b. Fibronectin

Extracellular matrix is composed of collagen, proteoglycan, and another class of proteins that were initially identified as "noncollagenous structural proteins" (Robert et al., 1970). The study of fibronectin began with the report of Morrison et al., (1948) showing a high molecular weight plasma protein that co-precipitated with fibrinogen at low temperatures. This protein was therefore termed Cold Insoluble Globulin (CIG). Later, other investigators reported the existence of a high molecular weight glycoprotein on the surface of cultured fibroblasts (Hynes, 1974; Hogg, 1974; & Vaheri & Ruoslahti, 1974). This protein was shown to be lost following transformation of cells in vitro (Hynes & Bye, 1974). Therefore it was deslgnated: Large, External, Transformation Sensitive Protein (LETS). It was later shown that CIG and LETS were the same protein (Ruoslahti & Vaheri, 1975). Today these proteins are designated plasma and cellular fibronectin respectively. While it is now known that they are largely identical in sequence, there are certain differences in amino acid sequence and glycosylation (Yamada & Kennedy, 1979).

Immunofluorescent studies have demonstrated that cellular fibronectin is arranged in a fibrillar network on the external surface of a wide range of cell types, including: fibroblasts from many different tissues, glial cells, endothelial cells, chondrocytes, hepatocytes, and macrophages (Hynes & Yamada, 1982). This fibrillar network appears to be formed by the polymerization of fibronectin dimers. Each subunit is composed of two 220 kDa fragments bound together by two disulfide bonds near the carboxy-terminal end. Plasma fibronectin occurs in the form of dimers, and cellular fibronectin is composed of dimers and hexamers called hexabrachions (Erickson & Inglesias, 1984). Fibronectin fibrils appear to be formed by polymerization of the molecules by disulfide bond exchange near the disulfide bond rich aminoterminal end (McKeown-Longo & Mosher, 1984) and by covalent crosslinkage by transglutaminase (Keski-Oja et al., 1976; Grinnell & Feld, 1979; Williams et al., 1983; and Fellin et al., 1988). Fibronectin in the dimer or hexabrachion form is secreted into the medium
of cultured fibroblasts, or may be removed from the cell surface by means of a wash with a low concentration of urea (Erickson & Inglesias, 1984). This form of fibronectin would therefore be extractable in the culture medium as a secreted protein. When fibronectin is covalently crosslinked into large molecular weight fibrils, it becomes insoluble - even in such detergents as Triton X-100 and sodium deoxycholate, and is therefore considered to be in a distinct pool from the secreted form.

The fibronectin molecule contains several domains that facilitate its specific interaction with various other extracellular matrix molecules. These binding domains are in turn connected by protease-sensitive regions that allow the molecule to display numerous functions depending on its domain composition after specific proteolysis. The domains include attachment sites for heparin, gelatin, collagen, proteoglycans, fibrin, and a site that interacts with a specific cell surface receptor (Furcht, 1983; Yamada, 1983).

Numerous studies indicate that fibronectin plays an important role in growth and development. For example, it is present in undifferentiated mesenchyme of embryonic kidney (Wartiovaara et al., 1978a). However, when these cells differentiate into the epithelial cells of the renal tubules, this fibronectin is lost (Wartiovaara et al., 1976). Similarly, there is a marked alteration in the distribution of fibronectin during myoblast differentiation into myotubes (Furcht et al., 1978) and exogenous fibronectin addition to myoblasts in vitro can retard myoblast differentiation and fusion (Podleski et al., 1979). The use of teratocarcinoma cells has demonstrated that pluripotent stem cells synthesize and secrete fibronectin into pool I but do not incorporate a significant amount into pool II (Wolfe et al., 1979). However, when these cells differentiate into endothelial cells, they incorporate a large level into pool II (Wartiovaara et al., 1978b).

The expression of fibronectin in dividing cells, such as fibroblasts, appears to vary with the cell cycle. Little, if any, fibronectin is detectable on the surface of fibroblasts during mitosis when the cells round up and are relatively unattached to the substrate (Hynes & Bye, 1974).
Levels are constant throughout S and G$_2$ phase, and markedly elevated during G$_1$. The mechanisms by which the cycling cell periodically frees itself from this fibrillar mesh by the redistribution of fibronectin and rounds up is an interesting problem and is thought to involve plasma membrane proteolytic enzymes (Chen et al., 1985).

An understanding of fibronectin biochemistry is thought to be necessary for an understanding of several pathological processes, such as cancer and atherosclerosis. Cellular transformation frequently results in a loss of normal cell-cell interactions with the result that cultured cells lose contact inhibition of growth (Vogt & Oulbecco, 1960; Borek & Sachs, 1966). This altered social behavior has been correlated with changes in proteins associated with the plasma membrane and the extracellular environment (Burger, 1969; Benjamin & Burger, 1970; Eckhart et al., 1971). Urea treatment (150mM-1.0M) has also been shown to cause reversible transformation (loss of contact inhibition) of cultured cells apparently by the removal of inhibitory peptides from the cell surface (Weston & Hendricks, 1972 Weston et al., 1979) (Pool I). The observation that fibronectin is frequently lost from the surface of transformed cells, led Ali et al., (1977) to ask whether it could be the inhibitory activity removed by urea. They demonstrated that exogenously-added fibronectin did return virally-transformed cells partly to a normal phenotype.

3. Secreted Proteases

The maintenance and restructuring of the extracellular matrix lattice in growth, development, adult homeostasis, and finally aging, involves multi-step processes including the proteolysis of existing extracellular matrix, alterations in resident cell cytoskeleton, cell translocation, and the redeposition of new extracellular matrix (Liotta et al., 1983; Ruoslahti, 1984). Increased proteolysis appears to be the initial step in this process in both normal metabolism (Eisen, 1969; Roswit et al., 1983; Blair, et al., 1986) and pathological metabolism
(Bauer & Eisen, 1978; Kronberger et al., 1982; Birkedal-Hansen, 1980). The degradative side of the activity is controlled by the action of extracellular proteases. This proteolytic pathway appears to involve numerous proteases. However, for the purposes of this review, I will discuss some of the most abundant and best-characterized of the enzymes and their respective inhibitors. These will include serine proteases that are capable of selectively activating plasminogen into its active form, plasmin, and a metalloprotease capable of initiating the breakdown of collagen; namely, collagenase.

**a. Plasminogen Activators/Inhibitors**

The potent proteolytic capacity of fibroblasts was first observed in the early days of tissue culture when the cells were cultured on clotted blood. It was observed that cultured cancer cells caused the dissolution of the fibrin clots (Carrel & Burrows, 1911; Lambert & Hanes, 1911; Losee & Ebeling, 1914). Fischer (1946) suggested that this proteolytic activity was derived from the activation of an inactive proenzyme in the serum. Goldhaber et al., (1947) called this inactive proenzyme profibrinolysin (plasminogen) and the activated form fibrinolysin (plasmin). The existence of plasminogen and its conversion to plasmin was demonstrated by Christensen (1945) and Kaplan, (1944, 1946). It was later shown that plasminogen is activated by limited proteolysis by a family of enzymes called plasminogen activators (Robbins et al., 1967). This understanding of the mechanism of plasminogen activation led to an early recognition of a correlation between cancer and fibrinolytic activity (Tagnon et al., 1953; Astedt et al., 1971). When it was reported that there is not a uniform correlation between plasminogen activator activity and the malignant phenotype, much of the early enthusiasm was lost (Astrup, 1978). However, the discovery of the complex regulation of plasminogen activators and the discovery that transformation by oncogenic viruses caused a marked increase in activity, has stimulated renewed interest (Reich, 1973; Unkeless et al., 1973; Quigley et al., 1974).
Fig. 3 Extracellular Protease Activity in Human Skin Fibroblasts. Schematic representation of the growth factor-responsive proteases regulating the turnover of connective tissue matrix. Solid arrows indicate protease activity while shaded arrows indicate inhibitory activity. (PAI - Plasminogen Activator Inhibitor; TIMP - Tissue Inhibitor of MetalloProteinases; PA-PAI – Plas-minogen Activator - Plasminogen Acti-vator Inhibitor complex; PA - Plas-minogen Activator; Pro-PA – Pro-enzyme form of Plasminogen Activator; Pro-CL - Procollagenase; Pro-SL – Pro-stromelysin).
It was later reported that plasminogen activators were also released by normal cells (Fleischer & Loeb, 1915; Santesson, 1935). Plasminogen activators were then isolated from a number of tissues (Astrup & Permin, 1947; Albrechtsen, 1957), urine (Williams, 1951; Astrup & Stage, 1952) and blood (Lewis & Furguson, 1951; Sherry et al., 1959). These studies led to the recognition of two types of plasminogen activator in human tissues that could be distinguished on the basis of molecular weight and antigenicity (Kucinski et al., 1968; Granelli-Piperno & Reich, 1978; and Nielsen et al., 1983). The first of these was isolated from urine, and was therefore designated u-PA or urokinase. The second was isolated from tissue extracts and was therefore designated t-PA. These enzymes have relative molecular weights of approximately 50 kDa and 70 kDa respectively. Like most secreted proteases, u-PA is secreted as an inactive proenzyme. The proenzyme is a single-chain protein that is converted to an active two-chain form by limited proteolysis (Nielsen et al., 1982; Eaton et al., 1984). It has been demonstrated that the proenzyme is unable to activate plasminogen (Skriver et al., 1982; Nielsen et al., 1982), but after activation by such proteases as plasmin itself, it acquires full proteolytic activity toward plasminogen (Nielsen et al., 1982; Eaton et al., 1984). There is, in other words, a positive feedback mechanism operating in plasminogen activation.

The heavy chain of the active u-PA molecule is also called the B-chain and is approximately 30 kDa, and the light chain (or A-chain) is approximately 20 kDa. A variant form of u-PA is frequently isolated from urine. This variant has an identical B-chain, and a proteolytically cleaved A-chain. This gives the variant form a relative molecular weight of about 30 kDa (Soberano et al., 1976a, 1976b). The plasminogen activator isolated from tissues, t-PA, also exists in two forms, a one-chain and two-chain form. The one-chain form is converted into the active two-chain form by limited proteolysis (Binder et al., 1979; Wallen et al., 1982, 1983).
Regulation of plasmin and plasminogen activator activity is also controlled by the synthesis and secretion of inhibitors; namely, α2-antiplasmin and plasminogen activator inhibitors (PAIs) respectively. PAIs can be classified into three immunologically distinct groups: endothelial-type inhibitors (PAI-1), placental-type inhibitors (PAI-2), and protease nexin I. PAI-1 is synthesized by endothelial cells (Loskutoff et al., 1983; Van Mourik et al., 1984), fibroblasts (Laiho et al., 1986a), vascular smooth muscle cells (Laung, 1985), and other cell types. PAI-1 is a 48kDa glycoprotein that forms a covalent complex with plasminogen activator, thereby inactivating it (Levin, 1983; Thorsen & Phillips, 1984). PAI-1 has been identified in both Pool I and Pool II of cultured fibroblasts (Levin & Santell, 1987) and deposition is increased markedly after exposure to TGF-β (Laiho et al., 1986a).

The early assay methods of plasminogen activator activity did not distinguish the different types of plasminogen activators or the effects of plasminogen activator inhibitors (Christman et al., 1977). More recently-described techniques allow the use of assays with plasminogen as the substrate and with u-PA and t-PA distinguished by the use of anticatalytic antibodies (Kucinski et al., 1968; Ossowski & Reich, 1983). The most widely used assay system uses a fibrin/plasminogen impregnated gel onto which samples are applied (Astrup & Mullertz; Lassen, 1953; Haverkate & Brakman, 1975). Quantitation is accomplished by means of the co-application of plasminogen activator standards. Saksela (1981) described a similar system utilizing casein instead of fibrin as the final substrate. It is also possible to detect plasminogen activators and plasminogen activator inhibitors after electrophoresis in a Laemmli gel (Granelli-Piperno & Reich, 1978; Heussen & Dowdle, 1980; Erickson et al., 1984). The approach allows the simultaneous observation of different molecular weight proteins and allows the determination of the relative levels of activity in individual protein bands.

Initially, it was thought that the only substrate for plasminogen activators was plasminogen. However, it has recently been demonstrated that fibronectin binds plasminogen and t-PA (Salonen et al., 1985) and is specifically cleaved by plasminogen activator derived from
transformed cell-conditioned medium from 220 kDa to approximately 200 kDa (Quigley et al., 1987). Another substrate was reported by Keski-Oja and Vaheri (1982) found that one target of u-PA is a 66 kDa protein observed in the deoxycholate-insoluble extracellular matrix. They report that treatment of the matrix with exogenous u-PA resulted in a shift in molecular weight of the 66 kDa band to approximately 62 kDa. While the identity of this protein remains unknown, its abundance and specific lability to plasminogen activator suggest an important role in the molecular pathways of matrix metabolism.

I will now discuss putative roles for the plasminogen activators in normal connective tissue metabolism. As mentioned above, the activation of plasminogen activators leads to site-specific proteolysis by the plasminogen activators themselves, and the activation of plasminogen. Once activated, plasmin is a wide-spectrum serine protease with sequence homology to trypsin and pancreatic elastase (Robbins et al., 1981; Castellino & Powell, 1981). An important target for plasmin appears to be procollagenase (Werb et al., 1977). Partial proteolysis of procollagenase leads to the activation of the enzyme. Similarly, plasmin appears to activate other proteolytic proenzymes; such as proelastase (Chapman & Stone, 1984). Plasminogen activators are therefore considered to be important activators of a proteolytic cascade leading to tissue destruction. Important examples of plasminogen activators in action are: their role in mammary gland involution following lactation (Ossowski et al., 1979), follicle wall dissolution during ovulation (Strickland, 1978), and the implantation of trophoblastic cells in the uterus (Strickland et al., 1976; Strickland & Mahdavi, 1978).

b. Neutral Proteases/Inhibitors

I will now discuss a family of neutral metalloproteinases distinguished by their ability to directly initiate the degradation of many extracellular matrix structural components. These include a collagenase from fibroblasts (Stricklin et al., 1977; Wilhelm et al., 1986; and Goldberg et al., 1986) and granulocytes (Lazarus et al., 1968; Macartney & Tschescche, 1983;
(Hasty et al., 1986) capable of initiating breakdown of interstitial collagen, a collagenase specific for type IV basement membrane collagen and denatured interstitial collagens (Collier et al., 1988), and stromelysin, an enzyme capable of degrading elastin, proteoglycans, and other extracellular matrix components, but not collagen (Chin et al., 1985; Okada et al., 1986; Whitham et al., 1986). Okada et al., (1986) has suggested a system of terminology for these enzymes. These proteases are designated matrix metalloproteinase-1 (MMP-1), MMP-2, and MMP-3 respectively. In this report I will refer to them as collagenase (CL), 66 kDa gelatinase, and stromelysin (SL) respectively. Interstitial collagenase (MMP-1) cleaves Types I, II, III, and X collagen (Harris et al., 1984; Schmid et al., 1986). The 66 kDa gelatinase (MMP-2) cleaves Types IV and V collagens and the denatured collagen that result from the action of MMP-1 (Murphy et al., 1982; Seltzer et al., 1981; Collier et al., 1988).

Gross & Lapiere (1962) described the first vertebrate collagenase in extract derived from the tailfin of tadpoles undergoing metamorphosis. Since then, collagenolytic activity has been observed from numerous sources including fibroblasts (Bauer et al., 1975), osteoblasts (Otsuka et al., 1984), monocytes (Campbell et al., 1987), keratinocytes (Petersen et al., 1987), and postpartum uterus (Jeffre et al., 1971). The collagen triple helix confers resistance to proteolysis by all but a specific class of collagenases (Gross & Nagai, 1965). Mammalian collagenases possess the unique capacity to initiate collagen degradation by cleaving the triple helix at a single locus 3/4 of the distance from the amino-terminal end yielding the 3/4-length amino terminal fragment TC\textsuperscript{A} and the 1/4-length carboxy-terminal fragment TC\textsuperscript{B} (Gross & Nagai, 1965; Eisen et al., 1968; Harris & Crane, 1974). At temperatures below 34\textdegree C, these proteolytic fragments maintain their triple-helical structure and resist further proteolysis. However, at physiological temperatures, the fragments denature into gelatin which in turn is susceptible to degradation by a variety of proteases including two characterized human skin gelatinases of 68 (MMP-3) and 95 kDa. (Werb and Reynolds, 1974; Seltzer et al., 1981).
Human skin collagenase (MMP-1) has been purified and characterized (Stricklin et al., 1977, 1978). It is a major constituent of secreted protein and has been reported to total 3% to 6% of the secreted proteins from human skin fibroblasts (Valle and Bauer, 1979). It is secreted as a proenzyme, procollagenase (proCL) or in two forms with relative molecular masses of 52 and 57 kDa, as measured on SDS-PAGE gels. The 57 kDa band represents an N-linked glycosylated form, and the 52 kDa represents a relatively less glycosylated or unglycosylated form (Chua and Ladda, 1987; Wilhelm et al., 1986). Other molecular masses have been reported, including 50 kDa and 45 kDa bands (perhaps representing the proteolytically-activated forms (Stricklin et al., 1977, 1978).

Collagenase is a neutral proteinase consistent with its role in degrading collagen in the extracellular, neutral pH environment. However, this fact requires that CL activity be tightly regulated to prevent widespread tissue damage. For example, overabundant CL production is known to play an important role in pathology associated with rheumatoid arthritis (Harris et al., 1970) and metastatic tumors (Hashimoto et al., 1973). Such pathology results from a loss of the proper regulation of collagenase activity. It is now known that collagenolytic activity is regulated at several levels including: transcription, packaging and secretion of proCL, activation of proCL, CL interactions with collagen, and inhibition of CL by specific inhibitors (Harris et al., 1984).

Another level of regulation is the activation of proCL into the active CL form. The first reported method of proCL activation was by limited proteolysis with trypsin (Vaes, 1972). Later, other proteases such as plasmin were shown to activate proCL (Werb et al., 1977). Plasmin-mediated activation of proCL would appear to be an important means of activation because of the highly regulated activity of plasmin (described previously). As mentioned earlier, unregulated proCL, activation would lead to connective tissue pathology. For example, metastatic tumor cells frequently secrete unusually high levels of PA (Ossowski et
al., 1973; Unkleless et al., 1973) which in turn activate proCL and allow tumor cell invasion through connective tissue barriers (Paranjpe et al., 1980).

Numerous agents have been identified that influence CL expression in cultured fibroblastic cells. These include phorbol-13-myristate acetate (PMA) (Brinckerhoff et al., 1979), exogenous proteases (Werb & Aggler, 1978), cytochalasin B (Harris et al., 1975), and poly(2-hydroxyethylmethacrylate) (Aggler et al., 1984b) which interfere with cell attachment and spreading. All of these agents share the property of possessing the capacity to disrupt cell attachment and spreading. Aggler et al., (1984b) noted the correlation of altered cell morphology and collagenase gene expression.

Stromelysin (MMP-3) is a wide-spectrum protease with substantial sequence homology to collagenase (Saus et al., 1988) and is apparently the human homolog of the rat protein transin (Matrisian et al., 1986). Its substrates include fibronectin, laminin, Type IV collagen, and cartilage proteoglycans. SL also appears to be an activator of proCL (Murphy et al., 1987). SL is seen as a doublet with 60 and 57 kDa bands corresponding to the more highly N-linked glycosylated and unglycosylated forms of proSL (Wilhelm et al., 1987). SL and CL are reported to be jointly regulated (Chin et al., 1985) and this is apparently due to coordinated activation of gene expression (Frisch et al., 1987).

There is at least one inhibitor of the active form of CL and SL. This inhibitor is a highly N-glycosylated protein of Mr= 28,500 which has been biochemically characterized (Stricklin & Welgus, 1983). When treated with N-glycosidase, the core protein has a relative molecular weight of 21 kDa (Stricklin, 1986). This protein has been designated by the acronym "TIMP" for Tissue Inhibitor of MetalloProteinases (McGuire et al., 1981; Murphy et al., 1981). Imbalances in TIMP/CL activity have been implicated in a number of pathological processes related to connective tissue morpholysis such as arthritis, cancer, and periodontal disease (Reynolds, 1986).
Collagenase activity may be assayed directly or by gel zymography as described for plasminogen activator. Hu et al. (1978) described a technique in which radiolabeled reconstituted collagen fibrils are reacted with samples. After varied times, the collagen fibrils are centrifuged into a pellet and the radioactivity remaining in the supernatant represents fragments cleaved by collagenase. Zymography is performed by electrophoresing samples in a gel impregnated with gelatin in a manner analogous to that described by Heussen and Dowdle (1980). This allows the visualization of both CL and the 68 and 95 kDa gelatinases. CL dissolves the gelatin substrate because it has a secondary gelatinolytic activity that is probably enhanced by SDS denaturation (Welgus et al., 1982). The use of casein as a substrate allows the visualization of SL as well (Saus et al., 1988). Modifications of this technique also allow the visualization of metalloproteinase inhibitors (Herron et al., 1986b).

4. Cellular Transformation and the Extracellular Matrix

Transformation alters the pattern of protein expression in manifold and diverse ways in different cells. However, one alteration that is almost universal is the decrease in collagen Type I and fibronectin synthesis in transformed fibroblasts (Bornstein & Sage, 1980; Setayama et al., 1985; Alema et al., 1985; Vaheri et al., 1978; Krieg et al., 1980; Hata and Peterkofsky, 1977; Levinson et al., 1975; Setoyama et al., 1985; Pearlstein et al., 1980) and an alteration in proteoglycan metabolism (Kramer et al., 1982).

The decrease in the production of Type I collagen appears to be regulated by a trans-acting transcriptional regulator (Howard et al., 1978; Sandmeyer et al., 1981). Setoyama et al. (1985) demonstrated this very clearly with NIH 3T3 cells transformed with transfected v-mos gene. These cells, when transfected with a collagen promoter linked to the neo gene, demonstrated G418 resistance under the control of v-mos.

Transformation of fibroblastic cells by SV40 virus produces severe alteration in extracellular matrix proteins (Krieg et al., 1980; Trueb et al., 1985). Krieg et al. (1980) reported less
collagen and fibronectin production in SV40-transformed fibroblasts. Cells transformed by
tumor viruses or other means also frequently express large quantities of proteinases; this,
combined with their lack of production of extracellular matrix, allows these cells to migrate
easily as would be consistent with cells involved in embryological development. This migration
or metastasis is frequently seen in histological sections of tumors where malignant cells
penetrate as "tongues" into the normal surrounding tissue (Robbins and Cotran, 1979).

5. Hormonal Regulation of Structural and Proteolytic Molecules

Serum growth factors are potent modulators of extracellular structural protein and protease
synthesis. This is in congruence with the fibroblasts role in remodeling the extracellular matrix
following tissue injury.

Fibronectin production is markedly induced following serum stimulation in cultured fibro-
blasts. Chen et al., (1977) reported that this induction appeared to be due to epidermal
growth factor; since only this factor, and not insulin, hydrocortisone, somatomedin B or C, or a
number of other factors and hormones were able to increase levels of fibronectin on the cell
surface. Blatte et al., (1988) reported that fibronectin mRNA transcription is induced within 10
min. of stimulation with FBS or epidermal growth factor in rat AKR-2B cells.

Serum growth factors also stimulate collagen synthesis and alter the relative production of
type III and V collagen (Narayanan & Page, 1977; 1983). PDGF, TGF-β, and IL-1 stimulate
collagen production (Narayanan & Page, 1983; Wrana et al., 1986; Ignotz & Massague, 1986;
Postlethwaite et al., 1988; Goldring & Krane, 1987).

Plasminogen activator gene expression is also stimulated by serum growth factors. Pro-
duction of the plasminogen activator, u-PA, is increased by exposure to epidermal growth
factor (Lee & Weinstein, 1978; Eaton & Baker, 1983) and colony stimulating factor (Hume &
Gordon, 1984). This stimulation is reflected by an increase in the level of u-PA mRNA in
mouse fibroblasts stimulated with both 10% FCS and epidermal growth factor (Grimaldi et al.,
1986). The potent tumor promoter phorbol 12-myristate 13-acetate (PMA) acts as a mitogen similar in many respects to PDGF (Rabin et al., 1986; Grunberger et al., 1984) by mimicking the action of diacylglycerol. PMA acts as a potent inducer of PA (Wigler & Weinstein, 1976; Waller & Schleuning, 1985; Stopelli et al., 1986), PAI (Eaton & Baker, 1983), and collagenase.

Metalloproteinases are also regulated at the level of transcription by various cytokines. For example, proCL synthesis is stimulated by EGF (Chua et al., 1985), PDGF (Chua et al., 1985; Bauer et al., 1985), β-TGF (Chua et al., 1985), and IL-1 (Oppenheim et al., 1982; Stephenson et al., 1987). In the case of PDGF-stimulation, proCL secretion occurred 8 to 10 hours after treatment (Bauer et al., 1985). TGF-β is unique in its ability to jointly inhibit the production of proCL and stimulate the production of TIMP (Edwards et al., 1987). One of the most potent inducers of proCL discovered to date is the phorbol ester 12-O-tetradecanoylphorbol acetate (TPA). Upon stimulation of rabbit synovial fibroblasts with TPA, up to 23% of the newly secreted protein was proCL (Aggeler et al., 1984).

6. Alterations in the Extracellular Matrix During Aging

Senescent cells have been reported to be defective in their ability to attach to extracellular matrix (Chandrasekhar et al., 1983a, b). It has been reported that senescent cells produce fibronectin (Chandrasekhar and Millis, 1980), though researchers disagree as to whether there are significant differences in the type of fibronectin produced. Vogel et al. (1981) report that there is a loss of FN from the surface of fibroblasts as they age in vitro.

The molecular basis of these observations is complicated by contradictory reports regarding FN content and distribution in young and senescent fibroblasts. A decrease in cell surface-associated FN measured by indirect immunofluorescence and an associated increase of FN in the CM as determined by radioimmunoassay has been reported (Vogel et al., 1981; Oka, 1985). In contrast to these studies, an increase in cell-associated FN, and a decrease in FN
from conditioned medium has been reported (Geuskens et al., 1986; Shevitz et al., 1986; Poot et al., 1986) Several of these studies are difficult to interpret because of a lack of data presented regarding cell density, cell feeding schedule, and cell protein content, all of which influence FN distribution (personal observation).

D. Statement and Testing of the Hypothesis

In the preceding review of the theories of aging I have presented evidence in favor of the programmed theory of aging. I reviewed the nature of normal biological aging and genetically-inherited premature aging syndromes and discussed how they lend support to the concept that aging may be due in large part to alterations in molecular pathways widely used in tissues throughout the body - in particular the connective tissues. On a cellular level, a loss of proliferative homeostasis is observed, characterized by a loss of cell number in some cases, and multifocal hyperplasia in others. I also discussed at some length evidence in support of the theory that there exists a dominant inhibitor of DNA replication associated with the plasma membrane and the extracellular matrix in senescent cells. In the review of characterized growth inhibitors I pointed out that many of these inhibitors have a potent effect on extracellular matrix protein synthesis in addition to inhibiting cell growth.

I discussed the evidence that normal fibroblasts early in their replicative lifespan respond to serum growth factors in a dynamic fashion by altering their synthesis of structural proteins and extracellular proteolytic enzymes. In contrast to normal cells, cellular transformation with immortalization leads to dramatic effects on extracellular matrix protein synthesis, frequently by boosting the synthesis of extracellular proteases. Senescent fibroblasts no longer respond to serum growth factors by entering the cell cycle, instead they appear to be blocked in a late stage of G1 which we have called Gs. These results lead me to the hypothesis that senescent fibroblasts are also unable to respond in a dynamic fashion to serum growth factors by elaborating extracellular matrix components in an appropriate fashion. This
unresponsiveness could abrogate the regulation of extracellular matrix protein expression and could lead to tissue matrix morpholysis.

I hypothesize that senescent fibroblasts are chronically fixed in a cell cycle phase distinct from G0. I further hypothesize that senescent fibroblasts are growth arrested by a genetically-programmed molecular pathway that interferes not only with the proliferative component of fibroplasia, but also with growth factor regulation of fibroblast function and connective tissue metabolism (such as the regulation of structural protein and proteolytic enzyme synthesis). I suggest that whereas a young fibroblast is induced to leave the quiescent phenotype to enter fibroplasia and thereby acquire a fibroclastic phenotype, the senescent fibroblast is permanently locked into a fibroclastic one. The corresponding result of cellular senescence in vivo would be connective tissue morpholysis and could have multiple sequelae, including a loss of proliferative homeostasis due to the destruction of healthy matrix structure and the release of proteolytic fragments of such proteins as fibronectin, elastin, and collagen, which are known to possess cytokine and chemotactic activity.

To test my hypothesis, I have chosen the cultured skin fibroblast as the model system for aging. Since it is well documented that aging fibroblasts lose their responsiveness to serum growth factors, I will study aging fibroblast function in response to stimulation with medium supplemented with 10% FBS as a source of growth factors. Specifically, I will harvest extracellular proteins in the 200 mM urea-extractable pool (Pool I) and the deoxycholate-insoluble pool (Pool II) from young cells that are subconfluent, and quiescent because of low growth factor concentration, young cells that are fully confluent and unable to growth because of density dependent inhibition, and senescent fibroblasts that are subconfluent and unable to grow due to senescent inhibition of growth. The characterization of alterations in extracellular matrix metabolism in young and senescent cells in these conditions should allow me to determine whether senescent cells are competent to enter G0 when placed in a relatively low concentration of serum growth factors and whether they are competent to
respond to serum stimulation by the appropriate alterations in extracellular protein synthesis. The use of serum deprivation with subsequent serum stimulation will also allow the characterization of cell cycle phase on the expression of extracellular proteins. Since the expression of all of the extracellular structural proteins and proteolytic enzymes discussed previously is dependent on cell density, serum concentration, and other cell culture variables, it will be necessary to control for these factors by appropriate cell plating and feeding schedules.

Specific objectives to test the hypothesis are:

1. Examine extracellular proteins from the 200 mM urea-soluble fraction (Pool I) and the deoxycholate-insoluble fraction (Pool II) of young-SC, young-DDI, and senescent-SC cells grown under steady-state conditions and identify possible markers for these different growth states.

2. Determine whether cellular quiescence and senescence can be distinguished using these extracellular protein markers.


4. Quantitate alterations in structural and proteolytic proteins in Pool I and II during serum stimulation.

5. Measure the net proteolytic activities of plasminogen activator and collagenase in Pool I and II from young and senescent cells during serum stimulation and visualize these activities by zymography.

Finally I will discuss these results in the light of the current literature to determine whether alterations in extracellular protein synthesis observed during cellular senescence in vitro aid in the understanding of connective tissue aging in vivo.
III. MATERIALS AND METHODS

A. Buffers

1. **BLOTTO**: 5% (w/v) non-fat dry milk, 0.01% (v/v) antifoam, 0.002% (w/v) thimerosal, 500 mM NaCl, 20 mM Tris-HCl, pH 7.5.

2. **CMF-PBS (Calcium and Magnesium-Free Phosphate Buffered Saline)**: 10 mM sodium phosphate buffer, pH 7.2, and 140 mM sodium chloride. [Also known as Dulbecco's phosphate-buffered saline (solution A)].

3. **RIPA**: 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.01 M Tris-HCl pH 7.2, 1% Trasylol.

4. **SALT WASH BUFFER A**: 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.2, 0.1% (v/v) NP40.

5. **SALT WASH BUFFER B**: 1.0 M NaCl, 1.0 mM EDTA, 0.01 M Tris-HCl, pH 7.2, 0.1% (v/v) NP40, 0.3% (w/v) SDS.

6. **SALT WASH BUFFER C**: 0.01 M Tris-HCl, pH 7.2, 0.1% (v/v) NP40.

7. **TE (Tris-EDTA Buffer)**: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

8. **TES (Tris, EDTA, SDS Buffer)**: 10 mM Tris-HCl, pH 7.4, 5.0 mM EDTA, 1.0% SDS.

9. **(Tris Buffered Saline)**: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5

10. **TTBS (Tris Buffered Saline with Tween 20)**: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% Tween 20.

11. **ZLB (Zymography Loading Buffer)**: (4X) 10% SDS, 4.0% sucrose, 0.25 M Tris-HCl, pH 6.8, 0.1% Bromphenol Blue.
B. Cells

**JAS-3 (CSC-303)** Normal diploid skin fibroblasts were designated JAS-3 and were obtained in this laboratory from a neonatal foreskin. Cells that are early in their in vitro lifespan and in a state of logarithmic growth will be designated "young". Fibroblasts that are fully arrested by contact inhibition and two weeks in the presence of 1% fetal calf serum will be designated "quiescent". Finally, fibroblasts that are subconfluent and arrested by cellular aging mechanisms while in the presence of 10% serum will be designated "senescent". Only the senescent cells were of clonal origin and those used in this study were designated "clone G".

**U-937** These cells (ATCC CRL 1593) were established from malignant cells in a pleural effusion from an adult male with histiocytic lymphoma. Upon treatment with phorbol myristate acetate, they assume a macrophage-like morphology, and secrete large quantities of collagenase and gelatinase into the medium (George Stricklin, personal communication) and can be used in the preparation of collagenase inhibitor zymography.

C. Tissue Culture

The cells were maintained in Eagle's minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 28 mM HEPES, 7.5% NaHCO$_3$, and 50 mg/ml gentamicin sulfate. The medium was changed weekly and confluent monolayers were serially propagated by trypsinization (Pereira-Smith et al., 1985).

D. Time Course Serum Stimulation/Deprivation

In studies of serum stimulation, young-SC and Senescent-SC cells were seeded at a subconfluent density (an approximately equal protein content per cm$^2$) in 10% FCS for four hours to promote attachment. Then, young-SC, young-DDI, and senescent-SC cells were changed to 0.5% FCS for five days. All cells were re-fed two days prior to experimental
**Fig. 4 Serum Stimulation/ Deprivation Protocol.** Schematic representation of the protocol used for the serum stimulation or deprivation of cell cultures in both Pool I and Pool II extractions.
Plate Cells

Serum Stimulation

<table>
<thead>
<tr>
<th>10% FCS</th>
<th>1/2% FCS</th>
<th>Re-feed 1/2% FCS</th>
<th>10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Hr</td>
<td>3 Days</td>
<td>2 Days</td>
<td>0-24 Hrs</td>
</tr>
</tbody>
</table>

Serum Deprivation

<table>
<thead>
<tr>
<th>10% FCS</th>
<th>10% FCS</th>
<th>1/2% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC 2 Days</td>
<td>Split Cells / Inc. 4 Hr</td>
<td>1 Day-2 Wk</td>
</tr>
</tbody>
</table>

Rinse Monolayer 2X PBS

Metabolically Label 2 Hr. 50 uCi/mL $^{35}$S-Methionine

Extract Protein
manipulation. The cells were then stimulated with pre-warmed 10% FCS-supplemented medium for 0, 0.5, 1, 4, 8.5, 12, or 24 hours.

Studies of serum deprivation were designed as above with the exception that they were maintained on 0.5% FCS and harvested after 2, 4, 7, 10, and 14 days. The cells were re-fed two days prior to the day of harvest.

**E. Pulse/Pulse-Chase Labeling**

Cells were rinsed twice with CMF-PBS and then incubated for two hours at 37 deg C. in serum-free MEM medium containing 1/15th the normal concentration of L-methionine (1 mg/L vs. 15 mg/L), and 50 µCi/ml [³⁵S] methionine (1,128 Ci/mmol; New England Nuclear). Cells were then harvested in pulse labeling experiments. Pulse-chase labeling was achieved by the addition of an equal volume of 1.0 g/L (final 500X excess) cold methionine in serum-free medium for varying lengths of time.

**F. Urea-Mediated (Pool I) Protein Extraction**

All urea extractions were performed two days subsequent to feeding. On the second day, cell monolayers in T-150 cell culture flasks were rinsed twice with CMF-PBS and then incubated for two hours at 37 deg. C. in the presence of serum-free medium. The rinse with CMF-PBS and the serum-free medium aid in the removal of fetal serum proteins from the surface of the fibroblasts. The serum-free medium is then removed and 2 ml /T150 of freshly made 200 mM urea in CMF-PBS was added. The flasks were then placed on a rocker at 37 deg. C. for two hours. The urea solution was then removed and immediately frozen at -70 deg. C.
Fig. 5  Comparison of Protein Extraction Methods. Schematic representation of the protein extraction methods used for the preparation of urea-extracted protein (Pool I) and deoxycholate-insoluble extracellular matrix protein (Pool II).
### SUMMARY OF EXTRACELLULAR PROTEIN EXTRACTION METHODS

<table>
<thead>
<tr>
<th>Urea Soluble Protein (Pool I)</th>
<th>Deoxycholate Insoluble Protein (Pool II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse 2X CMF-PBS</td>
<td>Rinse 2X CMF-PBS</td>
</tr>
<tr>
<td>Incubate Two Hours in Serum Free Medium (w or w/o $^{35}$S-Methionine)</td>
<td>Incubate Two Hours in Serum Free Medium (w or w/o $^{35}$S-Methionine)</td>
</tr>
<tr>
<td>Extract Protein in 200 mM Urea (2 Hr.)</td>
<td>Wash 3X 0.5% DOC, 10mM Tris (10 min, 0°C.)</td>
</tr>
<tr>
<td>Make 1 mM PMSF</td>
<td>Wash 3X 2mM Tris (5 min)</td>
</tr>
<tr>
<td>Dialyze w/ H$_2$O</td>
<td>Appropriate Buffer</td>
</tr>
<tr>
<td>Lyophilize/Appropriate Buffer</td>
<td></td>
</tr>
</tbody>
</table>
G. DOG-Mediated (Pool II) Protein Extraction

Extracellular matrix proteins were extracted using the method of Hedman et al., (1979). Cell layers were rinsed three times with CMF-PBS buffer at ambient temperature and then washed with 30 mL of 0.5% sodium deoxycholate (DOC), 1 mM phenylmethylsulfonylfluoride (PMSF, from 0.4M solution in EtOH), CMF-PBS buffer 3 X 10 min. on ice while on a rocking platform. The flasks were then washed in the same manner with 2mM Tris-HCl, pH 8.0 and 1 mM PMSF 3 X 5 min. The protein remaining attached to the flask was then removed in 2 mL of gel loading buffer with a rubber policeman.

Actin has been observed in the ECM of cultured fibroblasts (Hedman et al., 1979) using deoxycholate-resistant matrices as substrates for antiactin antibodies. In this case, actin was observed as "small amounts of aggregates and, in part, as distorted cables". It is not at all clear, however, whether the actin in the ECM is the result of secretion from fibroblasts, or debris from migrating or dying fibroblasts (Dunbar, 1987).

H. Protein Quantitation

Proteins were quantitated by the method described by Bradford (1976) using the reagent supplied by Bio-Rad (Bio-Rad Protein Assay Dye Reagent #500-0006). The quantitation of total cellular protein was performed by solubilizing the protein monolayer with 1.0 mL of 0.1% SDS and taking up with a rubber policeman. Samples with 200-1400 ug/mL of protein and bovine serum albumin standards were added (0.1 mL) to 5 mL of the Bio-Rad reagent. After 5 minutes, optical absorbence was measured at 595 nM against a blank. With extremely dilute samples (<25 ug/mL), 0.8 mL of samples and standards were added to 0.2 mL of the Bio-Rad reagent and the optical absorbence was determined on a microtiter plate.

Radioactivity was measured by adding 2.0 µL of the sample in Laemmli loading buffer to 10 mL of liquid scintillation cocktail (ScintiVerse Bio-HP, Fisher). Samples were counted for one minute and expressed as counts per minute after subtracting background counts.
I. SDS-Polyacrylamide Gel Electrophoresis

Samples containing measured amounts of protein, or radioactive cpm in the case of $^{35}S$-methionine labeled proteins, were electrophoresed on polyacrilamide gels as described by Laemmli, 1970. The stacking gel was 3% and the running gel was a 6-15% gradient gel unless otherwise stated. Molecular weight markers were myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Gels were then fixed in 10% glacial acetic acid (vol/vol) and 30% methanol (vol/vol) and either silver stained or prepared for autoradiography as described.

J. Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional gels were run as equilibrium and nonequilibrium pH-gradient electrophoresis (NEPHGE) gels according to the method of O'Farrell, 1975. For the first dimension, isoelectric focusing was performed using loading buffer containing 9.5M urea, 2% (v/v) nonidet P-40, 2% (v/v) ampholines [1.6% pH range 5-7; 0.4% pH range 3.5-10 (LKB)], and 5% (v/v) β-mecaptoethanol. The sample overlay buffer contained 8M urea and 1% ampholines [0.8% (v/v) pH range 5-7, 0.2% (v/v) pH range 3.5-10 (LKB)]. Gels were made from a stock acrylamide containing 28.38% (w/v) acrylamide (Biorad) and 1.62% w/v N,N'-methylene-bisacrylamide. In the case of equilibrium gels, the gel was prepared with 5.5 gm urea, 1.33 mL acrylamide stock, 2.0 mL of stock 10% NP40, 1.7 mL water, 0.6 mL ampholines (pH5-7), 0.115 mL of ampholines (pH3-10), 7 μL TEMED, and 10 μL of 10% ammonium persulfate. Nonequilibrium gels were made using 5.5 gm urea, 1.33 mL acrylamide stock, 2.0 mL of stock 10% NP40, 2.0 mL water, 0.25 mL ampholines (pH7-9), 0.25 mL of ampholines (pH 8-9.5), 14 μL TEMED, and 20 μL of 10% ammonium persulfate. Tube gels prepared for equilibrium isoelectric focusing were pre-run at 200V for 15 min., 300V for 30 min., and 400 V
for one hour. Tube gels were then equilibrated with SDS-polyacrylamide gel electrophoresis buffer for 10 minutes and frozen at -70 deg. C. until electrophoresed in the second dimension as described above for SDS-polyacrylamide gel electrophoresis.

K. Silver Staining

Gels were silver stained essentially by the ammoniacal silver technique described by Oakley et al., 1980. Gels were coomassie stained and then destained overnight in 33% methanol, 10% glacial acetic acid. They were then rocked for 30 minutes in 10% glutaraldehyde, and then washed at least four times in distilled water for at least eight hours. The gels were then vigorously shaken in the presence of 200 mL of ammoniacal silver nitrate solution, which was prepared as follows: 1.4 mL of concentrated ammonium hydroxide was added to about 170 mL of distilled water while stirring. Then, 1.75 mL of 1 N NaOH was added. Next, 8 mL of 20% silver nitrate was added dropwise. If the point comes where the dark metallic silver does not go into solution, then enough ammonium hydroxide is added to clarify the solution.

After shaking the gel for 15 minutes in the ammoniacal silver nitrate solution, the gel is developed in a solution of 0.005% (W/V) citric acid and 0.019% (V/V) formaldehyde. If the gel develops too quickly or too slowly, the amount of formaldehyde in the developing solution can be decreased or increased accordingly.

Other silver staining techniques such as those described by Sammons et al., (1981) and Poehling and Neuhoff, (1981) were tried. However, the method described above gave the most reproducible results.

L. Fluorography

Gels were fixed in 30% methanol (vlv), 10% acetic acid as described above. Those gels containing radiolabeled proteins were prepared for autoradiography essential as described by Bonner & Laskey, (1974) with the following modifications. Gels were impregnated with three
times the gel volume of Enhance (New England Nuclear) on a rocking platform for exactly one hour. The Enhance solution was decanted and disposed of as a radioactive solution. Deionized water at 4 deg. C. was then added to the gel, and it was allowed to precipitate the fluor for 30 minutes on a rocking platform. The gel was then transferred to 3MM paper and dried on a gel dryer. The gel was then exposed to pre-flashed Kodak XAR-2 film for the times indicated.

M. Quantitation of Radioactive Bands

Following fluorography, the region of the gel corresponding to the band of interest was excised and incubated in 0.5 mL of 30% H₂O₂ at 90 deg. C. for one hour. This was then added to 10 mL of ScintiVerse Bio-HP (Fisher) scintillation cocktail, and counted for 10 min. Background was measured with a similar sized gel fragment from an area not showing a band on the fluorograph. All data was presented as the net radioactivity over the background.

N. Immunoprecipitation

Proteins were labeled prior to immunoprecipitation with ³⁵S-methionine as described in section E above. Protein extracts were then solubilized in RIPA buffer. Typically one-half of lyophilized Pool I extract from one T-150 was solubilized in normal Laemmlili gel loading buffer, and the other half taken up in RIPA buffer. In the case of Pool II protein, 200 µL of Laemmli loading buffer was added to one flask, and 500 µL of RIPA buffer was added to a parallel flask. The solubilized protein in RIPA buffer was centrifuged in a microcentrifuge for three minutes.

Ten µL of washed staph A was then added to each 100 µL of supernatant. This was allowed to incubate at room temperature for 10 min. and was then microcentrifuged for five min. (pre-adsorption). For each 100 µL of supernatant, 10 µL of antiserum (immune or pre-immune) was added. This was then allowed to incubate at room temperature for one hour. Fifty µL of staph A was then added and incubated on ice for 15 min. and then
microcentrifuged for 5 min. The pellet was then washed in turn in 0.5 mL washes of salt wash buffer A, B, and C. The pellet was then resuspended in 50 µL of electrophoresis buffer, boiled for two min., and then frozen at -70°C.

O. Immunoblotting

After electrophoresis, gels were transferred to nitrocellulose following the procedure outlined by Dunbar (1987). Gels were placed in a Hoeffer transblot apparatus according to manufacturer's instructions while floating in 0.25M Tris, 1.92 M Glycine. Proteins were then transferred to Zeta Probe membrane (Bio-Rad) at 2.5 to 3 hours at 1.0 to 1.5 Amperes. The absence of methanol leaves SDS associated with protein and facilitates the transfer of high molecular weight protein (such as fibronectin) and the use of Zeta Probe membrane allows protein attachment without methanol.

Proteins were visualized using a Vectastain ABC kit following manufacturer's instructions with the following modifications: Nonspecific binding was blocked by shaking the nitrocellulose in 5% BLOTTO for at least one hour. Primary antibody was then added in 5% BLOTTO at various dilutions and shaken at room temperature overnight. Primary antibody was then removed and the membrane was washed with the following wash sequence: TBS-5 min., TTBS-5 min., TTBS-5 min., TBS-5 min. (hereafter referred to as the standard wash sequence). Secondary antibody was then added as suggested by the manufacturer in TBS and shaken at room temperature for at least one hour. The secondary antibody was then aspirated and the membrane was again washed according to the standard wash sequence. The filter was then placed in Vectastain ABC reagent in TTBS and shaken for 30 min. at room temperature. The membrane was then washed according to the standard wash sequence. Finally, the membrane was placed in the substrate solution for visualization. The substrate solution was prepared by adding the following to 5 mL of 100 mM Tris, pH 7.5: 100 µL of 40 mg/mL Diaminobenzidine tetrahydrochloride (stock solution), 25 µL 80 mg/mL NiCl₂ (stock
solution), and 15 µL of 3% H$_2$O$_2$. The protein bands were generally visualized within 15 minutes. The reaction was stopped by rinsing the immunoblot with TBS and air drying.

Relative molecular weights were calculated based on the mobility of radiolabeled protein markers (Sigma Chemical) as determined by exposing the immunoblot to x-ray film.

P. Identification of Collagenous Bands

Collagenous bands were identified by their digestion with collagenase isolated from *Achromobacter iophagus* (Boehringer-Mannheim) according to the protocol outlined by Berry and Shuttleworth (1988). Samples in collagenase sample loading buffer (CSLB) were heated at 100 deg. C. for 3 min., cooled to room temperature, and bacterial collagenase (in 25 mM Tris, pH 7.4, 0.1 M calcium acetate buffer) was added to a final concentration of 300 U/mg protein. Samples were incubated for 24 hours at 37 deg. C., then boiled for 3 min. to inactivate the collagenase, and then frozen until subjected to electrophoresis.

Nonspecific proteolysis was analyzed by electrophoresing both digested and undigested samples. Little proteolysis of protein bands other than those corresponding in size to that expected for procollagen was observed.

The procollagen bands were also identified using rabbit antiserum to type I procollagen on an immunoblot.

Q. Radial Caseinolytic Assay

Total plasminogen activator activity in Pool I and II protein extracts was assayed according to the method described by Saksela (1981). Serum-free medium conditioned by cells was dialyzed against water at 4 deg. C. for 18 hours, lyophilized, and redissolved in 2.5% SDS, 1% sucrose, 50 mM Tris-HCl, pH 6.8, and loaded for electrophoresis without boiling. The gel was prepared by making a 1% agarose solution in 0.1 M Tris-HCl, pH 8. After cooling to 45
deg. C., plasminogen was added to a final concentration of 2 µg/mL (0.03 U/mL), casein at 0.6%, a catalytic amount of plasmin at 20 ng/mL, and, finally, sodium azide at 0.1%. The gel was poured between preheated 15 x 17 cm glass plates with 1 mm spacers. After cooling, one plate was removed, a 6 X 7 matrix of 3.5 mm holes were punched in the gel, and the samples were loaded. The gel was then placed in a humidified atmosphere at 37 deg. C. for approximately 24 hours. Plasminogen activator activity could easily be observed as a clear disc of caseinolysis radiating from the well. Plasminogen activator-independent proteolysis was measured with a duplicate gel without added plasminogen. After proteolysis, the gel was placed on 3MM paper, dried, then removed in the presence of 30% methanol, 10% acetic acid, and coomassie blue stained as described above, destained, and photographed.

R. Plasminogen Activator/Inhibitor Zymography

Zymography for the detection of plasminogen activator activity was performed essentially as described by Granelli-Piperno & Reich (1978) with some modifications. Polyacrylamide gels were prepared with sucrose (10gm/100mL) added to the higher density polyacrylamide solution when making a gradient gel. Samples were applied to the gel in 1X ZLB and electrophoresed at 12 mA / gel at 4 deg. C. to reduce heat denaturation of the enzyme and interactions between the enzyme and the substrate. After electrophoresis, SDS was exchanged by rinsing the gel in 2.5% Triton X-100 with rocking at room temperature for two hours with one change. Excess liquid was then removed from one side with a paper towel, and the gel carefully rolled onto a casein-agar indicator gel so as to prevent any air bubbles from being trapped between the two gels. The casein-agar indicator gel was prepared as follows. The gel was prepared by making a 1% agarose solution in 0.1 M Tris-HCl, pH8. After cooling to 45 deg. C., plasminogen was added to a final concentration of 2 µg/mL (0.03 U/mL), casein at 0.6%, a catalytic amount of plasmin at 20 ng/mL and, finally, sodium azide at