REGULATION OF NEUTRAL PROTEINASE AND PLASMINOGEN ACTIVATOR SECRETION BY EPITHELIAL CELLS IN VITRO

by

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B.D.S., The University of Singapore, 1979

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

FACULTY OF GRADUATE STUDIES Department of Oral Biology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June, 1985

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ABSTRACT

The aim of this thesis was to study the regulation of proteinase secretion by epithelial cells (E-cells) derived from the epithelial cell rests of Malassez. Since these epithelial cell rests are present only in small numbers in-vivo, E-cells derived from porcine cell rests were cultured according to Brunette et al. (1976) and conditions chosen so that detectable amounts of the proteinases, neutral proteinase and plasminogen activator, could be obtained. The regulation of the secretion of these enzymes was investigated by varying the cell population density, adding E.Coli lipopolysaccharide to the cultures and altering the shape of the E-cells by both chemical and physical means.

Cell population density modulated both neutral proteinase and plasminogen activator secretion. Neutral proteinase secretion was highest at low cell population densities and the activity decreased with increasing cell population density. Plasminogen activator secretion followed a similar pattern.

Escherichia coli lipopolysaccharide (E.coli LPS) stimulated both neutral proteinase and plasminogen activator secretion. LPS extracted by the phenol method and LPS extracted by the trichloroacetic acid method caused similar increases in neutral proteinase activity but the increase in plasminogen activator activity was greater when the trichloroacetic acid extracted LPS was used. These findings support the proposal that bacterial LPS in contact with periapical tissues could stimulate the epithelial cell rests into increased production of proteinases, thereby contributing to the degradation of connective tissue associated with dental cyst formation.
E-cell shape was altered by physical and chemical means. Addition of cholera toxin and dibutyryl cAMP caused E-cells to flatten. Phorbol myristate acetate, however, caused the cells to retract slightly. Mechanical stretching was applied to the cells to cause cell flattening, and cell rounding was effected by mechanical relaxation. Another method made use of E-cells grown on a substrate with V-shaped grooves which caused the cells to adopt a rounder shape more frequently than cells grown on a flat substrate. In addition, dishes coated with increasing concentrations of poly(HEMA) solution, which altered dish adhesivity to the cell, caused the cells to become less well-spread. In all experiments, a more flattened cell shape correlated with a reduced level of neutral proteinase and plasminogen activator secretion while a more rounded shape correlated with increased amounts of neutral proteinase and plasminogen activator secretion.
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ACKNOWLEDGEMENTS

I am very grateful to Dr. D. Brunette, my supervisor, for his guidance throughout this project, patience in reading my numerous initial drafts, and suggestions while I was writing this thesis.

I wish to also thank all the people in the Department of Oral Biology who have made my introduction to research very pleasant: especially Holly Maledy and Dianne Price for their help and friendship; and Tony Ng for his generous help when 'things around the place broke down'. I am grateful to Lesley Weston who did the electron microscopic sections and Dianne who carried out the cAMP assays and did the figures.

And to Soo Inn, who believed in me and encouraged my venture into the laboratory, I am especially grateful.

I wish to also thank the Medical Research Council of Canada for their financial support.
CHAPTER 1 - INTRODUCTION

GENERAL INTRODUCTION

"Cells are normally very special and well behaved..... They follow rules and are receptive to signals and requests from one another. The rules govern both growth and territory, and they have to be followed perfectly. Otherwise we wouldn't end up divided into fingers and toes, eyes and brain; we would end up a hopeless and shapeless jumble."

L.L. Larison Cudmore (1977)

The rules governing cell growth have been the subject of investigation for many decades. Most of the work have concerned chemical mediators including macromolecules and simple compounds such as cyclic nucleotides, but physical stimuli such as mechanical stretching or gas tension can also affect cell division and behaviour. Of particular relevance to this thesis is the work of Folkman and Moscona (1978) who have shown that cell shape can regulate cell proliferation. For example, fibroblasts grown on tissue culture dishes flatten out and proliferate whereas the same population shows reduced growth when placed on dishes treated in such a way that the cells are more rounded. Folkman and Moscona concluded from these experiments that the shape of the cell regulates DNA synthesis and growth of normal cells.

In contrast, the growth of transformed cells or tumour-like cells is not regulated by individual cell shape. Folkman and Moscona found that SV 3T3 transformed cells, maintained at different degrees of flatness, grew at similar rates.

Another characteristic of transformed cells is their tendency to secrete elevated levels of plasminogen activator. For example, a good number of transformed cells lines and a variety of human cancers have
shown increased plasminogen activator secretion compared to normal cells.

It has been suggested that the elevated levels of plasminogen activator have an important role in tumour invasion and metastasis; plasminogen activator and its product plasmin act to aid tumour cells in detaching from their primary site and penetrating through the extracellular matrix (Reich, 1977; Ng & Kellen, 1983). Thus both the loss of growth control and increased plasminogen activator secretion are characteristics of transformed and neoplastic cells.

From the above, it appears that there is a complex but not necessarily causal relationship between growth, cell shape and proteinase secretion in tumour cells. It is thus of interest to determine whether cell shape regulates growth and or proteinase secretion in normal cells. For fibroblasts, this appears to be the case for the proteinase collagenase (Aggeler et al., 1984). The relationship between epithelial cell shape and proteinase secretion has not been studied directly and is the topic of this thesis.

The cells used in my study were epithelial cells (E-cells) derived from the epithelial cell rests of Malassez and the enzymes selected for study were neutral proteinase and plasminogen activator. By analogy with other cell types, it was hypothesized that proteinase secretion by epithelial cells would be regulated by cell population density, bacterial lipopolysaccharides and treatments which affect cell shape. The background information for these studies is presented in the following order.

I) Cell shape and its relationship to cell function
II) Epithelial cell rests of Malassez
III) Connective tissue degradation
   1) Intracellular degradation
   2) Extracellular degradation - proteolytic enzymes

IV Regulation of Proteinase activity
   1) Control of enzyme activity
   2) Control of enzyme synthesis

LITERATURE REVIEW
I) Cell Shape and its Relationship to Cell Function

Cell shape has been found to regulate a number of cell functions. Folkman and Moscona (1978) showed that the shape of endothelial cells was tightly coupled to DNA synthesis and growth. These investigators varied tissue culture dish adhesivity by precoating the dishes with different concentrations of poly (2-hydroxyethyl methacrylate) (poly (HEMA)) solutions. The extent of cell spreading was thus controlled and measured in terms of cell diameter or cell height. DNA synthesis, measured by the incorporation of $^3$H-thymidine, was found to be inversely proportional to the height of the cell. Thus, the more flattened the cell, the greater the DNA synthesis. Folkman et al further showed that the phenomenon of density dependent inhibition of cell growth may be mediated by cell shape. Endothelial cells grown to confluence on tissue culture dishes were compared with sparsely populated cells grown on poly(HEMA) coated dishes. Sparsely populated cells with the same height as their confluent counterparts showed similar rates of $^3$H-thymidine incorporation. Thus the crowded cells on plastic which were at a similar height as the sparsely populated cells on poly(HEMA) dishes showed similar rates of DNA synthesis.
Ben-Ze'ev et al. (1980) investigated the relationship between cell shape and DNA, rRNA, mRNA and protein synthesis. The authors showed that mouse fibroblasts in suspension demonstrated a strong inhibition of DNA, mRNA, rRNA and protein synthesis. Upon replating on plastic dishes which allowed the cells to flatten, the synthesis of these macromolecules was restored. Using the poly(HEMA) system of Folkman, these authors showed that the amount of DNA synthesis restored was correlated with cell shape over a certain range. Restoration of mRNA synthesis was sensitive to cell shape too. Reattachment of suspended cells on poly(HEMA) dishes (which caused cell rounding) resulted in a suppression of the mRNA synthesis compared to cells which were allowed to reattach to plastic dishes (which caused cell flattening). The ribosomal RNA synthesis was similarly affected by cell shape. Protein synthesis, however, was not affected by changes in cell shape; as long as the cell was in contact with the dish, protein synthesis proceeded at the same rate.

There are some indications that cell shape may play a role in enzyme secretion. Lawrence et al. (1979) reported that several treatments which caused rat ovarian granulosa cells to round up also raised plasminogen activator (P.A.) levels.

Aggeler et al. (1984) attempted to study the relationship between cell shape and collagenase secretion by using various chemical agents which alter cell shape. Cell rounding caused by trypsin, phorbol myristate acetate and cytochalasin B was correlated with an increase in collagenase secretion. Although suggestive, these results are not definitive because the chemical agents used are known to cause multiple effects on cell function.
Studies investigating the induction of differentiation of mouse mammary cells indicate that cell shape is an important factor in this process. Differentiation of mammary epithelial cells has been shown to be influenced by several hormones. Explants of mouse mammary glands maintained in defined medium containing lactogenic hormones result in morphological and biochemical changes indicative of functional differentiation (review of Saacke and Heard, 1974; Forsyth, 1971). But mammary epithelial cells grown on plastic or glass, in the presence of lactogenic hormones show little differentiation. However, Emerman et al. (1978) showed that mammary epithelial cells grown on floating collagen gels, in the presence of lactogenic hormones, show morphological and biochemical differentiation.

Floating collagen gels have three advantages over plastic or glass. 1) The floating gel permits accessibility of nutrients to basolateral cells surfaces. 2) There is epithelial cell interaction with collagen. 3) The substrate is flexible, allowing the cells to assume a cuboidal to columnar shape with rounded apical surfaces. In contrast, cells grown on plastic or glass are flattened and have broad apical surfaces.

Emerman et al. tested the significance of each of these features in relation to mammary cell differentiation. Substrate flexibility allowing for cell shape changes was found to be important for functional differentiation in mammary epithelial cells in-vitro. That the ability of these cells to assume a cuboidal or columnar shape plays an important role in the induction of differentiation has been confirmed by the experiments of Shannon and Pitelka (1981).
II) Epithelial Cell Rests of Malassez

The epithelial rests of Malassez (ERM) are a group of epithelial cells derived from Hertwig's epithelial root sheath which plays an important role in root formation. The development of the root is described by Bhussry (1976). The apical elongation of Hertwig's epithelial root sheath, which consists of an inner and outer epithelial layer, delineates the shape, size and number of roots. Cell proliferation at the tip of the short, horizontal, leading component of the root sheath results in root sheath elongation. At the same time, the adjacent connective tissue cells (both on its inner and outer surface) proliferate and the inner connective tissue cells adjacent to the root sheath differentiate into odontoblasts and form dentine. As soon as this happens, the epithelial cells of the root sheath becomes disrupted, leaving a network of epithelial cells. The connective tissue cells adjacent to the outer epithelial layer of the root sheath move through the openings to line up along the dentine surface as cementoblasts, secreting cementum. The remnants of the root sheath are present as cell rests throughout the lifetime of the tooth, their number decreasing with age, but reaching a plateau around age 30 years (Reeve and Wentz, 1962; Simpson, 1965).

There is general agreement that the ERM exists as in a network of cells (Provenza, 1972; Sicher and Bhaskaram, 1972) but whether this network is continuous with attachment epithelium or not is open to debate. Simpson (1965) and Valderhaug et al. (1966) show that part of the ERM appears to be continuous while Scott and Symons (1974) describe them as "isolated columns" or "an incomplete network". The relationship of the ERM to the attachment epithelium (or junctional epithelium)
at the marginal region has recently been investigated by Spouge (1984), who studied eight porcine molars and their supporting tissues. Spouge reports that the coronal margin of the network of epithelial rests was connected to the junctional epithelium in intermittent intervals. This relationship between the junctional epithelium and the network of ERM has been previously described by Spouge (1980) as a 'basketball hoop' with the junctional epithelium resembling the hoop and the network of cell rests suspended from the hoop, forming the basket. The detailed configuration of ERM in humans remains to be explored.

The physiological function of the ERM is not known. Suggested roles include an endocrine function (Higaki, 1931; Orban, 1953), formation of enamel pearls (Gottlieb, 1921), a protective role against root resorption (Waerhaug, 1958), and the maintenance of the periodontal ligament space (Loe and Waerhaug, 1961).

The ERM has been shown to be able to proliferate in inflammation and contribute to the formation of dental cysts (Valderhaug, 1972). Harris and Toller (1975) have suggested that the epithelial layer in the cyst serves as a barrier isolating pulpal irritants from the rest of the connective tissue. As dental cysts are the most common benign, destructive lesions of the skeleton (Harris and Toller, 1975), the study of the regulation of proliferation of the ERM would appear to be important.

In the light of the ability of the ERM to proliferate in response to the inflammatory stimulus, and the anatomical proximity of the cells to the attachment epithelium, Spouge (1980) has suggested that the apical migration of the attachment epithelium in chronic periodontitis could be facilitated by the proliferation of the ERM already present.
Thus the response of the ERM at the marginal region to inflammation could aid in pocket formation in periodontal disease.

Several aspects of ERM behaviour in-vitro have been studied. Grupe et al. (1967) were the first to observe ERM proliferation in tissue as opposed to cell culture. They found that ERM in tissue culture in contrast to ERM in-vivo showed DNA synthesis and a decrease in nuclear/cytoplasmic ratio. There was an absence of glycogen, little succinic dehydrogenase activity and an accumulation of fat droplets indicating that the metabolism had shifted from a citric acid cycle to a hexose monophosphate shunt.

Since then, it has been found that these epithelial cells can be isolated and grown in cell culture. Brunette et al. (1976) reported a technique for culturing epithelial-like cells from porcine periodontal ligament. Moreover the epithelial cells could be separated from fibroblasts by a trypsin-citrate saline solution (Owens, 1974). Fibroblast-like cells rounded up after 5 to 10 minutes while the epithelial-like cells took twice as long to detach from the dish.

Brunette et al. (1977) further observed that monkey periodontal ligament in culture produced outgrowths of multilayered epithelial and fibroblast cells. The epithelial cells in multilayers demonstrated attachment by desmosomes on the ventral and dorsal surfaces. No such structure was observed between epithelium and fibroblast layers. Epithelial cells were also found sandwiched between two fibroblast layers, mimicking their in-vivo relationship where epithelial cell rests of Malassez are surrounded by fibroblast cells of the periodontal ligament.

These porcine epithelial cells in culture were further
characterized by Brunette et al. (1979). The cells showed a diploid karyotype with 38 chromosomes, their size remaining constant over 9 subcultures. The epithelial cells also secreted several proteins and prostaglandins E and F.

Birek et al. (1983) showed that these epithelial cells in culture produce factor/factors which cause bone resorption in-vitro. The addition of indomethacin to the culture resulted in an inhibition of bone resorption indicating that the prostaglandin produced by the epithelial cells contributed to bone resorption. These results suggest that the ERM may play a direct role in the bone resorption seen in dental cysts.

These cells have also been found to be able to phagocytose collagen (Birek et al., 1980) and to secrete neutral proteinase and latent collagenase in-vitro (Pettigrew, Ph.D. Thesis; Limeback and Brunette, 1981). Thus the secretion of proteinases together with endocytosis by epithelial cells may be one of the mechanisms involved in connective tissue destruction observed in the pathogenesis of the dental cyst.

III) Connective Tissue Degradation

Collagen, the major component of connective tissue can be degraded through two pathways:

1) intracellular degradation following phagocytosis by the cell;
2) degradation of collagen by proteolytic enzymes secreted by the cell into the extracellular environment.
1) **Intracellular Degradation**

Whether cells can phagocytose extracellular collagen has been investigated by numerous workers. Electron microscopic evidence have led to the suggestion that fibroblasts (Ten Cate et al., 1976) and macrophages (Parakkal, 1969) can ingest and degrade collagen. However, the possibility of the intracellular collagen observed in electron microscopic sections resulting from endogenously synthesized collagen could not be discounted.

The in-vitro experiments of Svoboda et al. (1979) have shown clearly that fibroblasts can phagocytose collagen fibrils. Serial electron microscopic sections of fibroblasts which had been cultivated in the presence of rat tail collagen, showed large banded collagen fibrils within the cells. In contrast, fibroblasts cultured without exogenous collagen did not contain large intracellular accumulations of collagen fibrils.

Phagocytosis of collagen by fibroblasts has also been shown by Yajima and Rose (1977). These investigators cultivated human gingival fibroblasts on collagen coated coverslips. Electron microscopic sections of the cells showed the presence of collagen fibrils surrounded by dense bodies.

Using a similar experimental system as Svoboda et al, Birek et al. (1980) showed that E-cells derived from ERM could also phagocytose rat tail collagen. It had been previously reported that the intracellular degradation of collagen was carried out by hydrolytic enzymes in the lysosome (Deporter and Ten Cate, 1973). Birek et al. therefore tested for acid phosphatase, a lysosomal enzyme, and found a positive reaction in vesicles associated with intracellular collagen. Isolated lysosomal
fractions from E-cells were found to consist of cathepsin B₁, cathepsin D, and other lysosomal enzymes. Furthermore, the addition of this isolated lysosomal fraction to rat tail collagen resulted in the degradation of collagen into smaller fragments. Thus Birek et al. concluded that ERM can phagocytose collagen and that the ERM possess the mechanisms for the digestion of phagocytosed collagen.

The available evidence suggests that epithelial cells can contribute to connective tissue degradation by phagocytosis of collagen fibrils. In addition, there is evidence that a significant amount of newly synthesized collagen is degraded before it reaches the extracellular space (Bienkowski et al., 1978; Baum et al., 1980).

2) Extracellular Degradation - Proteolytic Enzymes

Extracellular degradation of collagen involves proteolytic enzymes, the most important being collagenase and neutral proteinase. Plasminogen activator is also thought to have a role in the degradation of connective tissue.

a) Collagenase

Collagenase is defined as an enzyme capable of degrading native collagen at physiological pH and temperatures (Harris and Cartwright, 1977). In-vivo, collagen exists as aggregates of fibrils, each fibril containing collagen molecules with cross-links between them. Collagen fibrils with intermolecular cross-links are more resistant to collagenases than are non-cross-linked fibrils, but it is not known whether other enzymes are needed to cleave the cross-links before the collagenase can act on the collagen molecule.
The collagenase cleaves the collagen molecule into 2 fragments: the 3/4 fragment, TC\textsuperscript{A}, and the 1/4 fragment, TC\textsuperscript{B}. The collagenase has been shown to be unable to degrade the 2 fragments further (McCroskery et al., 1975; Gross et al., 1974). Once cleaved, the fragments then denature and become solubilised as gelatin polypeptides (Sakai and Gross, 1967; McCroskery et al., 1973).

The collagenases are metallo-enzymes, Zn\textsuperscript{++} and Ca\textsuperscript{++} being essential for their activity. Thus agents like EDTA, inhibit collagenase activity, presumably by chelating Ca\textsuperscript{++} and or Zn\textsuperscript{++}.

b) **Neutral Proteinase**

While collagenases are defined as acting specifically on native collagen at physiological pH and temperatures, the neutral proteinases act to further degrade the products of primary collagenolysis (Harris and Cartwright, 1977). Neutral proteinase has been reported to be able to degrade gelatin, proteoglycan, azocoll and azocasein (Evans et al., 1981). It is not clear whether this represents a single enzyme with wide substrate specificity, or different enzymes with different substrate specificities but possessing similar characteristics.

There is one report (Sellers et al., 1978) that the neutral proteinase that degrades gelatin can be separated chromatographically from that which attacks cartilage proteoglycan. The gelatin-degrading enzyme did not degrade collagen, cartilage proteoglycan or azocasein but azocoll was degraded to a small degree. The other neutral proteinase degraded cartilage proteoglycan,
azocasein and azocoll but neither collagen nor gelatin. Other investigators have been unable to separate the gelatin degrading and the proteoglycan degrading neutral proteinases (Cartwright et al. (1983) and Murphy et al. (1981)).

Neutral proteinase shares many characteristics with collagenase. Both are metallo-enzymes, requiring Ca^{++} for activity and both are active at neutral pH. They are inhibited by the same agents, including EDTA, cysteine and some serum proteins. The latent forms can be activated by trypsin or APMA. Because the two enzymes are often found together, it has been suggested that collagenase and neutral proteinase are secreted together as a collagenolytic package (Pettigrew et al., 1978).

c) **Plasminogen Activator** (P.A.)

By definition, a plasminogen activator is an enzyme that converts plasminogen into the active form, plasmin. Plasminogen activators have usually been classified into four categories: circulating P.A., tissue P.A., urinary P.A. (urokinase) and tissue culture P.A. How these different P.A. are related to each other is not known but urokinase has been shown to be immunologically distinct from tissue P.A. Like other serine proteinases, P.A. is inhibited by diisopropyl phosphofluoridate. It is also inhibited by some serum proteins.

P.A. is thought to be involved in a host of physiological and pathological processes including: fibrinolysis, proteolytic processing of cellular and serum proteins (in complement activation, kinin formation and proinsulin formation), migration
of macrophages during inflammation, tissue remodelling, and neoplasia (Gelehter et al., 1983).

Of particular relevance to this thesis is the suggestion that P.A. is involved in extracellular proteolysis of connective tissue. Reich (1978) has observed that processes such as inflammation, ovulation, trophoblast implantation, and neoplasia share in common, events like tissue remodelling and cell migration, where connective tissue degradation takes place. Furthermore Reich has shown that there is a correlation between these events and increased secretion of P.A. Reich therefore suggests that the secretion of P.A. provides a mechanism which enables tumour cells and inflammatory cells to migrate, and allows the degradation of the ovarian follicle wall during ovulation. He further postulates that the secretion of P.A. could be part of a mechanism for localised extracellular proteolysis of connective tissue. Similarly, Ossowski et al. (1979) have also shown that there is a large increase in P.A. following the involution of mammary glands, during which there is degeneration of the secretory epithelium.

A second role for P.A. in connective tissue destruction has been put forward by Werb et al. (1977). Their finding that rheumatoid synovial cells could produce plasmin in the presence of plasminogen, and the ability of these cells in the presence of plasminogen to degrade collagen suggests that the plasmin activated the latent collagenase which is also secreted by these cells.

IV) Regulation of Proteinase Activity

Proteinases are subject to regulation on two levels: 1) control of the enzyme activity; 2) control of synthesis.
1. Control of Enzyme Activity
   a) Neutral Proteinase
      i) Tissue Inhibitor of Metalloproteinase

      Tissue inhibitors play an important role in regulating the activity of the neutral proteinase. Sellers et al. (1979) first showed that the collagenase inhibitor secreted by rabbit bone explants also inhibited the gelatin and proteoglycan-degrading neutral proteinase. The rabbit bone collagenase inhibitor purified by Cawston et al. (1981) migrated as a single band at Molecular Weight (MW) 28000 on the sodium dodecyl sulphate (SDS) polyacrylamide gel. This purified inhibitor named Tissue Inhibitor of Metalloproteinases (TIMP) blocked the activities of collagenase, gelatinase and proteoglycan-degrading neutral proteinase. Its activity was lost with trypsin treatment (250 \( \mu \)g/ml) for 30 minutes, heat treatment at 90°C for 1 hour and after reduction and alkylation. Inhibitors with similar properties as TIMP have been isolated from human amniotic fluid (Murphy et al, 1981a); from human synovium explants of normal, rheumatoid, and osteoarthritic patients (Murphy et al., 1981b); and from human gingival fibroblasts (Heath et al., 1982).

      The presence of considerable amounts of the free tissue inhibitor appears to be correlated with either the absence or low levels of the enzyme. Meikle et al. (1980) showed that explants of rabbit calvaria secrete elevated levels of the inhibitor in the first two days of culture when no neutral proteinase could be detected. With the appearance of the latent neutral proteinase, the level of the inhibitor dropped markedly. Cambray et al.
(1981) found that explants of normal rabbit synovium produced no active nor latent enzyme but considerable amounts of the inhibitor, whereas synovium from arthritic joints showed elevated levels of the active enzyme and a sharp decrease in inhibitor levels.

Thus Murphy and Sellers (1980) suggest that all connective tissues secrete tissue inhibitors to control the activity of the metalloproteinases. Only when there is excess enzyme over the inhibitor would tissue breakdown take place. Control over the activity can be exerted through different rates of synthesis and removal of the inhibitor.

11) **Latent Neutral Proteinase**

Neutral proteinase in a latent form has been reported from many tissue and cell culture systems. The latent enzyme can be activated by the action of proteinases such as trypsin or by the action of the thiol-binding reagents such as APMA. Most of the studies on the latent enzyme have been done on collagenase. Since the neutral proteinase presents as a latent form and is activated by the same agents, and the TIMP has been shown to inhibit both collagenase and neutral proteinase (Sellers et al., 1979), it is highly probable that the information on the latent collagenase applies to the neutral proteinase too.

Two mechanisms have been proposed to explain the latent form of the enzyme: 1) the latent enzyme is a proenzyme (Zymogen) that needs proteinases for activation; 2) the latent enzyme is an enzyme-inhibitor complex.
Different authors have proposed that the latent collagenase exists as a proenzyme form (Vaes, 1972; Stricklin et al., 1977). Stricklin et al. purified procollagenase from human skin fibroblasts and organ cultures of human skin. Two forms of procollagenase (60,000 and 55,000 daltons) were detected on sodium dodecyl sulphate polyacrylamide gels, and activation by trypsin resulted in 50,000 and 45,000 dalton forms, respectively. Auto-activation however, caused no detectable change in MW of the enzyme. Stricklin et al. suggested that this could be the result of a conformational change in the zymogen molecule, leading to the active site being exposed without change of peptide bonds.

Further evidence from Valle and Bauer (1979) has strengthened the hypothesis that the enzyme is secreted in a zymogen form. Valle and Bauer showed that both intracellular and extracellular collagenase proteins secreted by human skin fibroblasts in culture, comigrate with the two purified forms of procollagenase on the SDS polyacrylamide gel.

There is also evidence for the second hypothesis of an enzyme-inhibitor complex. Sellers et al. (1977) have found that latent collagenase from bone explants could be activated by trypsin as well as 4-amino-phenyl mercuric acetate (APMA) and other thiol-blocking agents, and this conversion was accompanied by a decrease in MW of 8,000 to 15,000. A collagenase inhibitor was identified and partially purified. Moreover, the combination of the inhibitor and APMA-activated enzyme resulted in a latent enzyme complex which could be reactivated by APMA or trypsin. Sellers et al. therefore suggested that the latent enzyme exists as an
enzyme-inhibitor complex and that reagents such as APMA and thiocyanate cause a dissociation of the enzyme-inhibitor complex while proteinases act by preferential degradation of the inhibitor. It was also significant that the disappearance of the inhibitor in the medium of bone explants coincided with the appearance of the latent enzyme. There is however, a discrepancy in MW data which is hard to explain. The combination of the inhibitor (MW 30,000) with the active collagenase (MW 30,000) yielded a latent enzyme of MW 40,000.

iii) **Serum Inhibitors**

Harris and Krane (1972) report that the neutral proteinase from rheumatoid synovial tissues is inhibited by serum proteins. Murphy et al. (1982) observed that α2-macroglobulin inhibited the activity of the purified gelatinase from human polymorphonuclear leucocytes. Thus serum inhibitors play a role in controlling the activity of the neutral proteinase. Therefore medium containing serum used for maintaining cells in culture makes it difficult for the presence of enzyme activity to be detected.

b) **Plasminogen Activator**

i) **Tissue Inhibitors**

Tissue inhibitors to P.A. have been found in a number of cell systems. Loskutoff and Edgington (1981) report the presence of an inhibitor to tissue P.A. and urokinase in the cytosol of rabbit endothelial cells. Bovine endothelial cells have also been shown to secrete an inhibitor to both human tissue P.A. and urokinase (Loskutoff et al., 1983) which has an apparent MW of 55,000 and which is stable to reducing agents, denaturants, and extremes of pH.
Protease nexin, an inhibitor secreted by human fibroblasts, has been extensively studied. It inhibits both urokinase and thrombin, and it resembles antithrombin III in having a high affinity heparin binding site (Baker et al., 1980). However, it is electrophoretically and immunologically distinct from antithrombin III. Phillips et al. (1984) have demonstrated an inhibitor different from protease nexin. They report an inhibitor secreted from human endothelial cells that showed a very low association rate with thrombin unlike protease nexin. Also while protease nexin has a high affinity heparin binding site, the endothelial cell inhibitor has a low affinity for heparin.

Coleman et al. (1982) report that hepatoma cells secrete an inhibitor to urokinase-type P.A. Subsequent studies showed that this inhibitor was also different from protease nexin, having no effect on thrombin activity.

Hoal et al. (1983) report a different mechanism which regulates P.A. activity. These authors found that melanoma cells which secrete P.A., when co-cultivated with fibroblasts, showed a decrease in P.A. activity. Evidence was presented which suggests that the P.A. secreted into the medium could become bound to fibroblasts and become inactivated.

ii) Inactive or Latent PA

Levin (1983) reported that human endothelial cells from the umbilical vein secrete a P.A. that is immunologically similar to tissue P.A. It is inactive in the media, has an apparent MW of 100,000 and is not sensitive to diisopropylfluorophosphate. Levin
suggests that this is a tissue P.A.-inhibitor complex based on the following evidence: 1) conditions that dissociate other serine protease-inhibitor complexes result in a reduction of anti-tissue P.A. immunoprecipitable material from 100,000 MW to 60,000 MW; 2) radiiodinated melanoma cell tissue P.A. mixed with the conditioned medium from endothelial cells showed an increase in MW from 70,000 to 110,000 indicating that the conditioned medium contained an inhibitor capable of complexing tissue P.A. Levin also showed that the inactive form of tissue P.A. could be activated by SDS treatment.

A tissue type P.A.-inhibitor complex has also been demonstrated to be secreted by human endothelial cells (Phillips et al., 1984). Serum free conditioned media showed no activity on film gels but SDS treatment converted the complex from a 95,000 to 135,000 MW to a 72,000 MW form which was active. The MW of the inhibitor component was estimated at 50,000 to 70,000.

iii) Serum Inhibitors

Serum has been shown to be able to suppress cell associated PA activity in endothelial cells in-vivo (Levin and Loskutoff, 1980). Urokinase has been found to be slowly inactivated by several plasma protease inhibitors: α2-antiplasmin, antithrombin III, and α1-antitrypsin (Moroi and Aoki, 1976; Clemmensen and Christensen, 1976). Tissue type P.A. was found to be inhibited by α2-antiplasmin and to a lesser extent by α2-macroglobulin (Korninger and Collen, 1981). In another study, Rijken et al. (1983) found that the tissue type PA in plasma formed a complex with α2-antiplasmin and α1-antitrypsin.
2. Control of Proteinase Synthesis

a) In Vivo and In Vitro Studies

A study that involved measuring the amount of neutral proteinase in extracts of tissue from patients has shown that the level of neutral proteinase was higher in gingival samples that were clearly inflammed compared to non-inflammed samples (Uitto et al., 1981). The usefulness of this study is in confirming that tissues in vivo secrete neutral proteinase and that the level is elevated in the diseased state. However, the limitations of such studies include difficulties in determining the cellular source of the enzyme and determining regulatory factors that modulate enzyme secretion.

In order to study factors that regulate proteinase secretion, in vitro cell culture provides a suitable model. Cell culture yields a defined population of cells and the effect of an added agent on cell behaviour can be studied and the level of enzyme secreted easily measured. However, the in vitro state is not necessarily identical to the in vivo state with its circulatory system and other physiological metabolites.

One of the goals of cell culture is to be able to grow cells in a serum-free medium because serum contains a host of factors which makes it difficult to interpret studies where an agent added to the culture results in a measurable effect. Of particular relevance to this study is the fact that serum contains $\alpha_2$-macroglobulin, an inhibitor of neutral proteinase activity, and other serum inhibitors which inactivate P.A. activity.
Recently Brunette (1984a) has developed a low serum media (0.5% dialysed foetal calf serum) containing low Ca\(^{++}\) and K\(^{+}\), which maintains epithelial cells in culture. This has made it possible to grow epithelial cells and to collect the secreted proteinases in this medium.

b) **Factors Implicated in the Regulation of P.A. Synthesis**

Cell culture conditions can affect P.A. secretion, one variable being serum. The presence of serum has been found to depress the P.A. activity in keratinocyte cultures, as measured by the rate of fibrin lysis (Birkedahl-Hansen and Taylor, 1983). Levin and Loskutoff (1980) also report that when 0.1% foetal calf serum was added to bovine aortic endothelial cell cultures, the cell-associated P.A. activity decreased by 50%. Serum from horse, goat, rat, and humans also caused a suppression in P.A. activity. Thus the collection of P.A. should preferably be done in serum free media which circumvents the problem of serum inhibition of P.A. activity.

Another important variable in cell culture affecting P.A. production is cell population density which refers to the number of cells per unit area. Liu et al. (1982) found that in their transformed cell line, there was an increasing amount of intracellular P.A. activity per cell with a decreasing cell density up to a point, when the P.A. activity per cell then became independent of the cell density. The authors showed that the decrease in P.A. activity at higher cell densities was not because of the release of soluble inhibitors and they suggest the effect is
mediated by cell-to-cell contact. Another study by Rohrlich and Rifkin (1977) showed that the amount of P.A. secreted by normal embryo fibroblasts increased with increasing cell density, reaching a plateau as the cells reach confluence. The data from these two studies cannot be directly compared because of the different cell lines used and the difference in P.A. location, one being intracellular and the other extracellular. However, the data indicates that cell population density should be taken into account in studies of P.A. activity. Also, the relationship between cell population density and P.A. activity can provide information for optimizing conditions to give maximum enzyme activity.

A third factor affecting P.A. production is the growth state of the cells. This effect has been studied by Chou et al. (1976). They measured the daily P.A. secretion as a group of cells grew to confluence and compared the P.A. activity of growing cells versus confluent cells which showed no growth. The secreted P.A. from confluent cells was six to ten fold less than the secreted P.A. from growing cells. Cell associated P.A. however, remained relatively consistent. This data is difficult to interpret because the cell population density effect has not been dissociated from the growth state of the cells which was of interest in this study.

Aggeler et al. (1982) found that extracellular P.A. activity increased during the G2 and M phases of the cell cycle of Chinese hamster ovary fibroblasts, indicating that increased P.A. is related to cell division.

Peritoneal macrophages obtained from mice that had been
injected with endotoxin showed more P.A. secretion compared to control macrophages (Gordon et al., 1974). Exposure to endotoxin and subsequent phagocytosis of latex particles had a strong stimulatory effect on the macrophage secretion of P.A. A number of other agents such as mineral oil, saline, BCG antigen, and foetal calf serum in combination with latex particles also induced increased secretion of P.A. but endotoxin proved to be the best priming stimulus.

Agents shown to stimulate P.A. secretion in human epidermal cells include colchicine, cholera toxin, and epidermal growth factor (Hashimoto et al., 1983).

P.A. secretion can be suppressed by glucocorticoids such as dexamethasone. Organ cultures of rat tongue, macrophages, human polymorphonuclear leucocytes, rat hepatoma cells, bovine endothelial cells, and tumour cells have been shown to respond to dexamethasone (Wunschmann-Henderson and Astrup, 1974; Vassali and Reich, 1976; Granelli-Piperno et al., 1977; Gelehrter et al., 1983; Levin and Loskutoff, 1982).

Laug et al. (1983) showed that bovine endothelial cells secrete both urokinase-type and tissue type P.A., and that dexamethazone, cortisone, and methyl prednisolone inhibit cellular and secreted P.A. Urokinase-type P.A. appeared to be selectively inhibited by dexamethasone while tissue P.A. seemed to be unaffected.

The inhibition of P.A. activity in rat hepatoma cells in the presence of dexamethasone has been shown to be due to the secretion of an inhibitor (Seifert and Gelehrter, 1978). Other
agents found to cause inhibition of P.A. secretion in bovine endothelial cells include thrombin which caused a decrease in both cellular and secreted P.A., and colchicine which blocked the secretion of P.A. but appeared to increase intracellular P.A. (Levin and Loskutoff, 1982).

c) Factors Implicated in the Regulation of Neutral Proteinase Secretion

The synovial joint system contains many different cell types but it has been shown that macrophages and chondrocytes are able to secrete neutral proteinase. Cell-to-cell interaction is one factor that regulates the neutral proteinase secretion in this system.

Lymphocytes, producing lymphokines (soluble lymphocyte factors) have been shown to stimulate macrophages into producing increased levels of neutral proteinase (Hauser and Vaes, 1979). In addition, macrophages have been shown to be able to stimulate other cell types to secrete proteinases. Deshmukh-Pradke et al. (1978) report that chondrocytes from normal rabbit articular cartilage and endotoxin-treated rabbit peritoneal macrophages separately secrete small amounts of neutral proteinase but when the conditioned medium from the macrophages was added to a culture of chondrocytes, increased secretion of neutral proteinase was induced. The stimulatory factor was heat stable and was lost by dialysis with tubing of MW cutoff 12,000. Ridge et al. (1980) also demonstrated that a macrophage factor could induce the synthesis of neutral proteinase from chondrocytes as well as articular cartilage in culture. Inhibition of the secretion of
this macrophage factor by cycloheximide indicated that it was a protein. It was also found to be heat stable (10 minutes at 60°C), stable to trypsin treatment but partially inactivated at pH 2.

Not only macrophages but synovial tissue of undefined cell types have been shown to be able to induce chondrocytes to secrete increased levels of neutral proteinase (Fell and Jubb, 1977). It is thought that the synovial tissue secretes a soluble factor, a protein which precipitated between 60 to 100% ammonium sulphate saturation, which was heat sensitive (at 70°C for 10 minutes) and was digested by chymotrypsin and pancreatic elastase, but not trypsin (Dingle et al., 1979).

Synovial cells themselves also respond to the macrophage factor by increasing their secretion of proteoglycan degrading neutral proteinase (Peeters-Joris and Vaes, 1984). This factor was non-dialysable through 8000 MW cutoff membrane and was partially precipitated by ammonium sulphate at 60% saturation. Inflammatory stimuli such as asbestos fibres, bacterial products (endotoxin, muramyl dipeptide, formyl-methionyl-peptide), phorbol myristate acetate, and concanavalin A were able to enhance the secretion of this factor by macrophages.

A variety of defined factors have also been shown to induce increased secretion of neutral proteinase from fibroblasts and tissue explants. Werb and Reynolds (1974) showed that the ingestion and subsequent intracellular storage of latex particles by rabbit synovial fibroblasts stimulated the release of neutral proteinase into the medium. The level of neutral proteinase
Porcine gingival explants in culture have been shown to respond to increased oxygen tension of the culture medium by increasing the level of neutral proteinase secreted (Pettigrew et al., 1978). The same explants responded to 5 μg/ml indomethacin by a decreased synthesis of neutral proteinase, indicating that prostaglandins may be involved in the regulation.

Pettigrew et al. (1981) also found that the addition of 30 μg/ml endotoxin into the culture media of the gingival explants resulted in an increased synthesis of neutral proteinase but not the tissue inhibitor, which was unaffected.

Meikle et al. (1980) reported that the application of continuous tensile stress to rabbit calvaria explants (organ cultures) resulted in an increase in secretion of both gelatin-degrading and proteoglycan-degrading neutral proteinase. This was accompanied by an increase also in the tissue inhibitor level. The authors had previously found (Meikle et al., 1979) that the same explants responded to tensile stress by a 1.5 to 3 fold increase in protein accumulation and a 2 fold increase in collagen. However, although there was increased secretion of proteolytic enzymes, this was not accompanied by an increase in the degradation of structural proteins as measured by 3H-proline release. The increased amount of inhibitor synthesized was able to complex the increased level of enzyme secreted, confirming that this is one level of control exerted on the activity of the proteinases.

Although epithelial cells have been shown to be able to
secrete neutral proteinase (Pettigrew, Ph.D. thesis), the factors regulating its secretion have not been investigated in detail.

**PROBLEM FORMULATION**

The aim of this thesis was to study the regulation, with emphasis on the role of cell shape, of neutral proteinase and plasminogen activator secretion by E-cells in culture. To this end, several techniques and experimental conditions had to be developed.

1) **Enzyme Assays**

The assays for neutral proteinase (N.P.) and P.A. activity had to be sensitive enough to detect the enzyme activities in the media.

2) **Cell Culture**

It was necessary to set up the cell culture system so that detectable amounts of the enzyme activity were obtained. Because of its importance in other systems, the effect of cell population density had to be investigated. The choice of the medium was also important as the relatively high concentrations of foetal bovine serum used in most cultures contains significant amount of α₂-macroglobulin and other factors which inhibit N.P. and P.A. activity. Moreover, I was interested not only in studying the effect of different factors on the secretion of N.P. and P.A. by epithelial cells but also the effects of cell proliferation on the regulatory process. Therefore conditions had to be devised which allowed a comparison between the cells in a proliferative and a non-proliferative state.
3) **Methods For Altering Cell Shape**

Various chemical agents known to cause morphological changes were applied to E-cells. Cholera toxin and dibutyryl cyclic AMP caused cell flattening while phorbol myristate acetate caused a slight cell rounding. The main problem associated with the use of these agents is that they affect more than just the cell shape; cholera toxin for example, causes an increase in cAMP and cell growth as well.

To circumvent this problem, mechanical means were also used to change cell shape, and their effects on proteinase secretion investigated. In addition, tissue culture dish adhesivity was altered by pre-coating the dishes with varying concentrations of poly(HEMA) (using the technique of Folkman and Moscona, 1978). An increasing concentration of poly(HEMA) solution resulted in a decrease in E-cell spreading. A detailed description of these techniques is given in the next chapter.
CHAPTER 2 - MATERIALS AND METHODS

I) **Cell Culture**

1) **Procedures**

Epithelial cells derived from porcine cell rests of Malassez were obtained from porcine periodontal ligament as described by Brunette et al (1976). The cells were cultured in αMEM plus 15% foetal calf serum (FCS) (Flow, Cockeysville, MD) with penicillin G (Sigma, St. Louis, MO) 100 μg/ml, gentamycin (Sigma) 50 μg/ml and amphotericin B (Gibco, Grand Island, NY) 3 μg/ml, at 37°C in a humidified atmosphere of air plus 5% CO₂.

To subculture the E-cells, the medium was removed from the dish and replaced with 10 ml of a trypsin solution (0.25% trypsin, Worthington Cat. No. 44521) in citrate saline (pH 7.8). This solution was removed and a fresh 10 ml of trypsin solution was added and the cells incubated at 37°C. After 4-5 minutes, when the cells had rounded and could be detached from the dish, the cells in trypsin solution were added to an equal volume of αMEM + 15% FCS in a sterile plastic tube (Falcon 2001) and the mixture centrifuged for 15 minutes at 750 g. The pellet of cells was resuspended in the medium and mixed well by pipetting the suspension up and down several times. This suspension was then used for inoculation of cultures.

For experiments, the E-cells were plated on 60 mm Falcon dishes at 4 x 10⁵ cells/dish unless otherwise stated and incubated overnight in αMEM + 15% FCS. The medium was removed and replaced with a modified low serum medium with 1 mM K⁺ and 0.1 mM Ca++
designated BMEM plus 0.5% dialysed FCS (described by Brunette, 1984a) for three to four days to allow sufficient enzyme secretion for the activity to be assayed. The number of cells in the culture were counted at each time point of medium collection.

2) **Determination of Cell Number**

One half ml of the cell suspension to be counted were added to vials containing 9.5 ml isotonic saline (Isoton, Coulter, Hialeah, Florida) and mixed thoroughly. Two readings were taken of each sample on an electronic cell counter (Coulter Electronics, Inc., Hialeah, Florida).

**II) Chemicals**

E. coli lipopolysaccharide serotype 0127:B8, phenolic extraction and trichloroacetic acid extraction (Sigma), cholera toxin (Sigma), dibutyryl cyclic - 3'5' - adenosine monophosphate (Bt$_2$ cAMP) (Sigma) and phorbol 12-myristate 12-acetate (PMA) (Sigma) were added to the medium to give the desired final concentration.

**III) Methods of Altering Cell Shape**

1) **Mechanical Stretching**

Mechanical stretching was applied using a modification of the method of Brunette (1984b) developed by Hasegawa et al. (in press). The flexible plastic bottom of a Petriperm dish (Tekmar, Cincinnati, OH) was stretched by putting a template with a convex surface underneath the dish and a lead weight on the top (see Figure 1). Twenty dishes could be stretched at the same time by
Fig. 1. Technique for mechanical stretching of cells grown on a petriperm dish. A convex template is placed underneath the petriperm dish and a lead weight placed on top.
placing a 40 x 26 x 0.55 cm plexiglass sheet over the dishes and then putting two 6 kg weights on the plexiglass. A template producing a 4% increase in surface area was used. The template was made by heating the centre of a 100 x 15 mm petri dish and placing the petri dish onto the curvature of a globe of radius 7.6 cm. The extent of the curved area was limited by applying a bottle cap of internal diameter 4.6 cm. The surface curvature produced was that of an arc of 36° with a radius of 7.6 cm.

The principle of the method is that stretching the flexible plastic membrane of the Petriperm dish, which is attached firmly to the cells by hemidesmosomes, results in stretching the cells and making them more flattened compared to the unstretched cells. Conversely, growing the cells on a stretched membrane and then taking the petriperm dish off the template allows the plastic to return to its initial state and the attached cells to become more rounded.

Sixty mm Petriperm dishes were plated with E-cells at $8 \times 10^5$ cells/dish in αMEM + 15% FCS overnight. The medium was replaced with fresh medium and the cells grown for an additional three days under the conditions below (see Figure 2). For the detection of proteinase secreted, the cells were washed twice with βMEM + 0.5% dialysed FCS (DFCS) and incubated in the same medium for 2 days.

1) Unstretched controls: The cells remained unstretched over the 5 day experimental period.
Fig. 2. Design of the mechanical stretching experiment. E-cells plated at $8 \times 10^5$ cells/dish were treated by either mechanical stretching or mechanical relaxation, with corresponding controls.
ii) Stretched: The cells were stretched during the two day proteinase collection period.

iii) Stretched controls: The cells were grown on a stretched membrane and remained stretched during the two day proteinase collection period.

iv) Relaxed: The cells were grown on a stretched membrane and then the dishes were taken off the templates during the two day proteinase collection period.

2) Preparation of Grooved Substratum

The amount of proteinase secreted by cells cultured on grooved substrates was compared to cells grown on flat substrates because time lapse cinemicrographic observations of cells grown on grooved substrates indicate that the cells assume a round shape more frequently than cells grown on a flat substrate (Brunette - personal communication). That cells grown on grooved substrates are more round than those grown on flat substrates has also been observed by Rovensky et al (1971). Silicon wafers with V shaped grooves were produced as described by Brunette et al. (1983). The grooved substrates had v-shaped grooves which were 79 microns wide at the top of the v and 60 microns deep. The grooves were separated by flat areas which were 13 microns in width.

Impressions of the groove pattern on the silicon wafers were made with Exaflex (C-C Dental Industrial Corp., Japan). The groove pattern was then reproduced onto Epotek resin (Epoxy technology Inc., Billerica, MA). Three ml of the resin were poured onto 60 mm dishes (Falcon) and the Exaflex impressions with a
diameter slightly smaller than the dish were lowered onto the surface of the resin. These were allowed to stand overnight at room temperature. The dishes were then placed in a 60°C oven for 3 days to allow complete curing. The control dishes were made with a flat surface.

3) **Preparation of Poly(HEMA) Plates**

Cell shape was also altered by changing the culture dish surface adhesivity. Following the method described by Folkman and Moscona (1978), 3 g poly(HEMA) (Polysciences, Inc., Warrington, PA) was dissolved in 25 ml 95% ethanol by stirring at 37°C overnight and the mixture was centrifuged for 30 minutes to remove undissolved particles. This stock solution was then diluted in 95% ethanol to give the desired concentrations. Following Folkman’s terminology, the poly(HEMA) concentrations stated are the amounts of dilution of the stock solution in alcohol. For example, to obtain a concentration of $10^{-3}$, 0.1 ml of the stock solution was added to 99.9 ml ethanol. Each 60 mm dish was precoated with 1.8 ml of poly(HEMA) solution, dried in a sterile environment overnight and the cells plated in αMEM + 15% FCS.

IV) **Morphological Techniques**

1) **Transmission Electron Microscopic Techniques**

E-cells grown on petriperm dishes at normal density were processed for electron microscopy. Each dish of cells was fixed in 2.5% glutaraldehyde (J.B. EM Services, Inc., Quebec) in 0.1M sodium cacodylate at pH 7.4 for 1 hour on ice, and then rinsed
three times with 0.1M sodium cacodylate buffer. Post-fixation was carried out in 1% osmium tetroxide (J.B. EM Services, Inc.) in 0.1M sodium cacodylate, pH 7.4, for 30 minutes on ice. The cells were washed three times with distilled water and stained in 2% aqueous tannic acid at room temperature for 20 minutes, followed by 5 washes in distilled water; and 1% aqueous osmium tetroxide for 30 minutes, followed by 3 washes in distilled water.

The cells were then dehydrated in graded ethanol, 30% to 100%, over a time period of 35 minutes. Infiltration was carried out in 50/50 mixture of ethanol and epon (J.B. EM Services, Inc.) and the dish allowed to stand overnight with the lid off. After 2 changes of fresh epon mixture for 2 and 4 hours respectively, the cells were left in epon mixture with the accelerator, tri (dimethyl aminomethyl) phenol (DMP 30) for 1 hour; replaced with a fresh mixture and placed in a 37°C oven overnight and subsequently in 60°C oven for 4 days. Once polymerized, the dish was turned over and a fresh mixture of epon and DMP 30 added to the bottom of the petriperm dish so that the cells and the plastic layer were sandwiched between two layers of epon.

The epon block was cut into 5 x 5 x 10 mm blocks by a diamond tip saw for electron microscopic sectioning. One micron sections were cut perpendicular to the plastic surface on a Sorvall MT2 Ultramicrotome (Sorvall Inc., Connecticut), using a glass knife (LKB Produkter AB, Stockholm), stained with Richardson's stain and examined to select for a suitable area. Thin sections were cut at 40 to 60 nm with a diamond knife (Diatome Ltd., Bienne,
Switzerland). Sections were picked up on 50 mesh copper grids (Fullam Inc., Schenectady, NY), coated with formvar and carbon, and stained with approximately 2% aqueous uranyl acetate for 20 minutes, followed by Reynolds' lead citrate for 5 minutes.

The sections were then examined under a Phillips 300 Transmission Electron Microscope at 80 kV.

2) **Richardson's Stain** (Richardson, Jarett & Finke, 1960)

Each dish of cells was fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate at pH 7.4 on ice. The solution was poured off and the cells washed in 0.1 M sodium cadodylate buffer three times and rinsed in distilled water three times. Richardson's stain consisting of 0.5% methylene blue and 0.5% Azure 2 in 1% borax solution was filtered before use. Three drops of the stain were placed onto the cells for 2 minutes and then the stain was washed off with distilled water.

3) **Scoring of Cell Spreading**

 Cultures stained with Richardson's stain to make the cytoplasm more evident were examined under the microscope. The extent of cell rounding was assigned a score on an arbitrary scale 0-3, based on the amount of cell area in contact with the dish. Cells grown on tissue culture plastic were assigned a score of 0. They were well spread. Cells which were slightly rounded compared to the cells which scored 0, were given a score of 1. Round cells which showed a cell area approximately a third of that of the cells scoring 0, were assigned a score of 3.
V) **Preparation of Media for Enzyme Assays**

The media in which the cells were incubated was centrifuged for 10 minutes at 500 rpm to pellet any cells that may have been present. Millipore CX-10 (MW cutoff 10,000) filters (Millipore Corp., Bedford, MASS) were pretreated by 1) passing through distilled water to remove preservatives and 2) passing through 100 ml of conditioned media per filter in order to saturate any sites on the membrane that could bind to the enzymes. To test whether the bound enzymes could be subsequently released, fresh medium was concentrated using a pretreated filter and the filtered medium assayed for neutral proteinase activity. No activity was found. Conditioned media were therefore concentrated ten to twenty times using the pretreated filters. The samples were kept at 0°C during processing, then dialysed in Spectrapore 2 membranes with MW cutoff 12,000 to 14,000 (Spectrum Med., Ind., LA) against 50 mM Tris buffer containing 200 mM sodium chloride and 5 mM calcium chloride at pH 7.5 at 4°C.

VI) **Assays**

1) **Cyclic AMP Assay**

Two dishes from each group were used for measurement of intracellular cAMP levels (Oey et al., 1974). The medium was removed and replaced with 2 ml of cold 5% trichloroacetic acid and the dishes incubated at 4°C for 1 hour. The mixtures were then centrifuged at 4°C for 5 min at 10,000 rpm (International Equipment Co., Needham Heights, Mass, USA), and the supernatant washed five times with 2 volumes of water saturated ether after which it was lyophilized. The pellet was collected for a protein
assay. Cyclic AMP levels in the supernatant were measured using the radioimmuno assay kit $^{[125\text{I}]}$ (Becton Dickinson Immuno-diagnostics, Orangeburg, NY).

2) **Assay for Neutral Proteinase (N.P)**

Neutral proteinase was assayed using azocoll (Calbiochem, San Diego, CA) as the substrate. Each assay included 450 µl of the activated sample, 4 mg azocoll and 1 ml of 50 mM Tris buffer containing 200 mM sodium chloride and 50 mM calcium chloride, pH 7.5. The assay mixtures were incubated in Reacti-Therm vials (Pierce Chem. Co., Rockford, IL) with stirring at 37°C. After 24 to 48 hours, the mixtures were transferred to 1.9 ml microcentrifuge tubes (Evergreen Scientific, LA, CA) and centrifuged for 5 minutes in an Eppendoff microcentrifuge (Brinkmann Instruments, Westbury, NY). The supernatant was read at 520 nm in an SP 800 spectrophotometer (Unicam, Cambridge, England) and compared to a standard curve of the digestion of azocoll by trypsin (Figure 4). All assays were done in duplicates and controls included 1) buffer only 2) fresh medium. The degradation of azocoll was linear with respect to enzyme concentration (Figure 5) as well as incubation time up to 56 hours (Figure 3). One unit of enzyme activity was defined as equal to 1 mg azocoll solubilised per hour at 37°C under these experimental conditions. Activities were expressed as Units per $10^5$ cells.
Fig. 3. Time course of neutral proteinase assay. Three hundred μl of conditioned medium were activated and incubated with azocoll for time periods shown. The background activity found in the control has been subtracted to give the values shown. Each point represents the average of two determinations.
Fig. 4. Standard curve for the digestion of azocoll. Different amounts of azocoll were incubated with excess trypsin at 37°C until all the azocoll had been digested. Each point represents the average of two determinations.

Fig. 5. Dependence of azocoll degradation on enzyme concentration. Increasing amounts of conditioned media were activated with mersalyl acid. The assays, done in duplicate, were incubated for 46 hours.
3) Activation and Characteristics of the Neutral Proteinase (N.P.)

The N.P was secreted in a latent form which could be activated by mersalyl acid or TPCK trypsin or 4-aminophenyl mercuric acetate (APMA). The reagents were made up as follows.

Mersalyl acid (Sigma) was added to water, the addition of a few drops of 1M NaOH helped to dissolve any remaining particles and the volume made up with water. APMA (Sigma) was dissolved in 1M NaOH, titrated with 1M HCl until a little precipitate is seen, then a few more drops of NaOH was added to dissolve it and the volume made up with water.

Activation with mersalyl acid or APMA was carried out by preincubating 300 µl of the concentrated media with 75 µl of 40 mM of the activator for 10 minutes, followed by an addition of 75 µl of 1 mg/ml Soybean trypsin inhibitor (STI) for a further 10 minutes. The STI inactivates any traces of trypsin that might have been left behind from the subculture procedure. Activation with trypsin was also carried out in a similar way using 75 µl of 0.1 mg/ml TPCK trypsin (Worthington Biochem. Corp., Freehold, NY) followed by a tenfold excess of STI (1 mg/ml).

Figure 6 shows that maximum activity was achieved with the addition of 75 µl of 20 mM mersalyl acid (1 mM final concentration). In contrast, 10 µg of TPCK trypsin (100 µl of 0.1 mg/ml TPCK trypsin) activated only 30% of the maximum activity. APMA at a final concentration of 2 mM activated 17% of the N.P. activity obtained by activating with an equivalent concentration of mersalyl acid.
Fig. 6. Comparison of mersalyl acid and TPCK trypsin as activators of neutral proteinase. Two hundred μl of the conditioned medium were preincubated with increasing amounts of mersalyl (20 mM)(o) or TPCK trypsin (0.1 mg/ml)(x). The assays were incubated for 46 hours. The background activity found in the control has been subtracted to give the values shown. Each point represents the average of two determinations.
The activated neutral proteinase was inhibited by EDTA (a metal chelating agent), cysteine and foetal calf serum (Table 1). These characteristics are similar to the neutral proteinase obtained from rheumatoid synovium, rabbit bone and rabbit synovial fibroblasts (Harris and Krane, 1972; Sellers et al., 1978; Werb & Reynolds, 1974).

4) **Plasminogen Activator (P.A.) Assay**

The two stage assay described by Jackson, Esmon and Tang (1981) was used. Plasminogen (Kabi, Stockholm, Sweden) was dissolved in 50 mM Tris buffer, pH 7.5 to a concentration of 5 U/ml and stored frozen in 200 µl aliquots. Before use, it was diluted to 1 U/ml with 50 mM Tris buffer + 0.5% bovine serum albumin (BSA), pH 7.5. Streptokinase (Behring Institute, Marburg, W. Germany) was made up to 100 IU/ml in 50 mM Tris buffer, pH 7.5 and stored frozen. Before use, it was diluted to 0.5 IU/ml with 50 mM Tris buffer + 0.5% BSA, pH 7.5. The synthetic substrate D-Val-Leu-Lys-p-nitroanilide (Kabi S2251) was dissolved in water to a concentration of 5 mg/ml. Before use, it was mixed with a 1.77 M NaCl in 32 mM Tris buffer solution (pH 7.5) in a 2:3 ratio.

The assay was carried out in 1.9 ml micro-centrifuge tubes (Evergreen) at 37°C in a shaking water bath. The first stage of the reaction was initiated by adding 20 µl of the sample to 40 µl Tris buffer and 40 µl of plasminogen (1 U/ml) solution. The first stage of the reaction was terminated and the second begun by adding 80 µl of the substrate S-2251 in NaCl solution at precisely
<table>
<thead>
<tr>
<th>Sample</th>
<th>$OD_{520}$</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
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<td>$0.59 \pm 0.01$</td>
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</tr>
<tr>
<td>Activated sample + EDTA (0.5 mM final concentration)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Activated sample + cysteine (0.5 mM final concentration)</td>
<td>$0.22 \pm 0.01$</td>
<td>34</td>
</tr>
<tr>
<td>Activated sample + dialysed FCS (0.5% final concentration)</td>
<td>$0.53 \pm 0.02$</td>
<td>89</td>
</tr>
<tr>
<td>Activated sample + dialysed FCS (10% final concentration)</td>
<td>$0.21 \pm 0.05$</td>
<td>35</td>
</tr>
</tbody>
</table>

Two hundred and seventy-five µl of the samples were preincubated with mersalyl acid (final concentration 2 mM) before the different agents were added. The assays were incubated for 21 hours. The OD reading represents tests minus background values. The estimated variation is given as ± SEM.
30 minutes after the reaction was started. The second stage of the reaction was terminated after a further 30 minutes by the addition of 0.8 ml of 40% acetic acid. The OD was then read at 405 nm. All assays were done in duplicate and included the following controls:

a) buffer only to measure any spontaneous degradation of the plasminogen;

b) controls without plasminogen to measure any S-2251 degrading enzymes other than plasmin (controls a and b should read zero);

c) A series of increasing volumes of Streptokinase solution to give the standard curve which was used as the basis for calculation of the amount of P.A. activity in the unknown samples (Figure 7). The assay was linear with respect to increasing amounts of the sample up to an absorbance of 0.9 (Figure 8).

Activities were expressed in terms of IU Streptokinase /10⁵ cells.

VII) Error Study and Statistical Analysis

In order to quantitate the amount of variation between groups, 5 groups of 3 dishes each were set up with cells plated at normal density. At day 3 of culture, the cells were counted and the medium assayed for N.P. and P.A. activity. The N.P. activity was 15.6 x 10⁻³ U/10⁵ cells ± 0.23 (SEM) and the P.A. activity was 55.9 x 10⁻³ IU
Fig. 7. Standard curve of Streptokinase activity. Each point represents the average of two determinations.
Fig. 8. Absorbance change as a function of the amount of added conditioned media. Increasing amounts of conditioned media from E-cells cultured on flat substrates (■) and from E-cells cultured on grooved substrates (▲) were assayed for P.A. activity.
Streptokinase/10^5 cells ± 1.4 (SEM). The coefficient of variation for N.P. and P.A. activity was 3% and 6% respectively.

In experiments where replicate cultures were used, the data was analysed using one way analysis of variance. In other experiments, the sign test (Zar, 1974) was used to test whether the differences observed were due to chance alone. The direction of the differences between the treated and control values was noted; a positive sign was assigned to the difference observed which was in the expected direction. For example, cholera toxin was expected to cause a reduced level of enzyme secretion. Therefore when a reduced value was observed, a + sign was assigned. A negative sign was assigned for a value that showed no difference from the control or showed a difference opposite to what was expected. A sign was given for each pair of values obtained from the treated and control groups. The probability owing to chance that the number of times + signs were obtained out of the total (N), was obtained from a table. The null hypothesis was rejected if the probability p < 0.05.
CHAPTER 3 - RESULTS

E-cells in culture secreted latent neutral proteinase which could be activated by either trypsin, APMA, or mersalyl acid. Inhibition of N.P. activity by EDTA, cysteine and FCS characterized it as a metallo-neutral proteinase similar to that secreted by other cell types (Harris and Krane, 1972; Sellers et al., 1978; Werb and Reynolds, 1974). No active form of the neutral proteinase was detectable. Tests for the free inhibitor of metalloproteinase in day 3 medium proved negative.

The presence of P.A. was detected by means of a chromogenic assay. The effect of cell population density, E.Coli LPS and cell shape on proteinase secretion by E-cells will be presented.

I. **Effect of Cell Population Density**

The effect of cell population density on proteinase secretion was investigated by culturing E-cells at 2, 4, 8, and 15 x 10^5 cells/ dish in MEM + 0.5% dialysed FCS. These conditions kept the cells in a quiescent state but also permitted growth when the cells were stimulated by cholera toxin or Bt2 cAMP. At days 3, 7, and 10 of the experiment, proteinase activity was assayed and the cells counted. Under these conditions, the number of cells per culture remained constant over the ten day period.

Figure 9 shows that neutral proteinase activity was highest at low cell population density, the activity decreasing with increasing cell population density. The relationship between cell population density and N.P. activity was similar at days 3, 7, and 10. P.A. secretion exhibited a similar pattern with the exception of day 3 (Figure 10).
Fig. 9. The effect of cell population density on N.P. activity. The cells were plated at 2, 4, 8, and 15×10^5 cells/dish. The medium was collected at days 3(o), 7(□) and 10(Δ), the cells were counted and the N.P. activity determined. The data shown are from one representative experiment that was repeated and the trends were the same in both experiments.
The effect of cell population density on P.A. activity. The cells were plated as described in Fig. 9 and the P.A. activity on days 3(o), 7(■) and 10(△) determined. The data shown are from one representative experiment that was repeated and the trends were the same in both experiments.
In contrast to N.P. activity, P.A. activity in the medium at day 3 was lower than that found on day 7 or day 10.

II. Effect of E.coli LPS

As bacterial lipopolysaccharide has been found to cause a variety of biological effects such as induction of the inflammatory response (Jensen et al., 1966) and the stimulation of macrophages and gingival explants to release increased amounts of metalloproteinases (Wahl et al., 1975; Pettigrew et al., 1981), it was of interest to investigate whether LPS had a similar stimulatory effect on E-cells.

E.coli LPS (phenol extract) was tested at 3, 30 and 60 µg/ml. Table II shows that N.P. secretion by E-cells was stimulated by E.coli LPS, 3 µg/ml being just as effective as 60 µg/ml.

Two preparations of LPS, a trichloroacetic acid (TCA) extract and a phenol extract, were compared to see if the method of LPS preparation affected proteinase secretion differently. Figure 11 shows that both preparations of E.coli LPS stimulated a 2 fold increase in N.P. activity compared to the control. One way ANOVA indicates that there was a statistically significant difference between the treated and control groups (p < 0.05). The TCA extract also caused an increased amount of P.A. secretion by E-cells; a cumulative increase of 3 1/2 times over the control was seen by day 10 of culture (Figure 12). Analysis of the data shows that the difference is significant (p < 0.05). The phenol extract, however, caused only a small increase in P.A. secretion (Figure 12).

Components of dental plaque have been shown to inhibit cell proliferation (Baloolol et al., 1970; Singer and Buckner, 1980). Dental
<table>
<thead>
<tr>
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<th>N.P. Activity 10^{-3} U/10^5 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 3</td>
</tr>
<tr>
<td>Control</td>
<td>2.2</td>
</tr>
<tr>
<td>LPS 3 µg/ml</td>
<td>14.4</td>
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<td>LPS 30 µg/ml</td>
<td>11.0</td>
</tr>
<tr>
<td>LPS 60 µg/ml</td>
<td>6.4</td>
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</table>

E-cells were plated in αMEM + 15% FCS overnight and then incubated in βMEM + 0.5% DFCS. The medium was collected on days 3, 7 and 10 and assayed for N.P. activity.
Fig. 11. Effect of E. coli LPS (TCA extract) and E. coli LPS (phenol extract) on N.P. activity. Duplicate cultures were incubated in βMEM+0.5% DFC; and the medium collected and assayed for N.P. activity, given as cumulative values. Control (○), LPS (TCA extract) 3 μg/ml (△) and LPS (phenol extract) 3 μg/ml (■) Error bars indicate SEM.
Fig. 12. Effect of E.coli LPS (TCA extract) and E.coli LPS (phenol extract) on P.A. activity. The experiment was described in Fig. 11. The medium was also assayed for P.A. activity, given as cumulative values. Control (o), LPS (TCA extract) 3 μg/ml (Δ) and LPS (phenol extract) 3 μg/ml (■). Error bars indicate SEM.
plaque has also been demonstrated to be able to alter the morphology of gingival fibroblasts in-vitro and to cause the degradation of cell surface proteins such as fibronectin (Larjava and Jalkanen, 1984). However, E.coli LPS at 3,30 and 60 μg/ml caused no changes in cell numbers. Furthermore, no change in cell morphology was observed.

Some studies have demonstrated that bacterial LPS can cause increases in cAMP levels in certain cell types (Watson, 1976; Naylor et al., 1978), but no significant effect was found in E-cells. Measurements of intracellular cAMP levels at day one of culture showed that E-cells exposed to 3 μg/ml E.coli LPS had 0.23 ± 0.05 (S.D.) pmol cAMP/10^5 compared to control cells which contained 0.18 ± 0.03 (S.D.) pmol cAMP/10^5 cells.

III. Cell Shape and Proteinase Secretion

To test the hypothesis that E-cell shape modulates proteinase secretion, different means of changing cell shape were used. Agents that increase intracellular cAMP levels such as cholera toxin and dibutyryl cAMP, which have been reported to cause E-cells to flatten out (Brunette, 1984a) were tested for their effect on proteinase secretion. A different effect was produced by phorbol myristate acetate at 10 ng/ml, which caused E-cells to retract slightly at the cytoplasmic edges. This morphological change caused by PMA is in agreement with the report by Wigler and Weinstein (1976) who observed a similar effect of PMA on normal chicken embryo fibroblasts.
1) **Cholera Toxin**

E-cells were exposed to cholera toxin at 1 ng/ml over a ten day culture period and the proteinase activity assayed at days 3, 7, and 10. The number of cells in each dish was also determined at each time point of the enzyme assay.

Table III shows that E-cells were plated at two different cell densities and at each density, cell numbers increased in the presence of cholera toxin. Control cultures showed a small decrease in cell number at day 10 compared to day 3. One way ANOVA indicates that the increased growth seen with cholera toxin was significant (p < 0.05).

Therefore, at the time that enzyme assays were done, the cultures treated with cholera toxin had a different cell population density compared to control cultures. Since it has been shown that cell population density modulates proteinase secretion, a correction for the cell population density effect was made on the basis of the data in Figs. 9 and 10.

E-cells in the presence of cholera toxin, produced on average, 69% of the amount of N.P. and 29% of the P.A. produced by control cultures (Table III). Statistical analysis using the sign test showed that the reduced amounts of N.P. and P.A. activity were significant (p < 0.05).

Since it is thought that cholera toxin mediates its effect on cells through cAMP, intracellular cAMP levels were measured in E-cells. At day 1 of culture, E-cells in the presence of cholera
### TABLE III: EFFECT OF CHOLERA TOXIN (C.T) 1 ng/ml

<table>
<thead>
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<th>Expt. 1</th>
<th>Expt. 2</th>
<th>% CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Counts</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$10^5$ cells/dish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>$4.2 \pm 0.04$</td>
<td>$2.16 \pm 0.06$</td>
<td>100</td>
</tr>
<tr>
<td>Day 7</td>
<td>$3.8 \pm 0.07$</td>
<td>$1.76 \pm 0.22$</td>
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<tr>
<td>Day 10</td>
<td>$4.0$</td>
<td>$1.24 \pm 0.02$</td>
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<td>Expt. 2</td>
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<tr>
<td>Day 3</td>
<td>$2.16 \pm 0.06$</td>
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<tr>
<td>Day 7</td>
<td>$1.76 \pm 0.22$</td>
<td>$2.49 \pm 0.07$</td>
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<tr>
<td>Day 10</td>
<td>$1.24 \pm 0.02$</td>
<td>$2.43 \pm 0.15$</td>
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<td><strong>N.P. Activity</strong></td>
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<tr>
<td>$10^{-3}$ U/$10^5$cells</td>
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<tr>
<td>Expt. 1</td>
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</tr>
<tr>
<td>Day 3</td>
<td>$6.4$</td>
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<td>Day 7</td>
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<td>Day 10</td>
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<tr>
<td>Day 3</td>
<td>$29.5$</td>
<td>$(23.2)$</td>
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<tr>
<td><strong>P.A. Activity</strong></td>
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</tr>
<tr>
<td>$10^{-3}$ IU</td>
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<td></td>
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<td>Expt. 1</td>
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<tr>
<td>Day 3</td>
<td>$0.7$</td>
<td>$(0.1)$</td>
<td>14</td>
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<td>Day 7</td>
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<td>Day 10</td>
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<td>$(0.5)$</td>
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<td>Day 3</td>
<td>$7.5$</td>
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<td>$(1.3)$</td>
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<td>Day 10</td>
<td>$3.7$</td>
<td>$(1.6)$</td>
<td>63</td>
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E-cells were plated in αMEM + 15% FCS overnight and then incubated in βMEM + 0.5% dialysed FCS for proteinase collection. C.T. was added at 1 ng/ml. Two dishes were used for cell counting at each time point of medium collection. The N.P. and P.A. activity from the C.T. treated cultures have been corrected for the cell population density effect. The values in brackets represent uncorrected numbers. Estimation of variation is given as ± SEM.
toxin (1 ng/ml) had 0.52 pmol cAMP/10^5 cells compared to control cells which had 0.18 ± 0.03 pmol cAMP/10^5 cells (± S.D). This difference in cAMP level was statistically significant (p<0.01).

2) **Dibutyryl cAMP (Bt$_2$ cAMP)**

The effect of Bt$_2$ cAMP on E-cell behaviour was similar to that of cholera toxin. Cell numbers increased four fold by day 14 (Table IV). N.P. secretion by E-cells exposed to Bt$_2$ cAMP averaged 8% of the N.P. secreted by control cultures (Table IV). Thus cell flattening caused by both cholera toxin and Bt$_2$ cAMP was accompanied by a reduction in proteinase secretion and an increase in cell proliferation.

3) **Phorbol Myristate Acetate (PMA)**

PMA caused a slight increase in cell number in comparison to control cultures. At day 4 of the experiment, cultures with PMA at 1 ng/ml showed a significant increase, 29% in cell numbers over the control (p<0.05). PMA at 10 ng/ml however, caused no significant increases in cell number. Table V shows that 10 ng/ml PMA stimulated a 1.4 to 4 fold increase in N.P. and P.A. secretion. PMA at 1 ng/ml caused a smaller increase in proteinase secretion.

The increase in proteinase secretion accompanying the slight cell rounding and the reduced proteinase secretion observed together with cell flattening (cholera toxin and Bt$_2$ cAMP experiment) suggests that cell shape influences proteinase secretion. The use of these chemical agents to modify cell shape however, can be criticized because they affect other cell functions as well.
<table>
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<th>(Bt_2\text{cAMP})</th>
<th>% CONTROL</th>
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<tr>
<td>10^5 cells/dish</td>
<td>Day 4</td>
<td>2.0 ± 0.04</td>
<td>2.6 ± 0.18</td>
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<tr>
<td></td>
<td>Day 7</td>
<td>2.2 ± 0.20</td>
<td>4.4 ± 0.20</td>
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<td></td>
<td>Day 11</td>
<td>1.7 ± 0.22</td>
<td>5.0 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>1.2 ± 0.01</td>
<td>5.0 ± 0.11</td>
</tr>
<tr>
<td><strong>N.P. Activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^-3 U/10^5 cells</td>
<td>Day 4</td>
<td>5.0</td>
<td>(0.4) 0.51</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>5.7</td>
<td>(0.74) 1.40</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>2.1</td>
<td>(0.06) 0.12</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>6.6</td>
<td>(0.4) 0.83</td>
</tr>
</tbody>
</table>

E-cells were plated in αMEM + 15% FCS overnight and then incubated in βMEM + 0.5% dialysed FCS. \(Bt_2\text{cAMP}\) was added at 0.5 mM. The N.P. activity from the \(Bt_2\text{cAMP}\)-treated cultures have been corrected for the cell population density effect. The uncorrected values are shown in brackets. Estimation of variation is given as ± SEM.
TABLE V: EFFECT OF PHORBOL MYRISTATE ACETATE (PMA)

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>PMA 1 ng/ml</th>
<th>PMA 10 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5 cells/dish</td>
<td>Day 1: 5.0 ± 0.04</td>
<td>5.85 ± 0.04</td>
<td>4.1 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Day 2: 5.45 ± 0.25</td>
<td>5.8 ± 0.07</td>
<td>5.6 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Day 4: 4.65 ± 0.04</td>
<td>6.0 ± 0.18</td>
<td>5.25 ± 0.18</td>
</tr>
<tr>
<td>N.P. Activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^-3 U/10^5 cells</td>
<td>Day 1: 5.3</td>
<td>17.4</td>
<td>21.3</td>
</tr>
<tr>
<td></td>
<td>Day 2: 6.2</td>
<td>9.9</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>Day 4: 7.6</td>
<td>11.5</td>
<td>10.6</td>
</tr>
<tr>
<td>P.A. Activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^-3 IUStrep-</td>
<td>Day 4: 1.8</td>
<td>4.4</td>
<td>6.7</td>
</tr>
<tr>
<td>tokinase/10^5 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E-cells were plated in αMEM + 15% FCS overnight and then incubated in βMEM + 0.5% DFCS for proteinase secretion. PMA was added at 1 ng/ml and 10 ng/ml. Two dishes were used for cell counting at each time point of medium collection. P.A. activity at days 1 and 2 were undetectable in both control and test groups. Estimation of variation is given as ± SEM.
For example, PMA is known to cause increased membrane transport (Boutwell, 1974) and stimulation of DNA, RNA, and protein synthesis (Boutwell, 1974). Therefore direct mechanical means of altering cell shape were tested and are described in the next section.

4) Mechanical Stretching

E-cells from the 4th to 8th subcultures were plated onto the flexible plastic bottoms of petriperm dishes. Electron microscopic sections of the interface between the cell and plastic show the presence of hemidesmosomes (See Figure 13) indicating tight attachment of the cells to the plastic surface. Thus as found by Brunette (1984b) stretching the dish also stretched the attached E-cells, resulting in the E-cells becoming more flattened. By removing the dishes from the template, a release in tension could be obtained allowing stretched cells to relax.

The amount of stretching was limited to 4% because stretching beyond this produced a loss of elasticity of the flexible plastic bottom. However, even stretching E-cells by 4% produced a 2 to 6 fold decrease in P.A. secretion compared to unstretched cells (Table VI). The effect of relaxing stretched cells compared to controls which remained stretched was an increase (40 to 210%) in P.A. secretion (Table VII). Statistical analysis was done using the sign test. The difference in P.A. values between the treated and the control on all five occasions that the experiments were carried out was in the expected direction. The probability of this occurrence happening due to chance alone is < 0.05.

The effect of stretching on N.P. secretion was investigated
Fig. 13. Electron microscopic section of E-cells grown on a petriperm dish. Cell attachment to the plastic surface (P) was through hemidesmosomes (HD). Magnification x 83,200.
### TABLE VI: EFFECT OF MECHANICAL STRETCHING ON E-CELLS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P.A. Activity (10^{-3}) IU Streptokinase/(10^5) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Unstretched Control</td>
<td>12.0</td>
</tr>
<tr>
<td>Stretched</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mechanical Stretching was applied as described in Materials and Methods.

The medium was collected on day 2 and assayed for P.A. activity.

### TABLE VII: EFFECT OF RELAXING E-CELLS CULTURED ON STRETCHED MEMBRANES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P.A. Activity (10^{-3}) IU Streptokinase/(10^5) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Stretched Control</td>
<td>1.5</td>
</tr>
<tr>
<td>Relaxed</td>
<td>3.2</td>
</tr>
</tbody>
</table>
using a parametric statistical test in a single experiment in which 10 groups of 4 dishes each were plated with cells at 8x10⁵ cells/dish. Five groups were stretched and the other 5 groups remained unstretched as controls. To get sufficient cells for this experiment, a 9th subculture was used, which was older than that normally used in these experiments. The cells from the 9th subculture did not demonstrate significant amounts of P.A. activity but detectable amounts of N.P. were present.

The stretched cells produced 5.9±0.16(SEM)x10⁻³ U of N.P./10⁵ cells compared to 7.7±0.16(SEM)x10⁻³ U/10⁵ cells produced by control cultures. The difference is statistically significant when tested by ANOVA (p<0.005).

There was no significant difference in cell numbers between the treated and control groups at the time of medium collection.

Thus, making the cells more flattened by stretching resulted in a decrease in proteinase secretion, while making the cells more rounded by relaxing stretched cells resulted in an increased proteinase secretion.

5) Grooved Substrata

This experiment makes use of an observation made previously in our laboratory, that cells grown on grooved substrates assume a round shape more frequently than do cells grown on a flat substrate (Brunette - personal communication). Rovensky et al. (1971) suggested that the cells probably attach to the bottom of the grooves less readily than to flat surfaces. Table VIII shows that the effect of the grooved substrate on proteinase secretion
TABLE VIII: EFFECT OF GROOVED SUBSTRATA

<table>
<thead>
<tr>
<th>N.P. Activity</th>
<th>FLAT</th>
<th>GROOVED</th>
<th>GROOVED/FLAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt. 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>0.15</td>
<td>0.39</td>
<td>2.6</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.13</td>
<td>0.33</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>6.5</td>
<td>11.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Day 7</td>
<td>2.5</td>
<td>6.2</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Expt. 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>7.45</td>
<td>9.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P.A. Activity</th>
<th>FLAT</th>
<th>GROOVED</th>
<th>GROOVED/FLAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt. 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>7.25</td>
<td>13.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.4</td>
<td>8.1</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>15.3</td>
<td>15.0</td>
<td>1</td>
</tr>
<tr>
<td>Day 7</td>
<td>14.3</td>
<td>17.5</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Expt. 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>4.2</td>
<td>7.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

E-cells were plated at 8 x 10⁵ cells/dish on flat epoxy substrates and epoxy substrates with v-shaped grooves (top width 79.3 µm, depth 60 µm, 13.3 µm apart). Proteinase activity was measured at days 3 and 7. P.A. activity from experiment 1 is also shown in Figure 8.
was, on average, a 2 fold increase in N.P. activity and a 1.6 fold increase in P.A. activity compared to the control. Analysis of the data using the sign test indicates that the increase in N.P. activity was significant (p < 0.05). No significant differences were observed in the cell counts between the test and control groups.

6) Poly(HEMA)-coated Dishes

Increasing concentrations of poly(HEMA) stock solution were used to alter tissue culture dish adhesivity. In agreement with the results of Folkman and Moscona (1978), E-cells became less spread out (that is, more rounded) on dishes coated with increasing concentrations of poly(HEMA) stock solution. Cultures stained with Richardson's stain were visualised through the microscope and the extent of cell spreading was assigned a score on an arbitrary scale 0-3, based on the amount of cell area in contact with the dish. Cells grown on plastic were well spread (score 0). The cells became less spread out, on dishes coated with $5 \times 10^{-4}$ dilution of stock poly(HEMA) solution. These were assigned a score of 1. A much reduced cell area compared to score 1 was observed when cells were grown on dishes coated with $10^{-3}$ dilution of the poly(HEMA) stock. These were given a score 3. Thus for E-cells, cell shape changes occurred over a narrow range of poly(HEMA) dilution.

E-cells were cultured in duplicate and the cell counts show that under the conditions used, there was no increase in cell number. N.P. activity increased with increasing poly(HEMA)
concentration (Figure 14). Cells cultured on poly(HEMA)-coated dishes at $10^{-5}$ dilution and greater showed a significant increase in N.P. activity compared to the control ($p < 0.05$ using the one way analysis of variance test). The sharpest increase in N.P. activity was observed between $5 \times 10^{-4}$ and $10^{-3}$ dilution of poly(HEMA) stock thus correlating with the sharp change in cell morphology noted. Although no obvious morphological change could be detected at $10^{-5}$ dilution of poly(HEMA), an increase in N.P. activity was observed. A more sensitive method needs to be developed to measure small changes in cell shape changes.

Figure 15 shows that P.A. activity also increased with increasing poly(HEMA) concentration, the increase being more gradual compared to N.P. activity.

The advantages of using poly(HEMA)-coated dishes to modify cell shape are: 1) the use of chemical agents with multiple effects is avoided; 2) the method allows different degrees of cell spreading to be achieved. The above results show that both N.P. and P.A. activity increased as the cells became less well-spread. A correlation between cell shape and proteinase secretion was thus shown.
Fig. 14. Effect of poly(HEMA) concentration on N.P. activity at day 3. E-cells were plated in duplicate at 4x10^5 cells/dish in dishes precoated with varying concentrations of poly(HEMA) solution. At day 3 of culture, medium was collected and assayed for N.P. activity. (○) represents data from experiment 1 and (▲) represents experiment 2. Error bars indicate SEM.
Fig. 15. Effect of poly(HEMA) concentration on P.A. activity at day 3. Medium collected on day 3 from the experiment described in Fig. 14 was assayed for P.A. activity (●) represents data from experiment 1 and (▲) represents data from experiment 2. Error bars indicate SEM.
CHAPTER 4 - DISCUSSION

E-cells in culture secreted latent neutral proteinase as well as plasminogen activator; the secretion of both proteinases was found to be regulated by cell population density, E.Coli LPS and cell shape changes. The significance of the results obtained will be discussed in the following order: I) cell population density; II) E.Coli LPS; III) cell growth; IV) cell shape; V) relationship between N.P. and P.A. secretion. Finally experiments which will build on these findings will be proposed.

I. Cell Population Density

In the experiments, cell growth was dissociated from cell population density by culturing E-cells in a modified medium, ßMEM + 0.5% dialysed FCS, with low Ca++ and K+. Under these conditions, cell numbers were maintained over the experimental period of ten days. Thus the relationship between cell population density and proteinase secretion could be analysed.

Both N.P. and P.A. secretion were dependent on cell population density. N.P. secretion was highest at low cell densities, decreasing as cell population densities increased. P.A. secretion showed a similar pattern except at day three, the day of the first medium collection. The regulation of proteinase secretion by cell population density is not confined to E-cells. Other cell lines have been reported to show density-dependent regulation of P.A. The work of Liu et al. (1982) and Rohrlich and Rifkin (1977) which have been briefly described in the Introduction indicate that different cell lines can respond to cell population density differently.
Unlike fibroblasts which were found to secrete increasing amounts of P.A. as cell population density increased, reaching a maximum at confluence (Rohrlich and Rifkin, 1977); E-cells produced more proteinase activity at low cell population densities. Thus, for studies investigating proteinase secretion by E-cells, the cells should be plated at appropriate low densities to allow a good harvest of proteinase activity in the medium.

The reasons for this density-dependent regulation of proteinase secretion are not clear. A possible explanation could be that at higher cell population densities, the proportion of cells secreting the proteinases decreases. Some indirect evidence for this hypothesis comes from the work of Birek et al. (1980) who found that the secretion of prostaglandins of the E series by E-cells was density-dependent with the highest amounts secreted at low cell population density and smaller amounts secreted at higher densities. Measurement of the percentage of hemolytic plaque forming cells (which indicates cells producing prostaglandin E) in relation to cell population density showed that the fraction of cells not secreting prostaglandin E was higher in dishes with higher cell population densities.

Another possible explanation for the density-dependent regulation of proteinases could be cell-cell contact. Cell-cell contact influences certain aspects of cell behaviour such as contact inhibition of locomotion, contact inhibition of extension, and contact inhibition of phagocytosis (Brown and Middleton, 1981). Cell-cell contact has also been reported to result in contact-induced spreading in a variety of epithelial cell types in culture (Brown and Middleton, 1981).

Cell-induced spreading is a phenomenon proposed and described by
Middleton (1977). He showed that isolated chick embryo pigmented retina epithelial cells lacking contacts with other cells, are normally poorly spread on the culture dish, display vigorous blebbing activity, and lack a well-defined leading lamella; but when such a cell comes into contact with one or more cells, it spreads out on the substratum, ceases blebbing, and develops a leading lamella from a part of the cell margin not in contact with the other cells. Thus the cells in contact with one or more cells become more flattened than the isolated cells.

Contact induced spreading has not been studied in detail in the E-cells used in this study but preliminary observations indicate that the phenomenon does occur in these cells (Brunette, in press). The phenomenon of contact spreading plus other considerations lead to the conclusion that the effect of cell population density on cell shape is complex. At the lowest cell densities, most of the cells would be isolated from the other cells. It would be expected that increasing the cell population density would result in more cell flattening because an increase in cell number per unit area would make contact between isolated epithelial cells more likely and thus promote spreading. At still higher densities, after confluence has been attained, however, increasing the cell population density results in more cells being crowded into the same area. This crowding normally causes the cells to become less spread. Finally, at higher densities, there is a tendency for keratinization to occur with the resultant production of squames or squame-like cells which would not be expected to contribute to proteinase secretion.

Because of these complexities, it is difficult to gain any insight into the mechanisms regulating proteinase secretion from the data given
in Figures 9 and 10 which nevertheless clearly show that cell population density modulates proteinase secretion. This data does have a practical application in the design of experiments investigating proteinase secretion because it shows that comparisons should be made when the cell population densities do not differ greatly or when the population densities are in the plateau region of the curves shown in Figs. 9 and 10.

II. *E. coli* LPS

The results show that *E. coli* LPS stimulated both N.P. and P.A. secretion by E-cells. LPS at 3 µg/ml was as effective at higher doses up to 60 µg/ml. These results are in agreement with those of Pettigrew et al. (1981) who showed that LPS at 30 µg/ml stimulated increased production of the metalloproteinases by gingival tissue explants. As described in the Introduction, the epithelial cell rests of Malassez are known to respond to the inflammatory stimuli by proliferating and participating in the formation of dental cysts. E-cells derived from the epithelial cell rests of Malassez have been shown to secrete N.P. (Pettigrew, Ph.D. Thesis) and collagenase in vitro (Limeback and Brunette, 1981). It is thus possible that endotoxins penetrating the periapical tissues could stimulate the cell rests into increased production of enzymes, thereby contributing to the degradation of connective tissue associated with cyst formation.

The LPS molecule consists of three distinct portions, the polysaccharide region, the core region and the lipid A region, linked together covalently. There is great variation in the side chains of the LPS molecule among different strains of bacteria. Even different methods of extraction result in differences in the structure of the
molecule. For example, the Westphal phenol-water extraction process results in a loss of protein which is retained during the TCA extraction process (Majde and Person, 1981). The lipid A region of the molecule is considered to be important for the expression of biological activity.

However, the mechanisms by which the LPS molecule causes these biological effects are not known. Endotoxin has been reported to cause altered mitochondrial function, increased activities of lysosomal enzymes, increased uptake of sugar, and increased formation of lactic acid (De Renzis and Chen, 1983). In addition, LPS has been shown to cause increased cAMP levels in certain cell types (Watson, 1976; Naylor et al., 1978), but not in others (Graber and Hellerquist, 1982). My results show that E.coli LPS at 3 µg/ml caused no significant changes in intracellular cAMP levels. Thus the increased production of N.P. and P.A. induced by LPS is likely to be independent of the cAMP pathway.

III. **Cell Growth and Proteinase Secretion**

Cholera toxin and dibutyryl cAMP affect cells by increasing intracellular cAMP levels but act by different mechanisms. Cholera toxin acts by activating adenylate cyclase, an enzyme that catalyses the conversion of adenosine triphosphate to cAMP. The cholera toxin molecule is made up of two different subunits: A and B. The function of the B subunits is to bind to the cell membrane, allowing subunit A to enter the cell by mechanisms which are still unclear. The role of the subunit A is to activate adenylate cyclase. Dibutyryl cAMP increases intracellular cAMP levels by inhibiting cAMP phosphodiesterase, an enzyme that degrades intracellular cAMP levels (Hsie, 1982).
As found by Brunette (1984a), cholera toxin and Bt$_2$ cAMP treatment significantly increased cell numbers. This increase in cell number necessitated increasing the values obtained for proteinase secretion to correct for the cell population density effect. Even after this correction, the flattened cholera toxin or Bt$_2$ cAMP treated-cells were still found to secrete less proteinase than untreated control cells. Moreover, although Aggeler et al (1982) and Chou et al. (1976) have suggested that cell proliferation is accompanied by increased P.A. secretion, this effect was not noted in these experiments, where the effect of cell shape appeared to be dominant. However cholera toxin and Bt$_2$ cAMP have multiple cellular effects and further investigation is needed to determine whether cell shape is the only mechanism regulating proteinase secretion here.

IV. Cell Shape and Proteinase Secretion

The results from the experiments using six methods of changing cell shape consistently show that irrespective of the method of changing cell shape, the more flattened cells produce reduced amounts of proteinase compared to the more rounded cells. This suggests that cell shape may be a means of regulating N.P. and P.A. secretion in E-cells. A more detailed discussion of these experiments follows.

As reported by Brunette (1984a), treatment of E-cells with cholera toxin or Bt$_2$ cAMP caused them to become more flattened. This morphological change was accompanied by a reduction in proteinase secretion; cholera toxin treated cells produced 69% and 29% of the N.P. and P.A. produced by control cultures. Dibutyryl cAMP-treated cells produced only 14% of the N.P. secreted by control cultures.
The morphological change in E-cells in the presence of $\text{Bt}_2 \text{cAMP}$ is in agreement with other studies. Different cells have been shown to become more flattened in the presence of $\text{Bt}_2 \text{cAMP}$ (Pastan and Willingham, 1978; Johnson et al., 1971; Hsie and Puck, 1971).

A study by Wilson and Reich (1979) showed that the addition of cholera toxin (1 ng/ml) or $\text{Bt}_2 \text{cAMP}$ ($10^{-3}$ M) to cultures of normal chick embryo fibroblasts caused only a small decrease in P.A. secretion. But both virus and PMA induced P.A. secretion were strongly inhibited by cholera toxin at 0.1 and 1.0 ng/ml and by cAMP at $10^{-3}$ and $10^{-4}$ M.

Vassali et al. (1976) found that thioglycollate-stimulated macrophages responded to cholera toxin ($10^{-12}$ M) by producing 10% of the P.A. secreted by control cultures. Dibutyryl cAMP-treated macrophages showed a 50% reduction in P.A. secretion. At the same time, macrophages unlike E-cells or fibroblasts, became more refractile and less well spread compared to the control cultures.

In contrast to cholera toxin and $\text{Bt}_2 \text{cAMP}$, PMA at 10 ng/ml caused the E-cells to retract slightly. The results show that both N.P. and P.A. secretion were increased compared to the control. This effect of PMA is in agreement with the report of Wigler and Weinstein (1976) who showed that a variety of cells including chick embryo fibroblasts, HeLa cells, and hamster embryo cells respond to PMA at $1.5 \times 10^{-8}$ M by increasing P.A. secretion. The elevation of P.A. secretion by PMA has also been reported by Troll et al. (1975) and Wilson and Reich (1979).

My data on the effect of cell shape on N.P. secretion is consistent with the work of Aggeler et al. (1984). In their study, the morphology of rabbit synovial fibroblasts was altered by different
agents and the degree of morphologic change was compared to the amount of collagenase induced. Agents like PMA, cytochalasin B, and D, trypsin, trifluoperazine, and poly(HEMA) coated dishes caused different degrees of cell rounding which was positively correlated with the increase in collagenase activity. Agents that did not alter cell shape did not induce collagenase secretion. However, no correlation between morphologic change and P.A. secretion was seen. A reason for the discrepancy between this data and mine could be the different cell types used.

Mechanical stretching made E-cells more flattened and conversely relaxing cells grown on stretched membranes made the E-cells more rounded. It was shown that stretched cells produced less proteinase and relaxed cells produced more proteinase than the controls. Even though the degree of stretching was small, 4%, a significant change in proteinase secretion was observed. This indicates that the control on proteinase secretion by cell shape is tight. The advantage of altering cell shape by mechanical means is that the multiple cellular effects produced by the use of some chemical agents are avoided. Thus the data from this experiment provides more direct evidence for the hypothesis that cell shape regulates proteinase secretion, with a more flattened cell shape favouring less proteinase secretion and a more rounded cell shape favouring increased proteinase secretion. The limitations of the present technique, however, is the small degree of mechanical stretching which can be applied.

There is one other study (Meikle et al., 1980) where the effect of tensile mechanical stress on the secretion of metalloproteinases by organ cultures of rabbit cranial sutures was investigated. Meikle et
al. found that mechanical stress stimulated 98.2% increase in gelatin-degrading N.P. and 35.9% increase in azocasein-degrading N.P. The explant consists of multiple cell types (bone, connective tissue, blood cells) and moreover, it is not clear what morphological effect the application of tensile mechanical stress had on the individual cells in different areas of the explant. It is therefore difficult to compare directly the results of this study and mine.

Another physical method of altering cell shape was the use of grooved substratum. Rovensky et al. (1971) suggest that cells attached to grooved surfaces are more round than those on flat surfaces. A similar finding has been made on E-cells grown in this laboratory. E-cells grown on grooved substratum demonstrated a two fold increase in N.P. and a 1.6 fold increase in P.A. activity. This result is consistent with the hypothesis that more rounded cells secrete increased amounts of proteinases.

Poly(HEMA)-coated dishes reduce dish surface adhesivity thereby affecting cell attachment and shape. The mechanism involved in the poly(HEMA) effect on dish adhesivity, however, is not known. Folkman and Moscona (1978) suggested that poly(HEMA), having a neutral charge, may act by reducing the negative electrostatic charge of the plastic dish. Another possibility was that increasing the poly(HEMA) concentration would decrease the number of available sites on the plastic to which the cells could attach.

It is clear that increasing the concentrations of poly(HEMA) solution decreases surface adhesivity. The degree of spread of E-cells, as indicated by the area of the cell in contact with the dish, decreases with increasing poly(HEMA) concentration.
E-cell shape change occurred over a narrow range of poly(HEMA) dilution: $10^{-5}$ to $10^{-3}$ of the stock. Measurement of the proteinase secretion showed that as the cells became less spread out (i.e. more round) the N.P. and P.A. secretion increased; the increase in P.A. being more gradual than that of N.P. A correlation between cell shape and proteinase secretion was thus shown; increased cell rounding correlated with increased N.P. and P.A. secretion. The advantages of the poly(HEMA)-coated dishes to modify cell shape are: (1) the addition of agents with known multiple biological effects is avoided; (2) the method allows different degrees of cell spreading to be achieved.

Thus, evidence from these experiments, where 6 different means of altering cell shape were used, have been presented which are consistent with the hypothesis that cell shape regulates N.P. and P.A. secretion; the more flattened cells produce less proteinase and more rounded cells produce increased amounts of proteinases.

However, the mechanisms involved in this regulation are not known although it is likely that the cytoskeleton is involved in some way. In the next section, the possible cytoskeletal changes brought about by the different methods of altering cell shape will be discussed and a hypothesis for the role of the cytoskeleton in regulating proteinase secretion will be presented.

1) **Cell Shape and the Cytoskeleton**

Electron microscopic studies have demonstrated that $\text{Bt}_2\text{cAMP}$ treatment of 3T3-4 cells results in a change in the distribution of microfilaments and microtubules. Willingham and Pastan (1975) showed that on cell flattening, microfilaments under the plasma membrane became more prominent, the number of microtubules increased, and became
aligned with the direction of the cell processes. 3T3-4 cells in the presence of $\text{Bt}_2 \text{cAMP}$ were demonstrated to become closely opposed to the irregular surface of the substratum and showed multiple sites of cell attachment. Dense microfilament bundles became visible at the edges of $\text{Bt}_2 \text{cAMP}$-treated cells as well as the attachment points. In contrast, control cells showed few attachment points; at the tips of cell processes, under the nucleus, and a few sites inbetween. Willingham and Pastan suggest that cAMP stimulates microtubule assembly promoting the extension of cell processes and at the same time cAMP inhibits microfilament-mediated contraction of the cell processes, therefore the final cell morphology is very flat.

Indeed, Li et al. (1975) and Hsie et al. (1977) have shown that the increased cAMP levels cause the activation of a cAMP-dependent protein kinase which results in protein phosphorylation and the polymerization of microtubules.

PMA is potent tumour promoter which has been found to cause a variety of effects on cultured cells, some of these changes correlating with the features of transformed cells. Of interest to us is the cytoskeletal changes caused by PMA.

Rifkin and Crowe (1979) reported that PMA at concentrations as low as $7.3 \times 10^{-10}$ M produced cytoskeletal changes. There was a loss in the normal structure of the actin containing fibres and a reduction in the length and thickness of these fibres. Sakiyama and Hiwasa (1984) also report that PMA at 20 ng/ml resulted in a loss of the structured actin fibres. These cells treated with anti-actin antibody and examined by indirect immunofluorescence microscopy showed a diffuse actin pattern.

Recent work by Fey and Penman (1984) indicate that PMA and other
tumour promoters affect the organization of the nuclear matrix-intermediate filament scaffold, a structure obtained after the removal of the soluble proteins, phospholipids, the cytoskeleton, and chromatin fractions. PMA resulted in morphological changes in Madin-Darby canine kidney (MDCK) colonies. Normally the cells in each colony display tight contact with one another. In the presence of PMA at 5 ng/ml, a breakdown in the organization of the cells was observed; the cells began to spread out and develop long processes. These morphological changes were reflected in the distribution of cytokeratins in the nuclear matrix-intermediate filament scaffold.

It is thus clear that PMA changes cell morphology by affecting the cytoskeletal elements.

In the experiment using mechanical stretching, E-cell shape was modulated by an applied external force and in the case of stretched cells being relaxed, there was a release in tension. In his experiments on E-cells, Brunette (1984b) found an increase in the volume fraction of microtubules and other filamentous structures in stretched cells compared to unstretched cells. Thus stretching the cells resulted in changes in the cytoskeletal elements.

The poly(HEMA)-coated dishes affect cell shape by reducing dish surface adhesivity to the cells. Increasing concentrations of poly(HEMA) resulted in E-cells being less spread out. The relationship between substratum adhesiveness and cell shape is in agreement with the results of Folkman and Moscona (1978) and Willingham et al. (1977). Willingham et al. (1977) measured substratum adhesiveness in terms of the length of time needed to remove 50% of the cells from the substratum. They found that a flatter cell morphology resulted in an
increase in substratum adhesiveness. On the other hand, a rounded cell morphology was correlated with a decrease in substratum adhesiveness.

Willingham et al. (1977) also showed that there was a strong correlation between the adhesive strength of the cell to the substratum, and the presence of microfilament bundles. Normal Balb/3T3 cells grown on an adhesive substratum showed a flattened morphology and numerous bundles of microfilaments. The same cells grown on a low adhesive substratum were round in shape and showed no discernible microfilament bundles. A mutant of Balb/3T3 cells, which is defective in its ability to acetylate glucosamine-6-phosphate adhered poorly to tissue culture dish and showed a rounded morphology. No microfilament bundles were seen. However, on addition of 10 mM N-acetyl glucosamine, normal adhesiveness was restored and the cells flattened out. Numerous bundles of microfilaments became evident. Furthermore, MC 5-5, a transformed cell line which showed rounded morphology, poor adhesion to the substratum also showed no bundles of microfilaments. When treated with cell surface protein, a factor mediating cell attachment, the cells flattened out and showed bundles of microfilament. It is thus clear that substratum adhesiveness affects cell shape and the microfilament bundles: a more flattened cell shape showing more bundles of microfilaments.

From the data above, it seems reasonable to conclude that changes in cell shape observed on poly(HEMA)-coated dishes involved changes in the cytoskeletal elements too.
2) Penman's Hypothesis on the Relationship Between the Cytoskeleton and Nuclear Metabolism

At present, there is little definitive evidence for the mechanisms that could be involved in the regulation of proteinase secretion by cell shape. However, a possible explanation comes from the hypothesis of Penman and colleagues (1983). These authors postulate that the gene activity of the cell responds to cell architecture. Benecke et al. (1978) showed that anchorage-dependent fibroblasts, when placed in suspension cultures, responded by shutting down all major macromolecular processes. Cytoplasmic protein synthesis could be restored by cell contact with a solid surface. Nuclear metabolism, such as mRNA and DNA synthesis, however, was subject to regulation by the degree of cell spreading (Ben Ze'ev et al., 1980). Furthermore, Cervera et al. (1981) showed that in HeLa cells, all actively translating message molecules were bound to the cytoskeleton. Upon infection with a virus, the ribosomes translated a mixed batch of host and virus messages, both being bound to the cytoskeletal structures. This suggested that mRNA was translated only when bound to the cytoskeletal framework and may be a means by which selection of mRNA occurs.

Further evidence comes from studies involving the interaction between the extracellular matrix and the cell in tissue development, in which there are indications that the cytoskeleton plays an important role. Bissell et al. (1982) stated in their review, "The extracellular matrix (ECM) is postulated to exert physical and chemical influences on the geometry and the biochemistry of the cell via transmembrane receptors so as to alter the pattern of gene expression by changing the association of the cytoskeleton with the mRNA and the interaction of the chromatin with the nuclear matrix". It is conceivable that changes
in cell shape alters the association of the cytoskeleton to the mRNA thereby regulating the amount of proteinase translated. But confirmation of this hypothesis awaits further research.

V. Relationship between N.P. and P.A. secretion

The secretion of N.P. and P.A. appeared to be linked under several of the conditions employed in these experiments. With the exception of P.A. activity from day 3 cultures which will be discussed later, E-cells respond to cell population density by secreting latent N.P. and P.A. in a similar pattern. N.P. and P.A. activity was highest at low cell population densities; the enzyme activity decreased with increasing cell population density. The secretion of latent N.P. and P.A. was stimulated by the addition of E-cell LPS to the cultures. In the experiments where different methods of altering cell shape were used, a more flattened cell shape correlated with a reduced level of N.P. and P.A. secretion while a more rounded cell shape correlated with an increased amount of N.P. and P.A. secretion.

There are, however, reports in literature that P.A. and the metalloproteinases show differences in regulation. Golds et al. (1983) report that synovial cells responded to mononuclear cell supernatant by secreting P.A. which stopped when the stimulus was removed. Collagenase secretion occurred only after a lag of one to two days and the secretion continued even after the stimulus was removed. In addition, all-trans retinoic acid caused an increase in P.A. but a reduction in latent collagenase. Gordon and Werb (1976) found that thioglycollate-induced macrophages responded to colchocine by increasing the secretion of metalloproteinases but decreasing that of P.A.
My experiments show that under certain conditions there are differences in the pattern of N.P. and P.A. secretion. For example, in day 3 cultures, cell population density modulated P.A. activity differently than N.P. activity. N.P. activity decreased continuously with cell population density whereas P.A. activity was low at $1 \times 10^5$ cells/dish, reached a peak at $2.4 \times 10^5$ cells/dish and then decreased gradually. A possible explanation could be that E-cells secreted inhibitors to P.A. in the early days of culture which reduced the activity of P.A. Figure 10 shows that the level of P.A. activity was lowest at day 3 compared to days 7 and 10. However, inhibitors to P.A. have not been assayed and this awaits further study.

Another difference observed is the different degree of stimulation of P.A. activity by the TCA extracted LPS compared to the phenol extract. The TCA extract caused a cumulative increase of 3 1/2 times in P.A. activity at day 10 compared to an increase of 1 1/2 times by the phenol extract. In contrast, N.P. activity was stimulated to the same degree by both TCA and phenol extract.

Thus, although the secretion of N.P. and P.A. appear to be linked together, there are differences in the pattern of secretion. This indicates that the two enzymes may not be secreted together in a proteolytic package, and they may respond to different stimuli differently. It has been shown that cell shape and E.Coli LPS regulate proteinase secretion. The mechanism involved in the LPS effect on proteinase secretion is not known but it is probably different from the cell shape effect since no morphological change was observed. Thus there is probably more than one mechanism that regulates proteinase secretion in E-cells.
Nonetheless, since P.A. has been found to be able to activate latent metalloproteinases in the presence of plasminogen (Werb et al., 1977; Paranjpe et al, 1980), the secretion of P.A. in-vivo may be a means which ensures the availability of N.P. in an active form.

VI. Future Work

Abnormal growth, changes in cell morphology, and elevated levels of P.A. are characteristics of transformed cells. The relationship of P.A. secretion to the other characteristics altered in transformed cells is not known. A long term goal would be to understand the role of P.A. in neoplasia and in particular, the possible relationship between cell morphology and P.A. in neoplasia. These broad questions require extensive research, but based on the existing techniques and findings in this thesis, two interesting and experimentally feasible questions can be posed. One would be to elucidate the exact nature of the relationship between cell shape and proteinase secretion. The second would be to determine whether the different methods of altering cell shape share similar mechanisms. The direction of future work to reach these goals will be presented in the following order: (1) Improvement in techniques; (2) Proposed experiments.

1) Improvement in Techniques

First, a sensitive method of measuring cell shape is needed. One possibility would be to measure the area of the cell by using a camera lucida to trace the cell outlines onto a digitizing tablet attached to a computer. This approach is currently being developed in Dr. Brunette's laboratory. If successful, cell shapes can be altered in a graded sequence using the poly(HEMA) method of Folkman, and the cell
area measured and correlated with proteinase secretion.

A second improvement needed is a method which can increase the desired range of mechanical stretching applied to the cells. The petriperm membrane is limited in the amount in which it can be reversibly stretched. The advantage of the mechanical stretching method is that, whereas the poly(HEMA) method makes the cells increasingly less spread out than the control (tissue culture plastic), the mechanical stretching method can be used not only to make the cells more flattened than the control but also to make the cells more rounded than the control. If the degree of stretching could be increased, it would be possible to plot the amount of stretching against proteinase secretion and a more complete picture of the relationship between cell shape and proteinase secretion obtained. One possibility is the use of polyurethane membranes. Preliminary experiments indicate the E-cells attach to these membranes and that they can be reversibly stretched to a greater extent than the petriperm membranes. However, a method has to be developed to fabricate dishes with the polyurethane membrane attached.

Thirdly, a difficulty encountered in this project has been the relatively large numbers of cells required for the experiments. For example, four 60 mm dishes of cells, plated at $4 \times 10^5$ cells/dish and incubated for 3 days in $\beta$MEM + 0.5% DFCS, are required for each experimental group in order to obtain sufficient enzyme for assay. The experiments would be simpler if the number of dishes of cells could be reduced. Preliminary tests indicate that cells in serum free Dulbecco’s medium produced 3 times more N.P. than cells grown in $\beta$MEM + 0.5% DFCS. In the experiments reported here, $\beta$MEM + 0.5% DFCS was used because it was of interest to compare proliferating cells (induced by cholera toxin or Bt$_2$cAMP) with non-proliferating cells. Thus where
cell proliferation is not required, Dulbecco's medium could be a better alternative.

2) Proposed Experiments

It has been shown that cell shape modulates proteinase secretion but the exact nature of this relationship is not known. Therefore one of the short term goals would be to elucidate whether this relationship is linear, that is, whether a change in cell shape on a quantitative scale would be accompanied by a proportional change in magnitude in the proteinase secretion. This could be done by altering cell shape in a graded manner, by the use of poly(HEMA) coated dishes, combined with a sensitive method of determining cell area. The cell shape and proteinase secretion could then be determined and the relationship analyzed.

A second goal would be to carry out some experiments to determine whether the mechanisms involved in the different methods of altering cell shape are similar. My results indicate that N.P. and P.A. secretion are modulated by changes in cell shape. But in the experiments using agents like cholera toxin, dibutyryl cAMP and PMA, there is the possibility that other mechanisms might be operative. To test the hypothesis, a standard curve of cell shape versus proteinase secretion would be obtained by the poly(HEMA) method and compared with other methods of altering cell shape. For example, PMA treated cells would have their cell areas and proteinase activity measured and compared to the standard curve. If the amount of increase in proteinase secretion accompanying the morphological change produced by PMA is substantially different from the proteinase secretion of cells showing an equal degree of morphological change using the poly(HEMA) technique, then the 2 mechanisms are probably at least partially independent of each other.
SUMMARY AND CONCLUSIONS

The regulation of proteinase secretion by E-cells derived from the cell rests of Malassez was studied in cell culture system.

1) It was established that E-cells secrete neutral proteinase in a latent form similar to the neutral proteinase produced by rheumatoid synovium, rabbit bone and rabbit synovial fibroblasts. P.A. was also secreted by E-cells.

2) The secretion of N.P. and P.A. was regulated by cell population density in the culture system. Proteinase activity was highest at low cell population densities.

3) E.coli LPS stimulated an increase in N.P. and P.A. secretion.

4) In contrast to other studies reporting a positive correlation between proliferation and proteinase secretion, a decrease in proteinase secretion was observed when E-cells were stimulated to grow by Bt$_2$ cAMP or cholera toxin. Proteinase secretion is thus not necessarily correlated with growth.

5) i) Cell shape was altered by both chemical and physical means. Cholera toxin and dibutyryl cAMP caused E-cells to flatten out; while PMA caused E-cells to retract slightly. Physical means included: mechanical stretching and relaxing stretched cells; growth of cells on grooved substratum and growth of cells on poly(HEMA)-coated dishes.
ii) In all experiments where these different methods of altering cell shape were used, flattening of the E-cells resulted in a reduced amount of N.P. and P.A. secreted whereas rounding of the E-cells caused an increased secretion of N.P. and P.A.
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