing the Rous sarcoma virus long terminal repeat which drives the expression of the bacterial β -galactosidase and cystatin C cDNA (LacZcysC) transgene. We were unable to regulate the transgene's constitutive expression by TGF β in transient transfection experiments. However, by the analysis of extended Northern blot exposures, an approximately 4 Kb message was detected that is regulated by TGF β ; this may be the primary cystatin C transcript based on its predicted size (Abrahamson et al, 1990), which would suggest that there may be regulation of cystatin C in the nucleus as well as the cytoplasm.

Previously, we reported that other growth factors,

including nerve growth factor, platelet-derived growth factor, and basic fibroblast growth factor do not induce cystatin C in SFME (Solem et al., 1990). We find that SFME cells respond to the addition of 10^{-7} M hydrocortisone with increased expression of cystatin C mRNA, and this induction is comparable to that found by TGF β . However, GFAP is not induced by hydrocortisone. We have found by Western blot analysis that DIA (differentiation inhibitory activity) induces GFAP in SFME (Collodi, Nishiyama and Barnes, personal communications). We were unable to detect an increase in cystatin C mRNA in SFME grown in the presence of DIA. Thus, these two genes may be differentially regulated in SFME.

METHODS

RNA isolation and Northern blot analysis:

SFME were grown under standard serum-free conditions (Loo et al., 1989); the addition of specific growth factors is as indicated. SFME stably integrated clones containing the pRSVcysC3' plasmid were selected in the presence of 200 $\mu\text{g/ml}$ G418 (Gibco/BRL). RNA isolation has previously been described (see Chapter 3, methods). RNA was size-fractionated on a 1.2% formaldehyde-agarose gel, and blotted onto nitrocellulose by standard methods (Southern, 1975; Davis et al., 1986). All RNA isolated from transient transfection experiments was DNased with RNase-free DNase (Promega) as described (see Chapter 3 methods in this dissertation).

Measurement of cystatin C mRNA stability:

SFME were grown 24 hours in the presence of TGF β (2 ng/ml), the medium was then changed to medium without TGF β , and α -amanitin (1 $\mu\text{g/ml}$) was added. TGF β was again added 15 minutes after α -amanitin addition to half of the plates. After 0, 3, 6, and 8 hours of incubation, RNA was isolated, DNases, and 20 μg RNA samples were run on a gel for Northern blot experiments. Hybridization was performed by standard methods (Davis et al., 1986), and cystatin C and β -actin mRNA was measured by exposing the blot to X-ray film. Quantitation

of the messages was determined using the AMBIS system that measures radioactivity, which is provided by the central service laboratory of the Center for Gene Research at Oregon State University.

Construction of the RSVLacZcysC plasmid:

pRc/RSV was purchased from Invitrogen, San Diego, CA. To produce a plasmid with the lacZ gene fragment under the direction of the RSV LTR, we cloned the 3.5 Kb lacZ fragment from a Not I digestion of pCMVlacZ (Clonetechn Laboratories, Palo Alto, CA.) into the unique Not I site in the polylinker region of pRc/RSV. The orientation of lacZ was determined by restriction enzyme digestion analysis. The 660 bp cystatin C cDNA containing Not I linkers was cloned into the Not I site at the 3' end of lacZ after first isolating a partial digest of pRSVLacZ that had been linearized at the 5' end Not I site, and removing the 5' Not I site by filling the recessive ends with Klenow and blunt-end ligating the pRSVLacZcysC plasmid. The cystatin C sequences of the transgene's mRNA should not be translated, since the LacZ open reading frame contains a stop codon. The cystatin C 3'- containing pRc/RSV plasmid (pRSVcysC3') was obtained by digesting cystatin C cDNA with Not I and Bgl II to yield a 340 base 3'half cystatin C fragment. The 5' half of cystatin C was further digested with Bst XI, which cleaves the fragment at position 229. The 3'- half cystatin C cDNA was identified and isolated by

fractionating on a low melting agarose gel (Gibco/BRL) and removing the agarose by phenol/chloroform extractions and precipitation with 95% ethanol. The 3'-half cystatin C was cloned at the 3'-end Not I site into the single Not I site in the polylinker region of the pRC/RSV plasmid, and the recessive ends were filled in with Klenow enzyme. The plasmid was blunt-end ligated to make the pRSVcysC3' plasmid. The direction of the 3' cystatin C insert was identified by digesting with Pst I, which cleaves the cystatin C cDNA at position 590, and a single site in the pRC/RSV plasmid in the polylinker region upstream of the Not I site.

Transient transfection and histochemical assay:

Calcium phosphate-mediated transfection was carried out as described (Helmrich et al., 1988). 50 µg plasmid DNA in 50 mM CaCl₂ (pH 7.2) was sheared with a 25 gauge needle and dropped into a solution of 50 mM HEPES (n-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid) (pH 7.2), 0.25 M sodium chloride, 1.8 mM sodium phosphate (pH 7.2). Approximately 10⁷ cells/ 75 cm² flask were transfected with the plasmid-containing solution. After four hours, the cells were isolated and replated into 50 cm diameter dishes in triplicate, and the cells were grown for an additional 4 hours. Staining of lacZ expressing cells was done as described (Sanes et al., 1986). Cell monolayers were gently rinsed three times with phosphate-buffered saline, fixed with 1.9% formaldehyde/0.2% glutaraldehyde in phosphate-

buffered saline, and stained with 0.5 mg/ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) in 44 mM HEPES (pH 7.2), 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, 15 mM sodium chloride, and 1.3 mM magnesium chloride in a final volume of 3 ml per 50 cm diameter dish.

RESULTS

mRNA stability regulation of cystatin C by TGF β :

The effect of TGF β on the stability of cystatin C mRNA was investigated in SFME. SFME were first incubated with TGF β for 24 hours to increase the basal level of message. Next, the cells were incubated with α -amanitin (1 μ g/ml) to block further transcription (Drew et al., 1989). The rate of depletion of the RNA was determined by Northern blot hybridization analysis of cells incubated with or without TGF β (2 ng/ml) in the culture medium. The rate was compared to the rate of depletion of β -actin and β 8, an 8.5 Kb mRNA specifically induced in SFME by TGF β (see Patrick Varga, PhD dissertation, 1992). Figure 4.1 represents the Northern analysis of cystatin C and β -actin mRNA. β 8 mRNA was also measured in a repeat experiment (data not shown), and TGF β had no effect on its stability. Although β actin mRNA shows no significant decay with and without TGF β after 8 hour transcription inhibition, there is an observable cystatin C mRNA reduction after 8 hours transcription inhibition when SFME are grown in the absence of TGF β . We have quantitated the radioactivity (Figure 4.2), and we find a 40% decline in cystatin C mRNA. In contrast, when TGF β is present after 8 hours there is no loss of cystatin C mRNA.

Measurement of LacZ-cystatin C transgene expression:

We wished to determine if sequences in the cystatin C mRNA could regulate a reporter gene by TGF β . We cloned the cDNA in the sense and antisense direction downstream of the β -galactosidase gene under the expression of the Rous sarcoma virus LTR. The plasmid also contains the neomycin resistance gene (neo), aminoglycoside 3' phosphotransferase, driven by the SV40 late promoter (Fig. 4.3). We transfected SFME grown in the presence and absence of TGF β with 50 μ g plasmid, and isolated mRNA after 24 hours. Fig. 4.4 is a Northern blot probed with cystatin C. The endogenous cystatin C mRNA is induced by TGF β , and can be detected after 8 hours exposure to X-ray film. After 5 days exposure, an additional band of approximately 4 Kb can be detected in the lanes containing RNA from SFME grown in the presence of TGF β . Initially, we believed this message represented the lacZ-cystatin C hybrid. However, probing with LacZ (Fig. 4.5) could not detect this 4 Kb message after 7 days exposure to X-ray film, although in a 100% LacZ-expression permanent SFME clone, the message was present. The neo message could be detected in all four transient experiments, as well as the control, after an overnight exposure (Fig.. 4.5). This suggests that neo mRNA is more prevalent in transient transfection experiments than the lacZ-cystatin C hybrid mRNA.

Also, we have cloned the 3' half of cystatin C into the

RcRSV plasmid, which does not contain the LacZ gene, and we have obtained permanent SFME clones resistant to G418. Probing for cystatin C in the total RNA from SFME grown with and without TGF β cystatin C also revealed a 4 KB TGF β -regulated message, although the 550 base 3' cystatin C transcript was not detected in all 4 clones (Fig. 4.6).

Since we could not measure the LacZcysC message by Northern analysis, we decided to see if we could detect the expressed protein by assaying for β -galactosidase activity by staining SFME. Table 4.1 contains data from several transient transfection experiments designed to see if TGF β treated SFME had higher β -galactosidase activity than SFME grown without TGF β . In the first experiment, approximately 10^7 cells were transfected with pRSVLacZcysC, after 4 hours the cells were replated into 50 cm diameter dishes, and TGF β was added to half the plates. After 4 hours, the cells were stained and the number of blue cells was determined after a 24 hour incubation. In both the serum free and TGF β -added plates, blue cells could be detected after 15 minutes with approximately the same frequency and color intensity. After 3 hours, the staining appeared to be complete. The frequency of transfection with and without TGF β was 272 blue cells/ 10^6 cells and 224 blue cells/ 10^6 cells, respectively. In the second experiment, 10^7 cells were grown in the presence of TGF β (2 ng/ml) for 24 hours. The cells were transfected with plasmid, and after 4 hours replated into dishes with and

without TGF β . After 4 hours, the cells were stained for β -galactosidase activity, and counted after 24 hours. Again, the number of blue stained cells was about the same with and without TGF β , indicating that the cystatin C sequences did not confer regulation by TGF β . As a control, SFME grown with and without TGF β were transfected with pRSVlacZ. TGF β did not affect the number of cells expressing β -galactosidase, as was determined by staining and counting the number of blue cells.

SFME are induced by hydrocortisone to express cystatin C:

After 48 hour of TGF β exposure, there was an approximate 2-fold induction of cystatin C in SFME. We have tested other growth factors and cytokines, including nerve growth factor, platelet-derived growth factor, basic fibroblast growth factor, (see Fig. 4.7), γ -interferon and T3. None induced cystatin C in SFME. However, SFME cells grown serum-free in the presence of hydrocortisone (10^{-7} M) for 24 hours had a strong induction of cystatin C (Fig. 4.7). GFAP is not induced in SFME under serum-free conditions. We tested the differentiation inhibitor, DIA, to see if cystatin C mRNA is increased in SFME. DIA induced GFAP in SFME as determined by Western blot analysis. After 48 hours treatment with DIA (10 ng/ml), we did not see an increase in cystatin C mRNA (Fig. 4.8).

DISCUSSION

TGF β has multiple effects on the expression of genes associated with the extracellular matrix; these include cell attachment proteins, like collagen and fibronectin, their receptors, and also several proteases and protease inhibitors. The regulation of these genes is both transcriptional and post-transcriptional (Massague, 1990). In this report, we present evidence that TGF β affects cystatin C expression by stabilizing the message. After 8 hours of transcription inhibition, there is a 40% decline in cystatin C mRNA when TGF β is removed from the culture medium, but no decline in message when it is present. Further analysis of the cystatin C mRNA half-life may be made by extending the period of transcription inhibition. The observation that β -actin mRNA shows no significant decay after 8 hours of transcription inhibition is not unusual; the average half-life of cytoplasmic mRNA is 16 hours, and also SFME may not contain high endogenous RNase activity compared to some cells.

We report our attempts to regulate a reporter gene (LacZ) by attaching cystatin C sequences to its 3' end. It is known (Ross and Kobs, 1986) that mRNA decay is initiated at or near the 3' terminus and proceeds 3' to 5'. Other experiments have demonstrated the regulation of the stability of a reporter gene's mRNA by attaching 3' regulatory elements from various

eukaryotic messages downstream of its sequences (Shaw and Kamen, 1986; Sato et al., 1989). Cystatin C mRNA contains several short, repeating sequences at its 3' end that are in close proximity to inverted repeat sequences. Therefore, we wished to determine if these sequences confer TGF β -regulated stability. Although we were unable to regulate the LacZcysC transgene expression, we believe that these sequences may still have some function in cystatin C regulated expression; they may operate with other sequences to control processing and stability of the primary transcript. Previous experiments report used β -galactosidase activity to measure regulated expression of LacZ-containing transgenes (Norton and Coffin, 1985; Swergold, 1990). Although in these experiments β -galactosidase activity was directly quantitated for enzymatic activity, Swergold (199) compared the quantitation with the number of cells stained by histochemical analysis of transient transfection experiments, and found that these two analyses corresponded.

We were unable to measure the LacZcysC hybrid mRNA by Northern analysis although the neo mRNA was readily detected. We do not know why the neomycin resistance gene mRNA is detected so readily: it may be more stable than the LacZcysC message. We have previously tested the ability of various promoters to direct LacZ expression by transient transfection of SFME, and we do not find significant differences in the strengths of the two promoters, SV40 and RSV LTR. We find

that many of our pRSVLacZ-containing and pRSVcysC3'-containing permanent SFME clones are G418 resistant, but do not express the genes driven by RSV LTR based on Northern and histochemical analysis. It is reported that the neo gene contains sequences that act as silencers of transcription (Artelt et al., 1991). Thus, removal of the neo gene before transfection may enhance the expression of RSV-driven genes.

An approximately 4 Kb message was detected after extended exposures of Northern blots containing RNA from transiently transfected SFME and RSVcys3'-containing SFME clones. This message is induced by TGF β , and is the correct size of the predicted cystatin C primary transcript (Abrahamson et al., 1989). We can detect this 4 Kb message in untransfected cells. Although they are inherently unstable, unprocessed messages have been detected in Northern experiments (Silver et al., 1990; Ferrari et al., 1992). If the 4 Kb message is indeed the primary transcript, this suggests that cystatin C is also regulated in the nucleus. The promoter region of human cystatin C has been described (Abrahamson et al., 1989), and it has house-keeping features. It is known that the transcription rates of constitutive genes do not correlate with the intracellular levels of their respective mRNAs (Cabrera et al., 1984). Thus, it may be that increased levels of the primary transcript in the presence of TGF β is due to a post-transcriptional event.

Cystatin C is markedly induced by hydrocortisone in SFME

although GFAP is not induced under serum-free conditions. Steroid compounds often regulate genes transcriptionally, but there are reports that they also operate post-transcriptionally. For example, estrogen and its receptor dramatically stabilizes vitellogenin mRNA (Brock and Shapiro, 1983). Also, in rat fibroblasts dexamethasone affects the stability of the procollagen mRNA (Raghow et al., 1986). Further analysis of the regulation of cystatin C by hydrocortisone will need to be made to begin to discern its effects on gene expression in SFME.

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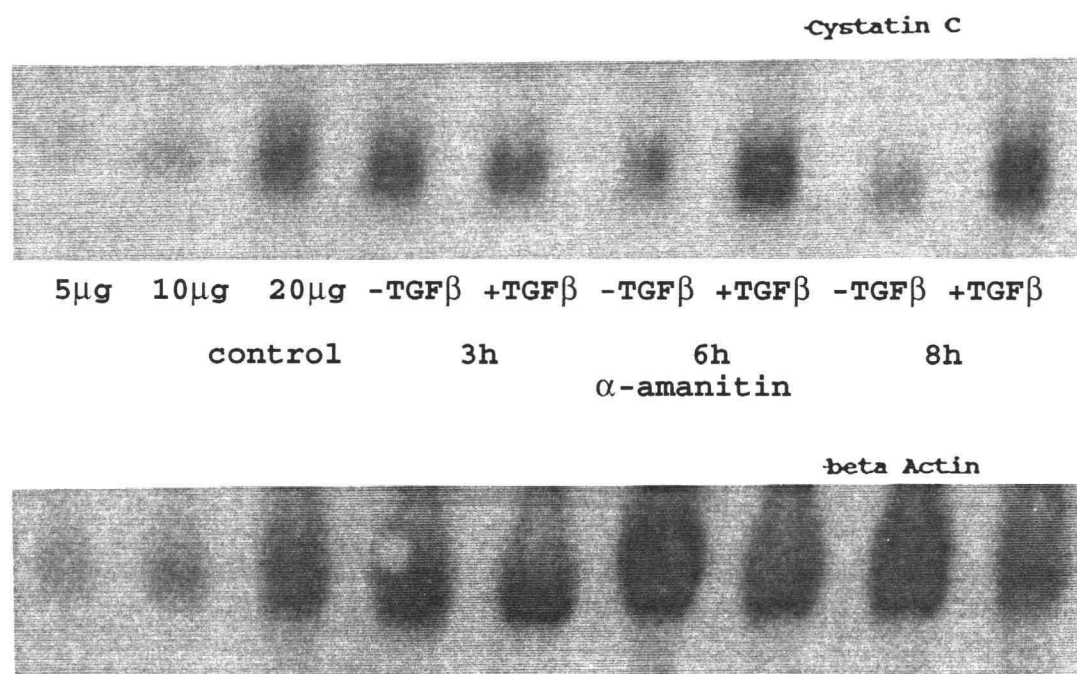


Fig. 4.1: mRNA stability of cystatin C and β -actin in SFME grown with and without TGF β in the presence of the transcription inhibitor α -amanitin.

TGF BETA AFFECTS CysC mRNA STABILITY

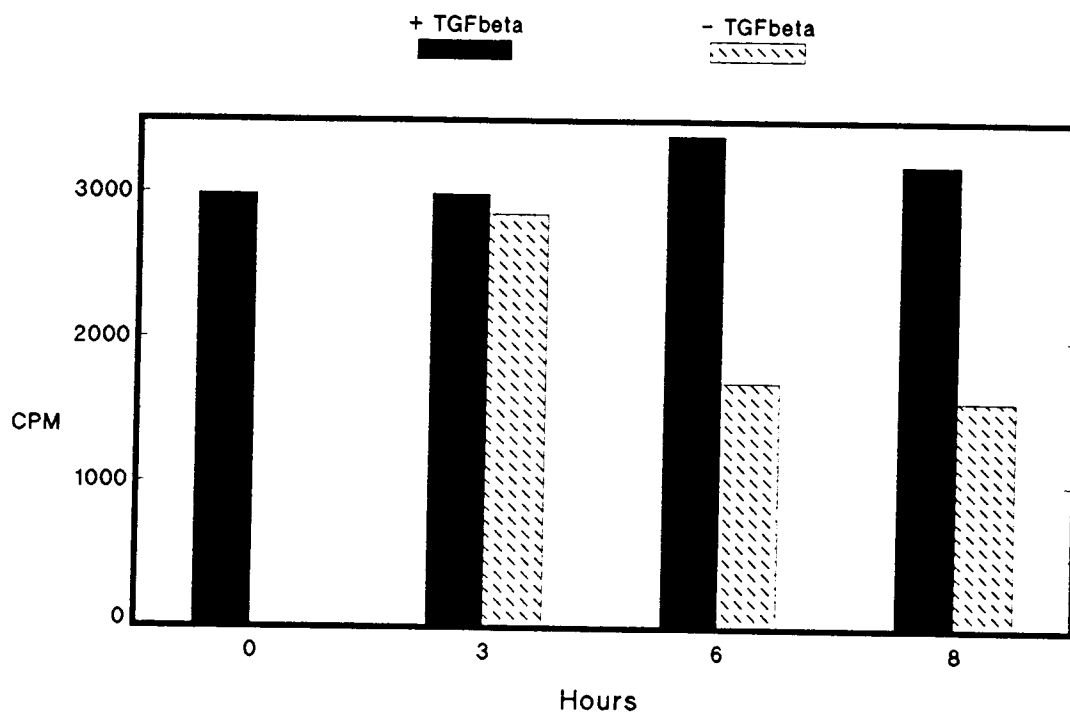


Fig. 4.2: Quantitation of cystatin C mRNA stability experiment. Quantitation in counts per minute (cpm) of cystatin C mRNA decay at 0, 3 h, 6 h and 8 h of transcription inhibition with and without TGF β .

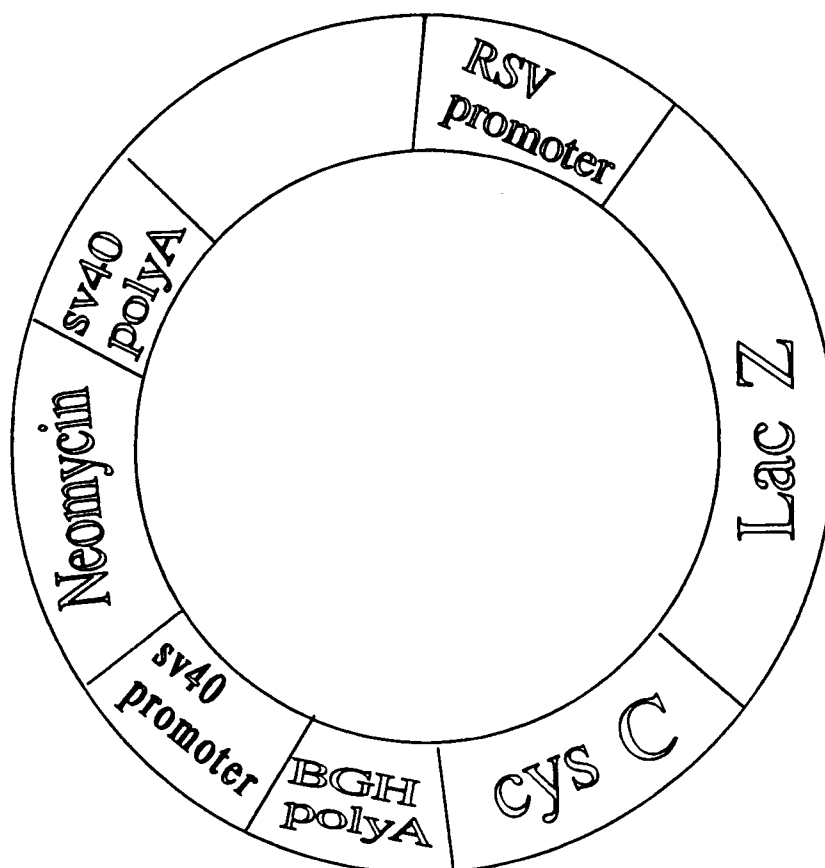


Fig. 4.3: Diagram of the pRSVLacZcysC plasmid. The LacZcysC transgene is driven by the Rous sarcoma virus long terminal repeat. The size of the β -galactosidase gene (LacZ) is 3.5 Kbp. Cystatin C cDNA is 660 bp. Also contained in the plasmid is the neomycin resistance gene under the direction of the SV40 promoter. The neomycin resistance gene (neo) is approximately 1.1 Kbp and is polyadenylated by the SV40 late polyadenylation sequences.

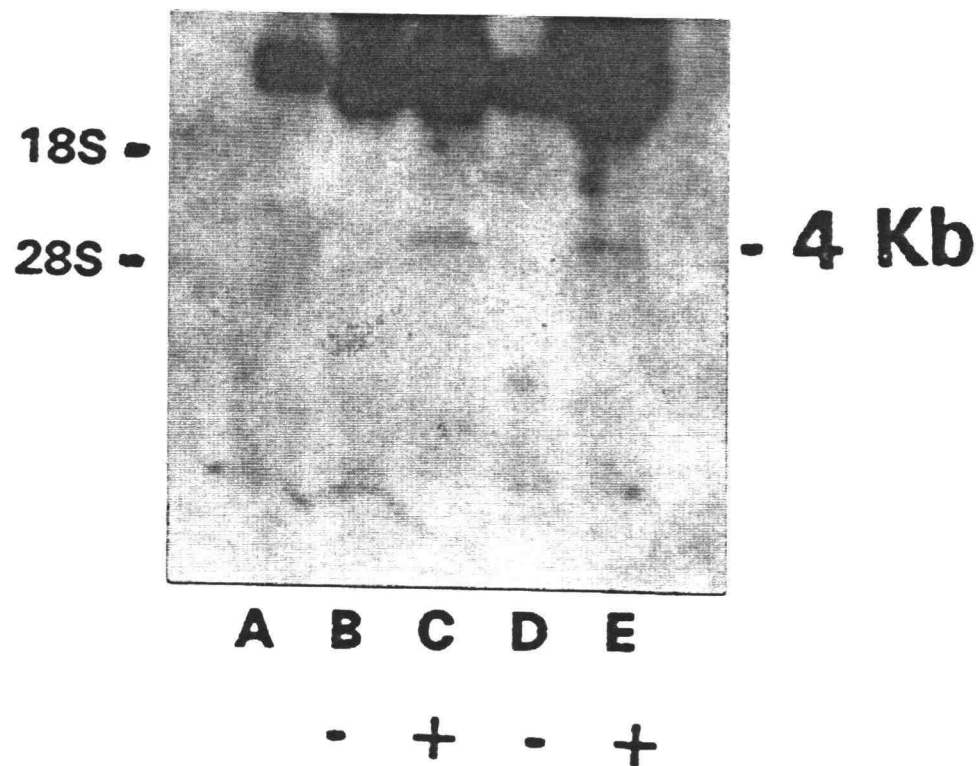


Fig. 4.4: Northern blot analysis of the transient transfection of SFME with pRSVLacZcysC probed for cystatin C. Cells were transfected with plasmid, then were grown for 24 hours in the presence and absence of TGF β . RNA was isolated, DNases and probed with ^{32}P -radiolabeled cystatin C cDNA. (A) RSVLacZ 100% stably integrated clone; (B) transiently transfect with RSVLacZcysC(Sense), w/o TGF β , 24h; (C) transiently transfect with RSVlacZcysC(sense), with TGF β , 24h; (D) transiently transfect with RSVlacZcysC(antisense), w/o TGF β , 24h; (E) transiently transfect with RSVlacZcysC(antisense), with TGF β , 24h.

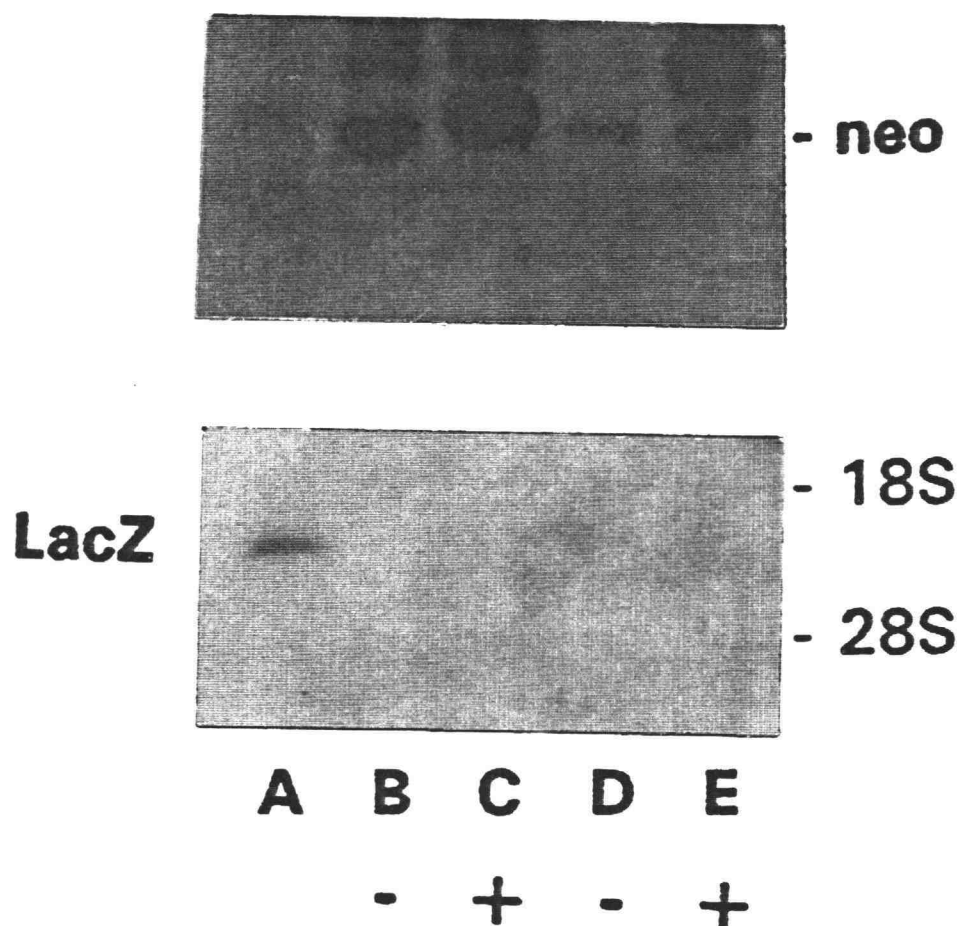


Fig. 4.5: Northern blot analysis of the transient transfection of SFME with pRSVLacZcysC probed for lacZ and neo. Cells were transfected with plasmid, then were grown for 24 hours in the presence and absence of TGF β . (A) RSVLacZ 100% stably integrated clone; (B) transiently transfect with RSVLacZcysC(Sense), w/o TGF β , 24h; (C) transiently transfect with RSVlacZcysC(sense), with TGF β , 24h; (D) transiently transfect with RSVlacZcysC(antisense), w/o TGF β , 24h; (E) transiently transfect with RSVlacZcysC(antisense), with TGF β , 24h. Probed with 32 P-radiolabeled β -galactosidase gene (lacZ) and neomycin resistance gene (neo) cDNA.

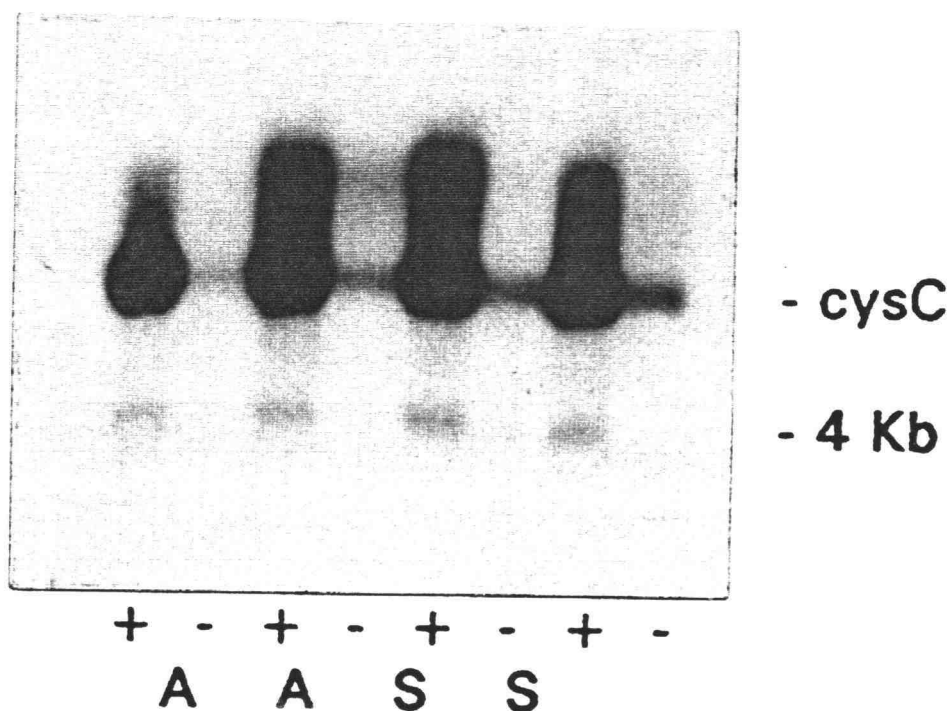


Fig. 4.6: Detection of 4 Kb transcript in RSVcysC3' SFME clones treated with TGF β . Total RNA was isolated from four independent, G418 resistant SFME clones transfected with pRSVcysC3'. 20 μ g RNA was isolated for Northern analysis of clones containing the 3' half of cystatin C in the antisense direction (A) and sense (S) direction downstream of the Rous sarcoma virus long terminal repeat. Each of the four clones were grown in the presence (+) and absence (-) of TGF β (2 ng/ml, 48 h).

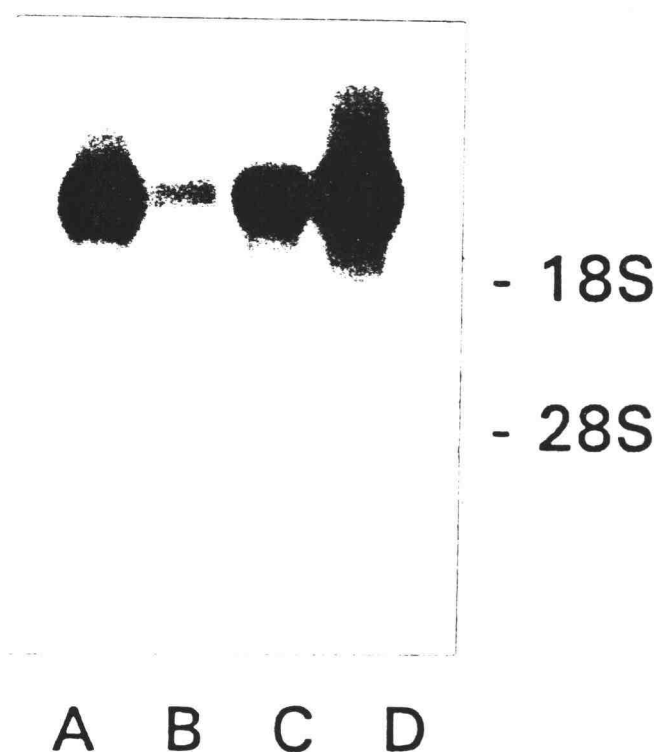


Fig. 4.7: Northern blot analysis of cystatin C induction in SFME by hydrocortisone. SFME were grown serum-free and treated for 24 hours with (A) hydrocortisone (10^{-7} M), (B) serum-free, (C) with TGF β (2 ng/ml), 24 hours, (D) with TGF β (2 ng/ml, 48 hours).

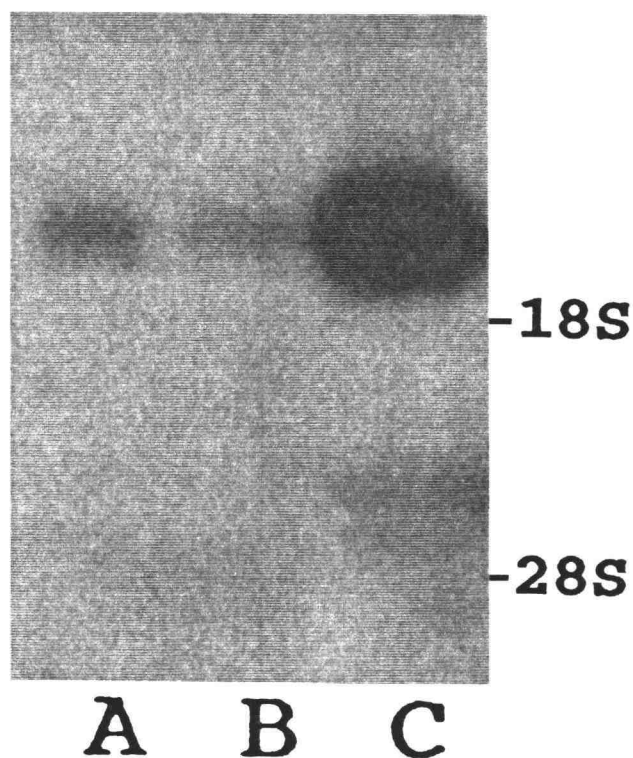


Fig. 4.8: DIA does not induce cystatin C in SFME. SFME were grown in the presence of differentiation inhibitory activity (DIA), 10 ng/ml for 48 hours. RNA was isolated and probed for cystatin C by Northern blot analysis. A: 20 μ g total RNA from SFME treated with DIA (10 ng/ml, 48 h). B: 20 μ g total RNA from SFME grown under serum-free conditions. C: 20 μ g total RNA from SFME treated with TGF β (2 ng/ml, 48 h).

Table 4.1: Transient transfection of SFME with pRSVLacZcysC in the presence and absence of TGF β . Measure of β -galactosidase activity in SFME. After transfection, the cells were incubated for 4 h with and without TGF β before staining and counting for blue cells.

Exp. design	TGF β	SF
	(# blue cells/10 ⁶ cells)	
1. pRSVLacZ control	1066/0.25x16	880/0.25x1 ⁶
2. transfect cells with pRSVLacZcysC w/o TGF β	224(1)*	272(1)
3. transfect TGF β - treated (24 h) cells with pRSVLacZcysC	486(3)	319(3)

*numbers in parentheses indicate the number of 50cm plates counted. Each dish was counted if there were less than 200 blue cells/dish, approximately 200 blue cells were counted and the number of blue cells was extrapolated from the area of the dish counted.

CHAPTER V

CONCLUSION and FUTURE PERSPECTIVES

The biologist may find interesting the research in this thesis about the species distribution of S-protein. We detect what appears to be a single copy gene in the seven mammals we examined. It may be interesting to more carefully analyze the conserved regions of this multidomain protein in mammals. Whether we will find a related S-protein gene in a more divergent species may depend on if there are fundamental and essential regions found in this protein. The gene for cystatin is ancient; it is over 1 million years old. A cystatin-related gene has been detected in *Drosophila* (Delbridge and Kelly, 1990). The gene exists in chicken, and the corresponding amino acid sequence is over 50% homologous with human cystatin C (Rawlings and Barrett, 1990). It may be interesting to see if cystatin is present in a genetically definable system, such as yeast, in order to characterize it's cellular functions.

What may interest the cell biologist, is the research that examines the patterns of expression of S-protein and cystatin C. S-protein, a serum glycoprotein, is a multifunctional protein that is expressed at high levels by

the liver, and also we detect its mRNA in the brain. Cystatin C, also found at high concentrations in serum, is expressed at varying levels by many different tissues, and its mRNA is most prevalent in the heart, lung and brain. Both genes have been associated with amyloid deposits in the brain (Grubb et al., 1984; Akiyama et al., 1991). Both have been found to be regulated in certain cell lines by transforming growth factor β . We would like to know more about the distribution of these two genes in the brain and their biological functions. Although our proastroblast cell line, SFME, can be strongly induced to express cystatin C, we are unable to detect S-protein produced by these brain cells. There are several glial and neuronal cell lines available to examine the expression and growth factor-regulation of these two genes. Continuing in-situ hybridization and immunochemical assay studies of various brain sections from young, aged, diseased and injured mouse brains may outline their location and give some hints about their possible functional roles in the central nervous system. There is evidence that cystatin C has a developmentally-regulated expression in the brain. Several proteases and protease inhibitors are known to regulate neurite outgrowth, and it may be that cystatin has some involvement in building neural networks during brain development, and maintaining them throughout life. Associated with this, is S-protein's ability to bind glia-derived nexins, which are known neurite outgrowth promoting agents. Cystatins

have been purified from bovine brain that inhibit substance P degradation by cysteine proteases (Aghajanyan et al., 1988). It may be that cystatin C plays a role in the processing and turnover of various neurotransmitters and neuropeptides during their secretion or in the extracellular space. Proenkephalin is known to be processed by a cysteine protease present in secretory vesicles. It has been determined that mature astrocytes secrete unprocessed proenkephalin, and it may be interesting to see if proastroblasts, like SFME, can process the neuropeptide. When SFME are induced to differentiate in SFME by TGF β , cystatin C may inhibit proenkephalin processing by inhibiting this cysteine protease.

As a biochemist, I am particularly interested in gene regulation of cystatin C in SFME. Cystatin C is markedly induced by TGF β in SFME, and the mRNA is increased after four hours of exposure to the growth factor. It is a secreted protein, and the release into the culture medium parallels the mRNA increase. We have tested other cell lines for TGF β -regulated expression, and have found them not to be regulated by TGF β for the expression of cystatin C. I present evidence in this thesis that TGF β stabilizes cystatin C mRNA, and although I was unable to discern the particular regions of the cystatin message involved in regulating the stability, the results are in agreement with the many findings that TGF β affects certain genes' post-transcriptionally. The observation that cystatin C can be induced by TGF β when

protein synthesis is inhibited is a clear suggestion that prior gene expression by immediate early genes, such as transcription factors, are not necessary. In contrast, GFAP induction by TGF β is abolished by cycloheximide treatment. Many reports describe GFAP's transcriptional regulation (Sarid, 1991; Mucke et al., 1991), and our finding that prior protein synthesis is necessary for this gene's induction by TGF β is evidence that initial induction of gene activators is needed to direct GFAP gene transcription.

We find a 4 Kb message is induced by TGF β that is detected by cystatin C probe. I believe this message is the primary cystatin C transcript, which suggests that there is regulation of the gene in the nucleus. It is not as likely that the message is instead a related gene that shares some homology with cystatin C; the cystatin gene family has been extensively investigated, and there are no such genes with mRNA of this size. To establish that it is unprocessed cystatin C message, we will need to clone the cystatin C gene and obtain intron sequences as probes. We will need to isolate nuclear RNA to make sure that the transcript is located in the nucleus, since it is possible that unprocessed message may leak into the cytosol, and be regulated by TGF β in this location. If it is indeed nuclear-derived, we may also want to determine the control point(s) of cystatin C gene expression in the nucleus, and the factors involved in this regulation. The signal transduction pathways involved in the

regulation of cystatin C are also interesting. Protein kinase C is probably not involved, since Northern blot analysis of TPA-treated SFME shows no increased cystatin C mRNA levels. TPA induces a morphology in SFME similar to TGF β . Whether GFAP is induced by TPA has not been determined.

BIBLIOGRAPHY

Abraham, C.R., Selkoe, D.J. and Potter, H. (1988), Immunochemical identification of the serine protease inhibitor α 1-antichymotrypsin in the brain amyloid deposits of Alzheimer's disease. *Cell* 52:487-501.

Abrahamson M., Grubb, A., Olafsson, I. and Lundwall, A. (1987), Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine protease inhibitor cystatin C. *FEBS Letters*. 216:229-233.

Abrahamson, M., Olafsson, I., Palsdottir, A., Ulvsback, M., Lundwall, A., Jensson, O., Grubb, A. (1990), Structure and expression of the human cystatin C gene. *Biochem. J.* 268:287-294.

Aghajanyan, H.G., Arzumanyan, A.M., Arutunyan, A.A., Akopyan, T.N. (1988), Cystatins from bovine brain: purification, some properties, and action on substance P degrading activity. *Neurochem. Res.* 13:721-727.

Akiyama, H., Kawamata, T., Dedhar, S., McGeer, P.L. (1991), Immunohistochemical localization of vitronectin, its receptor and β -3 integrin in Alzheimer brain tissue. *J. Neuroimmun.* 32:19-28.

Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., Hauser, H. (1991), The prokaryotic neomycin-resistance encoding gene acts as a transcriptional silencer in eukaryotic cells. *Gene* 99:249-254.

Barnes, D. and Sato, G. (1980a), Serum-free cell culture: a unifying approach. *Cell*. 22:649-655.

Barnes, D.W., Wolf, R., Serrero, J., McClure, D. Sato, G. (1980b), Effects of a serum spreading factor on growth and morphology of cells in serum-free medium. *J. Supramol. Structure*. 14:47-63.

Barnes, D.W., Silnutzer, J., See, C., Shaffer, M. (1983a), Characterization of human serum spreading factor with monoclonal antibodies. *Proc. Natl. Acad. Sci., U.S.A.* 80:1362-1366.

Barnes, D.W., Silnutzer, J. (1983b), Isolation of human serum spreading factor. *J. Biol. Chem.* 258:12548-12552.

Barnes, D., Foley, J., Shaffer, M., Silnutzer, J. (1983c), Human serum spreading factor; relationship to somatomedin B. *J. Clin. Endocrinol. Meth.* 59:119-121.

Barnes, D.W., Reing, J. and Amos, D.B. (1985a), Heparin binding properties of human serum spreading factor. *J. Biol. Chem.* 260:9117-9122.

Barnes, D.W., Reing, J. (1985b), Human spreading factor; synthesis and response by HepG2 hepatoma cells in culture. *J. Cell Physiol.* 125:207-214.

Barrett, A.J. (1987), The cystatins: a new class of protease inhibitors. *Trends Bioch. Sci.* 12:193-196.

Brawerman, G. (1989), mRNA decay: Finding the right targets. *Cell.* 57:9-10.

Brock, M.L., Shapiro, D.J. (1983), Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. *Cell* 34:207-214.

Brooks, R.F. (1975), Growth regulation in vitro and the role of serum. In: Allison, A.C. (ed): *Structure and Function of Plasma Proteins*, Plenum, NY, 1-112.

Cabrera, C.V., Lee, J.J., Ellison, J.W., Britten, R.J., Davidson, E.H. (1984), Regulation of cytoplasmic mRNA prevalence in sea urchins (*Strangylocentrotus purpuratus*) embryos: Rates of appearance and turnover for specific sequences. *J. Mol. Biol.* 178:869-880.

Cantley, L., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S. (1991), Oncogenes and signal transduction. *Cell.* 64:281-302.

Cataldo, A.M. and Nixon, R.A. (199), Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer's brains. *Proc. Natl. Acad. Sci., U.S.A.* 87:3861-3865.

Chapman, H., Reilly, J.J.Jr., Yee, R., Grubb, A. (1990), Identification of cystatin C, a cysteine proteinase inhibitor as a major secretory product of human alveolar macrophages in vitro. *Am. Rev. Respir. Dis.* 141:689-705.

Chen, K.C., De Falco, M., Maloy, W.L., Liem, H.H., Muller-Eberhardt, U. (1988), Peptide-specific antibodies employed in determining the interspecies immunological cross-reactivity of hemopexin. *Comp. Bioch. Physiol.* 91:467-472.

Cohen, D.H., Heiner, H., Jensson, O., Frangione, B. (1983), Amyloid fibril in Hereditary Cerebral Hemorrhage with Amyloidosis (HCHWA) is related to the gastroentero-pancreatic neuroendocrine protein, gamma-trace. *J. Exp. Med.* 158:623-628.

Cole, T., Dickson, P.W., Esnard, F., Averill, S., Risbridger, G.P., Gauthier, F. and Schreiber, G. (1989), The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for β 2-microglobulin in rat brain. *Eur. J. Biochem.* 186:35-42.

Cross, M. and Dexter, M. (1991), Growth factors in development, transformation, and tumorigenesis. *Cell.* 64:271-28.

Davis, L.G., Dibner, M.D., Battey, J.F. (1986), *Basic Methods Mol. Biol.*, 222-226, Sci. Publ. Co., New York.

Declerck, P.J., De Mol, M., Allessi, M.C., Baudner, S., Preissner, K.T., Muller-Berghaus, G., Collen, D. (1988), Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. *J. Biol. Chem.* 263:15454-15461.

Delbridge, M.L., Kelly, L.E. (1990), Sequence analysis, and chromosomal localization of a gene encoding a cystatin-like protein from *Drosophila melanogaster*. *FEBS*, 274:141-145.

Denhardt, D.T., Hamilton, R.T., Parfett, C.L.J., Edwards, D.R., St. Pierre, R., Waterhouse, P., Nilsen-Hamilton, M., (1986), Close relationship of the major excreted protein of transformed murine fibroblasts to thiol-dependent cathepsins. *Cancer Res.* 46:4590-4593.

Deuel, T.F. (1987), Polypeptide growth factors: roles in normal and abnormal growth. *Ann. Rev. Cell Biol.* 3:443-492.

De Vellis, J., Wu, D. and Kumar, S. (1986) in *Biochemistry, Physiology and Pharmacology of Astrocytes*. (S. Fedoroff and A. Vernadakis, eds.) Vol. 2, 29-237. Academic Press, Orlando, FL.

Drew, P.D. and Ades, I.Z. (1989), Regulation of the stability of chicken embryo liver γ -aminolevulinate MRNA by hemin. *Biochem. Bioph. Res. Comm.* 162:102-106.

Edwards, D.R., Murphy, G., Reynolds, J.J., Whitham, S.E., Docherty, A.J.P., Angel, P. and Heath, J.K. (1987), Transforming growth factor β modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* 6:1281-1286.

Fedoroff, S. and Vernadakis, A. (eds), (1986), *Astrocytes*, vol 1-3, Academic Press, Orlando, FL.

Ferrari, S., Tagliafico, E., Manfredini, R., Grande, A., Rossi, E., Zucchini, P., Torelli, G., Torelli, U. (1992), Abundance of the primary transcript and its processed product of growth-related genes in normal and leukemic cells during proliferation and differentiation. *Cancer Res.* 52:11-16.

Frederickson, R.C.A. (1992), Astroglia in Alzheimer's disease. *Neurobiol. Aging.* 13:239-253.

Fuquay, J., Loo D., Barnes, D. (1986), Human serum spreading factor; binding to *Staphylococcus aureus*. *Inflam. and Immun.* 52:714-717.

Ghiso, J., Jensson, O. and Frangione, B. (1986), Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of γ -trace basic protein (cystatin C). *Proc. Natl. Acad. Sci., U.S.A.* 83:2974-2978.

Gladson, C.L., Cheresh, D.A. (1991), Glioblastoma expression of vitronectin and the α -V β -3 integrin. Adhesion mechanism for transformed glial cells. *J. Clin. Inv.* 88:1924-1932.

Grubb, A., Jennson, O., Gudmunsson, G., Arnason, A., Lofberg, H., Milam, J. (1984), Abnormal metabolism of the gamma-trace alkaline protein: The basic defect in Hereditary Cerebral Hemorrhage with Amyloidosis. *N. Eng. J. Med.* 311:1547-1549.

Gubler, U. and Hoffman, B.J. (1983), A simple and very effective method for generating cDNA libraries. *Gene.* 25:263-269.

Hartwell, L.H. and Weinert, T.A., (1989), Checkpoints: controls that ensure the order of cell cycle events. *Science.* 246:629-634.

Hayman, E.G., Pierschbacher, M.D., Ohgren, Y., Ruoslahti, E. (1983), Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc. Natl. Acad. Sci., U.S.A.* 80:4003-4007.

Heine, u.I., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H.Y.P., Thompson, N.L., Roberts, A.B., Sporn, M.B. (1987), Role of transforming growth factor β in the development of the mouse embryo. *J. Cell Biol.* 15:2861-2876.

Helmrich, A., Bailey, G.S., Barnes, D.W. (1988), Transfection of cultured fish cells with exogenous DNA. *Cytotech.* 1:215-221.

Jenne, D., Stanley, K. (1985), Molecular cloning of S-protein; a link between complement, coagulation and cell-substratum adhesion. *EMBO J.* 4:3153-3157.

Jenne, D., Stanley, K. (1987), Nucleotide sequence and organization of the human S-protein gene: Repeating peptide motifs in the 'Pexin' family and a model for their evolution. *Biochem.* 26:6735-6742.

Kerr, L.D., Olashaw, N.E. and Matrisian, L.M. (1988), Transforming growth factor β 1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. *J. Biol. Chem.* 263:16999-17005.

Knauer, D.J. and Cunningham, D.D. (1984), Protease nexins: cell-secreted proteins which regulate extracellular serine proteases. *Trends Biochem. Sci.* 9:231-233.

Knox, P., Griffiths, S. (1979), A cell spreading factor in human serum that is not cold-insoluble globulin. *Exp. Cell Res.* 123:421-424.

Koli, K., Lohi, J., Hautanen, A., Keski-Oja, J. (1991), Enhancement of vitronectin expression in human HepG2 hepatoma cells by transforming growth factor β 1. *Eur. J. Biochem.* 199:337-345.

Lah, T.T., Clifford, J.L., Helmer, K.M., Day, N.A., Moin, K., Honn, K.V., Crissman, J.D. and Sloane, B.F. (1989), Degradation of laminin by human tumor cathepsin B. *Biochim. Biophys. Acta.* 993:63-73.

Laiho, M., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M., Massague, J. (1990), Growth inhibition by TGF β linked to suppression of retinoblastoma protein phosphorylation. *Cell* 62:175-185.

Leibold, E., Munro, H. (1988), Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy and light subunit MRNAS. *Proc. Natl. Acad. Sci., U.S.A.* 85:2171-2175.

Leung-Tack, J., Tavera, C., Martinez, J. and Colle, A. (1990a), Neutrophil chemotactic activity is modulated by human cystatin C, an inhibitor of cysteine proteases. *Inflammation* 14:247-259.

Leung-Tack, J., Tavera, C., Gensac, M.C., Martinez, J. and Colle, A. (1990b), Modulation of phagocytosis-associated respiratory bursts by human cystatin C. *Exp. Cell Res.* 188:16-22.

Lillien, L.E. and Raff, M.C. (1990), Differentiation signals in the C.N.S.: type 2 astrocyte development in vitro as a model system. *Neuron.* 5:111-119.

Lindholm, D., Hengerer, B., Zafra, F., Thoenen, H. (1990), Transforming growth factor β 1 stimulates expression of nerve growth factor in rat CNS. *Neuroreport*. 1:9-12.

Loo, D.T., Fuquay, J.I., Rawson, C.L. and Barnes, D.W. (1987), Extended culture of mouse embryo cells without senescence: inhibition by serum. *Science* 236:200-202.

Loo, D.T., Rawson, C.L., Ernst, T., Shirahata, S. and Barnes, D.W. (1989a) in *Cell Growth and Cell Division*, (R. Baserga, Ed.) 17-34. IRL Press, London.

Loo, D., Rawson, C., Helmrich, A. and Barnes, D. (1989b) Serum-free mouse embryo cells: growth responses in vitro. *J. Cell Physiol.* 139:484-491.

Loo, D., Rawson, C., Schmitt, M. Lindburg, K. and Barnes, D. (1990), Glucocorticoid and thyroid hormones inhibit proliferation of serum-free mouse embryo (SFME) cells. *J. Cell Physiol.* 142:210-217.

Lund, L., Riccio, A., Andreassen, P., Nielsen, L., Kristensen, P., Laiho, M., Saksela, O., Blasi, F. and Dano, K. (1987), *EMBO J.* 6:1281-1286.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning Manual*. Cold Spring Harbor Press, New York.

Maruyama, K., Ikeda, S., Ishihara, T., Allsop, D. and Yanagisawa, N. (1990), Immunohistochemical characterization of cerebrovascular amyloid in 46 autopsied cases using antibodies to β protein and cystatin C. *Stroke* 21:397-403.

Massague, J. (1990), The transforming growth factor β family. *Annu. Rev. Cell Biol.* 6:597-641.

Meier, R., Spreyer, P., Ortmann, R., Harel, A. and Monard, D. (1989), Induction of glial-derived nexin after lesion of a peripheral nerve. *Nature* 342:548-550.

Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J., Stein, G. (1986), Involvement of the 5'-leader sequence in coupling the stability of a human H3 histone mRNA with DNA replication. *Proc. Natl. Acad. Sci., U.S.A.* 83:981-985.

Mucke, L., Oldstone, M.B., Morris, L.C., Nerenberg, M.I. (1991), Rapid activation of astrocyte-specific expression of GFAP-lacZ transgene by focal injury. *New Biol.* 3:465-474.

Muller, T., Moller, T., Berger, T., Schnitzer, J., Kettenmann, H. (1992), Calcium entry through kainate receptors and resulting potassium-channel blockage in Bergmann glial cells. *Science*. 256:1563-1566.

Mullner, E.W., Neupert, B., Kuhn, L.C. (1989), A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell* 58:373-382.

Murray, A.W. and Kirshner, M.W. (1989), Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339:275-280.

Neugebauer, K.M., Tomaselli, K.J., Lilien, J., Reichardt, L.F. (1988), N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes in vitro. *J. Cell Biol.* 107:1177-1187.

Nielsen, D.A., Shapiro, D.J. (1990), Estradiol and estrogen receptor-dependent stabilization of a mini-vitellogenin mRNA lacking 5,100 nucleotides of coding sequence. *Mol. Cell Biol.* 10:371-376.

Nurse, P., (1990), Universal control mechanism regulating onset of M-phase. *Nature* 344:503-508.

Penttinen, R.P., Kobayashi, S., Bornstein, P. (1988), Transforming growth factor β increases mRNA for matrix proteins both in the presence and absence of changes in mRNA stability. *Proc. Natl. Acad. Sci., U.S.A.* 85:1105-1108.

Podack, E.R., Kolb, W.P., Muller-Eberhard, H.J. (1977), The SC5b-7 complex formation, isolation, properties and subunit composition. *J. Immunol.* 119:2024-2029.

Podack, E.R., Kolb, W.P., Muller-Eberhard, H.J. (1978), The C5b-6 complex: Formation, isolation and inhibition of its activity by lipoprotein and the S-protein of human serum. *J. Immunol.* 120:181-184.

Podack, E.R., Muller-Eberhard, H.J. (1979), Isolation of human S-protein, an inhibitor of the membrane attack complex of complement. *J. Biol. Chem.* 254:9908-9914.

Podack, E.R., Dahlback, B., Griffin, J.H. (1986), Interaction of S-protein of complement with thrombin and antithrombin III during coagulation. *J. Biol. Chem.* 261:7387-7392.

Price, J., Hynes, R.O. (1985), Astrocytes in culture synthesis and secrete a variant form of fibronectin. *J. Neurosci.* 5:2205-2211.

Raff, M.C., Miller, R.H., Noble, M. (1983), A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature*. 33:390-396.

Raff, M.C. (1989), Glial cell diversification in the rat optic nerve. *Science*. 243:1450-1455.

Raghow, R., Gossage, D., Kang, A.H. (1986), Pretranslational regulation of type I collagen, fibronectin and a 50 Kd noncollagenous extracellular protein by dexamethasone in rat fibroblasts. *J. Biol. Chem.* 261:4677-4684.

Raghow, R., Postlethwaite, A.E., Keski-Oja, J., Moses, H.L., Kang, A.H. (1987), Transforming growth factor β increases steady state levels of type I procollagen and fibronectin RNAs posttranscriptionally in cultured human dermal fibroblasts. *J. Clin. Inv.* 79:1285-1288.

Ramon y Cajal, S. (1909), The neuron and the glial cell. de la Torre, J., Gibson, W.C, eds.), revised (1984), Charles C. Thomas Publisher, Springfield, Il.

Rauvala, H., Pihlaskari, R., Laitinen, J., Merenmies, J. (1989), Extracellular adhesive molecules in neurite growth. *Biosci. Reports* 9:1-11.

Rawlings, N. and Barrett, A. (1990), Evolution of proteins of the cystatin superfamily. *J. Mol. Evol.* 30:60-71.

Rawson, C., Shirahata, S., Collodi, P., Natsuno, T., Barnes, D. (1990a), Oncogene transformation frequency of nonsenescent SFME cells is increased by c-myc. *Oncogene* 6:487-490.

Rawson, C., Smith C. and Barnes, D. (1990b), Death of serum-free mouse embryo cells caused by epidermal growth factor deprivation is prevented by cycloheximide, 12-O-tetradecanoylphorbol-13-acetate, or vanadate. *Exp. Cell Res.* 186:177-181.

Rawson, C., Loo, D., Helmrich, A., Ernst, T., Natsuno, T., Merrill, G. and Barnes, D. (1991), Serum inhibition of proliferation of serum-free mouse embryo cells. *Exp. Cell Res.* 192:271-277.

Rizzino, A. (1988), Transforming growth factor β : multiple effects on cell differentiation and extracellular matrices. *Dev. Biol.* 130:411-422.

Rosenthal, A., Lindquist, P.B., Bringman, T.S., Goeddel, P.V., Derynck, R. (1986), Expression in rat fibroblasts of a human transforming growth factor α cDNA results in transformation. *Cell*. 46:301-309.

Ross, J., Kobs, G. (1986), H4 histone messenger RNA decay in cell-free extracts initiates at or near the 3' terminus and proceeds 3' to 5'. *J. Mol. Biol.* 188:579-593.

Rovelli, G., Stone, S.R., Preissner, K.T., Monard, D. (1990), Specific interaction of vitronectin with the cell-secreted protease inhibitor glia-derived nexin and its thrombin complex. *Eur. J. Bioch.* 192:797-803.

Saad, B., Constam, D.B., Ortmann, R., Moos, M., Fontana, A., Schachner, M. (1991), Astrocyte-derived TGF β_2 and NGF differentially regulate neural recognition molecule expression by cultured astrocytes. *J. Cell Biol.* 115:473-484.

Sakai, Y., Rawson, C., Lindburg, K. and Barnes, D. (1990), Serum and transforming growth factor β regulate glial fibrillary acidic protein in serum-free mouse embryo cells. *Proc. Natl. Acad. Sci., U.S.A.* 87:8378-8382.

Sanes, J.R., Rubenstein, J.L.R., Nicolas, J.F. (1986), Use of recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* 5:3133-3142.

Sarid, J. (1991), Identification of a cis-acting positive regulatory element of the glial fibrillary acidic protein gene. *J. Neurosci. Res.* 28:217-228.

Sato, G., (1979), *Hormones and Cell Culture*. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Sato, K., Yagi, T., Kobayashi, K., Hosokawa, K. (1989), Stability of CAT transcript depends on the 3'-end structure. *Nuc. Acid Res.* 21:23-24.

Seiffert, D., Keeton, M., Eguchi, Y., Sawdey, M., Luskutoff, D.J. (1991), Detection of vitronectin mRNA in tissues and cells of the mouse. *Proc. Natl. Acad. Sci., U.S.A.* 88:9402-9406.

Selkoe, D.J. (1990), Deciphering Alzheimer's disease: the amyloid precursor protein yields new clues. *Science* 248:1058-1060.

Shaffer, M.C., Foley, T.P., Barnes, D.W. (1984), Quantification of spreading factor in human biological fluids. *J. Lab. Clin. Med.* 103:783-790.

Shaw, G., Kamen, R. (1986), A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*. 46:659-667.

Shaw, P.A., Barka, T., Woodin, A., Schacter, B.S., Cox, J.L. (1990), Expression and induction by β -adrenergic agonist of the cystatin S in submandibular glands of rat. *Biochem. J.* 265:115-120.

Shirahata, S., Rawson, C., Loo, D., Chang, Y. and Barnes, D. (1990), ras and neu oncogenes reverse serum inhibition and epidermal growth factor dependence in serum-free mouse embryo cells. *J. Cell Physiol.* 144:69-76.

Sierra, F., Fey, G.H., Guigoz, Y. (1989), T-kininogen expression is induced during aging. *Mol. Cell Biol.* 9:5610-5616.

Silnutzer, J., Barnes, D.W. (1984), A biologically active thrombin cleavage product of human serum spreading factor. *Bioch. Biophys. Res. Comm.* 118:339-343.

Silver, G., Krauter, K.S. (1990), Aryl hydrocarbon induction of rat cytochrome P-450d results from increased precursor RNA processing. *Mol. Cell Biol.* 10:6765-6768.

Solem, M., Rawson, C., Lindburg, K. and Barnes, D. (1990), Transforming growth factor β regulates cystatin C in serum-free mouse embryo (SFME) cells. *Bioch. Biophys. Res. Comm.* 172:945-951.

Solem, M., Helmrich, A., Collodi, P., Barnes, D. (1991), Human and mouse S-protein detected in Northern blot experiments and evidence for the gene encoding S-protein in mammals by Southern blot analysis. *Mol. Cell Bio.* 100:141-149.

Southern, E.M. (1975), Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

Sporn, M.B., Roberts, A.B., Wakefield, L.M., Assoian, R.K. (1987), Transforming growth factor β : biological function and chemical structure. *Science* 233:532-534.

Sporn, M. and Roberts, A. (1988), *Nature* 332:217-219.

Stenn, K.S. (1981), Epibolin: a protein of human plasma that supports epithelial cell movement. *Proc. Natl. Acad. Sci., U.S.A.* 78:6907-6911.

Sun, Q. (1989), Growth stimulation of 3T3 fibroblasts by cystatin. *Exp. Cell Res.* 18:150-160.

Suzuki, S., Oldberg, A., Hayman, E., Pierschbacher, M.D., Ruoslahti, E. (1985), Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J.* 4:2519-2524.

Swergold, G.D. (1990), Identification, characterization, and cell specificity of a human LINE-1 promoter. *Mol. Cell Biol.* 10:6718-6729.

Tavera, C., Leung-Tack, J., Prevot, D., Gensac, M.C., Martinez, J., Fulcrand, P., Colle, A. (1992), Cystatin C secretion by rat glomerular mesangial cells: autocrine loop for in vitro growth-promoting activity. *Bioch. Bioph. Res. Comm.* 182:1082-1088.

Temin, H.M., Pierson, R.W., and Dulak, N.C. (1972), The role of serum in the control of multiplication of avian and mammalian cells in culture. In: Rothblat, G. and Cristofalo, V.J. (eds): "Growth, Nutrition and Metabolism of Cells in Culture," Academic Press, NY, 1:50-81.

Thomas, T., Schreiber, G., Jaworowski, A. (1989), Developmental pattern of gene expression of secreted proteins in brain and choroid plexus. *Dev. Biol.* 134:38-47.

Varmus, H. (1987), Cellular and Viral Oncogenes. In the Molecular Basis of Blood Diseases (G. Stamatoyannopoulos, A.W. Nienhuis, P. Leder, P.W. Majerus, eds.), 271-345, Philadelphia, Saunders.

Wagner, S.L., Geddes, J.W., Cotman, C.W., Lau, A.L. (1989), Protease nexin I, an antithrombin with neurite outgrowth action is reduced in Alzheimer's disease. *Proc. Natl. Acad. Sci., U.S.A.* 86:8284-8288.

Wahl, S.M., Allen, J.B., McCartney-Francis, N., Morganti-Kossmann, M.C., Kossmann, T., Ellingsworth, L., Mai, U.E., Mergenhagen, S.E., Orenstein, J.M. (1991), Macrophage and astrocyte-derived transforming growth factor β as a mediator of central nervous system dysfunction in acquired immune deficiency syndrome. *J. Exp. Med.* 173:981-991.

Yarden, Y. and Ullrich, A. (1988), Growth factor receptor tyrosine kinases.. *Ann. Rev. Biochem.* 57:443-478.

Yankner, B.A., Dawes, L.R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M.L., Neve, R.L. (1989), Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science.* 245:417-420.

Yankner, B.A., Duffy, L.K., Kirschner, D.A. (1990), Neurotrophic and neurotoxic effects of amyloid β protein: reversal by Tachykinin neuropeptides. Science 250:279-282.

Zucker-Franklin, D., Warfel, A., Grusky, G., Frangione, B. and Tzeitel, D. (1987), Novel monocyte-like properties of microglial/astroglial cells. Constitutive secretion of lysozyme and cystatin C. Lab. Inv. 57:176-185.

APPENDIX

Cystatin C release into SFME conditioned medium

INTRODUCTION

We have detected a message that encodes the cysteine protease inhibitor, cystatin C, that is strongly induced by TGF β in serum free mouse embryo cells. Cystatin C is a secreted protein and an inhibitor of the cathepsins B, H, and L, as well as the cysteine protease, papain. We wished to determine the time course of cystatin C's release into the conditioned medium of SFME treated with TGF β by measuring it's ability to inhibit papain protease activity.

METHODS

Cystatin C was quantitated as described (Lah et al., 1989) using chicken egg white cystatin (Sigma) as a standard. SFME were grown in the presence of TGF β (10 ng/ml) for 0, 3, 8, 18, 28, 48, and 72 h. The conditioned medium was harvested and was immediately freezed at -88°C until it was used for the experiments. Commercial papain (Type III) was purchased from Sigma. A stopped assay was performed to fluorimetrically measure the inhibition of papain cleavage of the substrate Z-Phe-Arg-Methoxy- β -naphthylamide (Enzyme System Products). The enzyme (final concentration, 1.0 nM) was preincubated for 10 min. at 25°C with the appropriate dilution of the standard inhibitor, chicken cystatin (final concentrations 0, 0.15, 0.30, 0.60, 1.0, and 3.0 nM) in the presence of 10 mM DTT, 0.2 M citrate buffer (pH 6.2), 1.0 mM EDTA, 2.0% F12/DMEM (final volume, 800 μl). Similarly, the TGF β -treated SFME conditioned medium (2.0%) for each timepoint was incubated with the enzyme. After 10 min. at 25°C , the substrate was added (final concentration, 0.5 mM), and the reaction was allowed to proceed for 15 min. at 25°C . The reaction was stopped by addition of 400 μl of 1 N HCl. The increase in fluorescence was measured at an excitation wavelength of 292 nm and emission wavelength of 410 nm. Buffer blanks were prepared by adding buffer instead of enzyme and inhibitor.

RESULTS

A stopped assay was performed to determine the cysteine protease inhibitory activity in the culture media of TGF β -treated SFME. It was assumed that papain-inhibition was completely due to the release of cystatin C into the media, since cystatin C was determined to be induced by TGF β by Northern blot analysis. A standard curve was made using chicken cystatin, and it was assumed that the inhibitory activities of cystatin C and chicken cystatin are equivalent under the experimental conditions. After 15 minutes of enzyme incubation with the substrate, the reaction was terminated and papain activity was measured. Chicken cystatin at concentrations ranging from 0 to 3.0 nM linearly reduced papain's cleavage of the substrate from 0 to 50%, as determined by spectrofluorimetric analysis. At higher concentrations of chicken cystatin the standard curve was non-linear. Using 2.0% SFME culture media, these samples gave values that fit into the linear portion of the standard curve. There appears to be a linear increase with time of cysteine protease inhibitory activity released into the TGF β -treated SFME conditioned medium. After 48 hours of TGF β treatment, the culture medium of SFME had approximately 34 nM amounts of cystatin C (Fig. 3.8).

DISCUSSION

After 48 hours of TGF β exposure, cystatin C mRNA is increased 20-fold in SFME. We also see an increase in the cysteine protease inhibitory activity present in SFME culture medium. Our initial attempts to quantitate the activity may be further improved by several means: The concentration of papain was used according to the assigned values of the manufacturer. We may increase the purity of the enzyme by gel filtration, and a microscale titration analysis of the active site can be gotten using synthetic irreversible inhibitors, like α -bromo-4-hydroxy-3-nitroacetophenone. Similarly, we may increase the purity of the chicken cystatin standard by gel filtration or affinity chromatography, and determine it's molar concentration and inhibitory activity once the precise concentration of papain is known. We may want to examine the relative stability of cystatin C released into the culture medium; this may be reduced by secreted proteases, or proteases released into the medium from degenerating cells. The effects of storage on the protease inhibitory activity of the conditioned medium may be examined by measuring it's activity before and after freezing the medium. We may want to repeat the experiment and isolate media from TGF β -treated SFME at many time points in triplicate, since the concentration values we have assigned are from a single data

point at all the timepoints we previously examined. A determination of the inhibition constant of cystatin C in SFME media may be made by a continuous assay of papain activity to measure the rate constants in the absence and presence of inhibitor. A comparison with the known values for papain and cystatin C activity constants should be made. Finally, cystatin C can be purified from the culture media by standard methods (Lah et al., 1989) to demonstrate that TGF β induction of cystatin C involves the secretion of the final product.