Effects of Substratum Surface Chemistry and Topography on Extracellular Matrix Gene Expression

By
Laisheng Chou
D.M.D., Shanghai No. 2 Medical University, Shanghai, 1978

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Department of **ORAL BIOLOGY**

The University of British Columbia
Vancouver, Canada

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The reactions at tissue-implant interfaces are key elements in the study of dental implantology. Most previous studies have been limited to assessment of the interface at the morphological and histological levels. The mechanisms underlying the tissue-implant interface reaction are largely unknown. In this thesis I hypothesized that the surface chemistry and microtopography of biomaterials could serve as signals to the attached cells and effectively regulate the interface reaction at the molecular level. I predicted that expression of genes coding for specific molecules involved in cell attachment and matrix assembly could be altered in vitro by substratum surface chemistry and topography. If the hypothesis and prediction were accurate, the results from this thesis would support the development of biomaterial-controlled interface reactions by modifying the signals of surface chemistry and topography.

The hypothesis and prediction were tested in this thesis by examining the mRNA level, mRNA stability, gene product secretion and activity of two major extracellular proteins, fibronectin and 72 kDa gelatinase (MMP-2), in human gingival fibroblasts cultured on smooth or micromachined grooved titanium substrata to determine the effects of surface topography. Tissue culture plastic substrata were compared with smooth titanium substrata to determine the effects of surface chemistry.

The study revealed that a grooved substratum surface significantly altered the shape and orientation of normal fibroblasts. Fibronectin mRNA levels, mRNA stability, amounts of secreted proteins and matrix-assembly activity were significantly increased in the cells on grooved titanium surfaces in comparison to the cells on smooth titanium surfaces. Compared to cells on smooth tissue culture plastic, the cells on smooth titanium surfaces showed altered levels of fibronectin mRNA, secretion and matrix-assembly activity, as well as reduced mRNA stability. The substratum surface topography and chemistry also altered the MMP-2 mRNA levels and stability. Cell
proliferation and total secreted protein levels were essentially unchanged in the cells cultured on the different types of surfaces studied. These results indicated that the effects of surface topography and chemistry can sensitively and selectively regulate specific molecules at several levels. Thus, the techniques of molecular biology provide a sensitive and specific approach to the assessment of cell response to biomaterials.
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<thead>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>cytochalasin B</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscope</td>
</tr>
<tr>
<td>CS buffer</td>
<td>cytoskeletal stabilizing buffer</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-dichloro-1-b-D-ribofuranosyl benzimidazole</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FN-</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GAPD</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GT buffer</td>
<td>guanidinium thiocyanate buffer</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT</td>
<td>microtubules</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>RFGD</td>
<td>radio-frequency glow-discharge</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acids</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TCP</td>
<td>tissue culture plastic</td>
</tr>
<tr>
<td>Ti</td>
<td>titanium</td>
</tr>
<tr>
<td>VN</td>
<td>vitronectin</td>
</tr>
<tr>
<td>VRC</td>
<td>vanady-ribonucleoside complex</td>
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VTi titanium with v-shaped grooves
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PREFACE

PUBLICATIONS CONTAINING THESIS WORK

Papers:


Abstracts:


AWARDS

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1991 Medical Research Council (Canada) Dental Research Fellowship

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CHAPTER 1
INTRODUCTION

I. Overview

A critical problem in dentistry is the replacement of missing teeth for the edentulous patients. A survey by the National Institute of Dental Research (NIDR) has shown that 42% of the population over the age of 65 is totally edentulous, and that a substantial number of other patients are partially edentulous with an average of 10 teeth missing (NIH, 1988, Kohn, 1992). The use of dental implants to provide support for replacement of missing teeth has become an important component of modern dentistry as evidenced by a dramatic increase in the use of dental implants in Canada and the United States over the last decade. Meanwhile, in the past 20 years, a large number of sophisticated techniques have been developed for analyzing the chemical and mechanical properties of implant biomaterials (Young, 1988). With these techniques, the compositions of implant biomaterials and the chemical states and locations of the atoms present can be precisely evaluated. But, the "bio" portion of "biomaterials" for dental implants has been much less studied (Shulman, 1988). The mechanisms underlying the tissue-implant interface reaction are poorly understood because the complete characterization of biological properties of implant materials at the biochemical and molecular levels is lacking.

Many medical and dental implants of various designs are made of titanium in either its pure or alloyed form. A high degree of biocompatibility, strength, and corrosion resistance make these metals ideal for implants. However, the success of implanted titanium devices depends upon the interaction of cells with the surface of the titanium device. These interactions are controlled by the surface chemistry and topography of the implant material. My hypothesis is that the surface chemistry and topography of biomaterials may serve as signals to the attached cells. These signals may effectively regulate the activity of genes responsible for cell attachment, proliferation, differentiation and mineralization at the transcriptional and post-transcriptional levels. Therefore,
cell behavior at the implant interface can be controlled at various levels from cell attachment to gene expression by modifying the signals from material properties, namely material surface chemistry and/or topography.

The study of cell response at the molecular level to biomaterials should provide sensitive indicators of cell behavior at tissue-biomaterial interfaces. The evaluation of gene activities in response to the surface properties of biomaterials could be a valuable approach to determining the molecular biocompatibility of biomaterials in conjunction with conventional clinical and histological profiles. This thesis focuses on delineating the influence of biomaterial surface chemistry and topography on the cell shape and expression of genes encoding major extracellular proteins. Fibronectin, the major adhesive molecule, and matrix metalloproteinase-2 (MMP-2 or 72-kDa gelatinase), the major secreted proteinase of fibroblasts, were selected as the target proteins in studies on effects of surface topography and chemistry on gene expression.

This chapter will review some of the background of biomaterial properties on cell adhesion (Section II), the mechanism of cell attachment and tissue reorganization around implant materials (Section III), and the theory of topographic guidance underlying cell behavior in response to the topography of substrata (Section IV). The structure, functional activities and regulation of fibronectin and 72-kDa gelatinase will be briefly reviewed in Section V and VI as the introduction to the studies on their regulation by surface chemistry and topography. As the technique of double-labeling microtubules and fibronectin mRNA was developed in this thesis, a brief review on microtubules and intracellular mRNA distribution are described in Sections VII.
II. Physical Properties of Artificial Cell Culture Substrata

The *in vitro* study of tissue-implant interface reaction has generally been performed using cell cultures on the defined artificial surfaces of biomaterials that are commonly used for implant devices. The general rule that has emerged from studies of cell attachment to various biomaterials is that, with some exceptions, the ability of cells to attach to the substrata is dependent on the physical properties as described below (reviewed by Brunette, 1986a).

A. Mechanical Rigidity

Both fibroblasts and epithelial cells may exert tensile forces on deformable substrata while they attach to and migrate on the substrata (Harris, 1982 and 1984). Thus, substrata require mechanical rigidity to withstand the tensile forces exerted by the cells (Maroudas, 1973). The nature and dependency of cell attachments upon the rigidity of the substrata may differ depending on the extent of traction forces exerted by different cell types (Curtis and Clark, 1990).

B. Wettability or Surface Energy:

The wettability of the surface is measured by the critical contact angle formed by liquid droplets spreading on the surface (Baier and Meyer, 1988). Surface wettability is closely related to the free energy of a material. Materials may be classified into two broad groups: high surface energy materials such as glass and metals which are highly wettable and hydrophilic, and low surface energy materials such as waxes and silicone which are hydrophobic.

The wettability of a material can be temporarily increased by a procedure termed radio frequency glow discharge (Baier, et al., 1984 and 1988), in which plasma clouds of argon gas are used to bombard the surface. Treatment by radio frequency glow discharge can remove the organic contaminants away the material’s surface and yield sterilized and highly hydrophilic substrata for cell adhesion (Doundoulakis, 1987; Gombotz and Hoffman, 1987).
The wettability of a material has a direct influence on the adsorption of macromolecules onto the surface which, in turn, facilitate cell attachment. A thin layer (2-5 nm) of macromolecules can adsorb onto a surface within a minute of exposure of the surface to cell culture medium (Baier, 1986). The mechanism underlying the high surface energy of substrata for cell culture has been attributed to the conformation of adsorbed proteins in addition to their absolute amounts (Lewandowska et al., 1989). It has been suggested that on substrata with a high surface energy, macromolecules may bend out of shape and develop more points of attachment for cells (Baier, 1970; Kasemo and Lausmaa, 1988). Therefore, the efficiency of cell attachment to the substrata depends on both the quantity and the conformation of adsorbed macromolecules.

C. Material Surface Chemistry

Many oral implant of various designs are made of titanium or titanium alloys. Commercially pure titanium also contains trace amounts of elements which markedly improve the mechanical properties of pure titanium (Parr et al., 1985). The most commonly used titanium alloy contains 6% aluminum and 4% vanadium (Ti-6Al-4V).

The surface chemical properties of titanium are determined by the oxide layer composed primarily of titanium dioxide (Kasemo, 1983; Healy and Ducheyne, 1989). The surface oxides grow by inward transport of oxygen atoms, either gaseous or dissolved, and/or outward metal atom transport from the oxide-metal interface. There are concentration gradients for oxygen and titanium. Titanium forms several stable oxides such as TiO₂, TiO, and Ti₂O₃, with Ti₂O being the most common. All titanium oxides have high dielectric constants (higher than most other metal oxides) in the range of 50 to 150.

Oxide formation on the titanium surface is extremely rapid (Kasemo, 1983; Toth et al., 1985).
Exposure to air of a machined titanium surface results in an oxide layer greater than 10 Å in less than a millisecond, and within a minute the oxide thickness can be of the order of 50 to 100 Å. An oxide formed in this way will be quite different from the corresponding bulk oxide, with expectations for a high degree of nonstoichiometry, variations in crystal structure, and a richness of defects in the surface oxide (Kasemo, 1983). The formation of titanium oxides can be significantly affected by the sterilization procedures when the titanium is used for implantation. For example, autoclaving can produce thicker oxide layers and be contaminated by Fe and Cl (Keller et al., 1990). The exact effects of variations in the oxides formed on titanium surface on cell adhesion and cell phenotypic behavior still remain largely unknown.

D. Material Surface Topography

Biomaterial surfaces differ greatly in topography depending on the fabrication procedure. Methods of producing a rough surface include grinding or blasting the surface with abrasives (Salthouse, 1984), bombarding the surface with charged ions (Polanski et al., 1983; Wasfie et al., 1984), or sintering spherical particles onto the surface of solid biomaterial to make a porous surface (Meffert et al., 1987).

Micromaching is a versatile technique that provides a flexible means to fabricate wafers of single-crystal silicon into a variety of surface shapes (Angell et al., 1983). With this technique, three-dimensional topographies including grooves, pits, holes, trenches, walls, pyramids and hemispheres can be precisely generated. Such micromachined grooved surfaces have been used as cell culture substrata to manipulate cell behavior (Brunette et al., 1983; Brunette 1986 a, b; Dunn and Brown, 1986). Previous studies on cell behavior in response to micromachined grooved substrata will be reviewed in the following sections.
III. Cell Adhesion to Artificial Substrata in vitro

Adhesion of cells in the presence of serum is a multistep process which involves adsorption of attachment molecules from the medium to the substrata, contact between the cells and substratum, attachment of cells to the coated surface, spreading of the attached cells and formation of stable contact structures (Grinnel, 1979). The structures of cell attachment and their functions are reviewed below.

A. Structures of Cell Attachment at the Light and Electron Microscopy level

Three types of regions of cell adhesion depending upon the distance between cells and their substrata can be identified with interference reflection microscopy (IRM) (Curtis, 1964; Izzard and Lochner, 1976).

1. Extracellular Matrix Contacts

Extracellular matrix contacts (ECM contacts) appear grey or white in IRM with distances of 100 nm or more between the cell membrane and the substrata, typically located under the center of the cell (Izzard and Lochner, 1976).

2. Close Contacts

Close contacts appear as broad grey areas in IRM and about 30 nm from the substrata (Izzard and Lochner, 1976). Close contacts are mainly distributed at the peripheral regions of the leading lamellae in highly mobile cells.

3. Focal Contacts

Focal contacts appear black by IRM and about 10 to 15 nm between the cells and substrata, 2-10 μm long and vary in width between 0.1-0.5 μm (Izzard and Lochner, 1976; Opas, 1987). Focal
contacts are generally located under peripheral sites of leading lamellae and near edges of non-spreading regions of the cell margins in moving and stationary cells (Oakley, 1995), and oriented parallel to the direction of movement of the cell (Izzard and Lochner, 1976). As the adhesion plaques observed by the electron microscope have been recognized as the same structure as the focal contacts by IRM, the term “focal adhesion” was suggested because it conferred an adhesive function to a discrete focal region of the plasma membrane (Burridge et al., 1988).

B. Structural Components of Cell Attachment Sites

The discussion in this section is limited to focal adhesions, as close contacts have not been well-characterized on a molecular basis. Focal adhesions can be associated with extracellular matrix molecules, including fibronectin (discussed in detail in Section V) and vitronectin (Grinnell, 1980, 1986; Singer and Paradiso, 1981), transmembrane integrin receptors and a group of cytoplasmic components including vinculin, talin and actin.

1. Integrins

Integrins comprise a diverse family of matrix receptors that are composed of α and β subunits, both of which contain a transmembrane segment, a short cytoplasmic domain and a large extracellular domain. The integrins function as a transmembrane linker to mediate interaction between the cytoskeleton inside the cell and the extracellular molecules. Different combinations of subunits possess various binding affinity for different extracellular ligands (Hynes, 1992; Haas and Plow, 1994). The fibronectin receptor (α5β1 integrin) is one of the best-characterized matrix receptors. There are about 1-5 x 10^5 fibronectin receptors per fibroblastic cell (Akiyama and Yamada, 1985). The β subunit of the fibronectin receptor appears to bind to cytoplasmic proteins whereas the α subunit regulates the binding of the ligand (Hynes, 1992). Binding of fibronectin to
α5β1 integrin causes the integrins to be relocated from a diffused distribution in the plasma membrane to a localized accumulation within the focal adhesions. The location of the fibronectin receptor in the plasma membrane differs in motile, stationary, and malignant cells (Chen et al., 1986; Duband et al., 1986). In addition to cell attachment, the integrins are involved in signal transduction through triggering intracellular events (Hynes, 1992; Horwitz and Thiery, 1994), such as tyrosine phosphorylation via integrin β subunit (Kellie et al., 1991), Ca\(^{2+}\) sequestration and diacylglycerol (DG) turnover via phosphoinositide phosphate (PIP) cycle (Platefaber and Lander, 1991; Cooper, 1991).

2. Extracellular Matrix Associated with Focal Adhesions

The involvement of extracellular matrix (ECM) components is critical in cell adhesion (reviewed by Burridge et al., 1988; McCarthy et al., 1991; Woods et al., 1993). Fibroblasts adhere well to surfaces coated with various ECM proteins, such as fibronectin (FN) or vitronectin (VN). The cells spread and develop focal adhesions on these ECM-coated substrata. In contrast, normal fibroblasts adhere poorly and fail to develop focal adhesions when plated on naked glass in the absence of serum or on glass coated with nonspecific proteins, such as bovine serum albumin.

The codistribution of FN and VN at the substrate surface of fibroblasts was localized at focal adhesion sites in cultures with low serum (0.3%) (Singer and Paradiso, 1981; Singer, 1982). Although FN and VN are structurally quite different, they share along with several other ECM proteins two features that appear to be important for the formation of focal adhesions. The first is a cell-binding domain containing the amino acid sequence Arg-Gly-Asp (RGD), which has been identified in many ECM proteins that interact with cells (Ruoslhti and Pierschbacher, 1987). Secondly, both proteins contain a heparin-binding domain. The role of the RGD sequence in focal adhesion formation was investigated in several studies. Fibroblasts were plated on cell-binding
fragments of FN or on short synthetic peptides containing the RGD sequence alone, resulted in that the cells adhered well, spread and developed focal adhesions on the substrata with the cell-binding fragment of FN, but failed to develop focal adhesions on the substrata with the RGD sequence alone (Singer et al., 1987a). This second group of cells could be induced to form focal adhesions and associated stress fibers if the heparin-binding domain of FN was also adsorbed to the substrata or even if it was added in solution (Woods et al., 1986). These experiments with ECM proteins and their fragments define the substrate requirements for focal adhesions; the ECM protein often contains an RGD sequence and usually a heparin-binding domain (Singer et al., 1987b; LeBaron et al., 1988).

3. Cytoplasmic Proteins Associated with Focal Adhesions
A group of cytoplasmic molecules are linked to integrins forming the adhesion plaque. Talin and \( \alpha \)-actin can bind to cytoplasmic domain of \( \beta 1 \) integrin subunit (Beckerle and Yeh, 1990). Talin can also bind to vinculin which, in turn, binds paxillin and \( \alpha \)-actin (Otto, 1990). Alpha actinin binds and crosslinks actin. Tensin can bind vinculin, and may also bind and cap the plus ends of actin filaments. Other cytoplasmic molecules, such as radixin, have also been localized to focal adhesions (Luna and Hitt, 1992; Schaller and Parsons, 1994). Clustering and interactions of cytoplasmic proteins at the focal adhesions may stabilize their association with adhesion sites (Haas and Plow, 1994; Hitt and Luna, 1994). However, the exact configuration and functional significance of the complex interaction of cytoplasmic proteins at the focal adhesions remain unclear (Opas, 1987; Uitto and Larjava, 1991; Luna and Hitt, 1992).

C. Formation and Maturation of Focal Adhesions
Focal adhesions usually form within or just behind the ruffling leading edge of motile cells (Izzard and Lochner, 1980). There is often a correlation between ruffling and the later formation of a focal
adhesions at the same site (Rinnerthale et al., 1988). The formation of focal adhesions is usually preceded by a structural precursor consisting of a microspike or bundle of actin filaments oriented radially within the leading edge of a cell (Izzard and Lochner, 1980; DePasquale & Izzard, 1987; Rinnerthale et al., 1988). The precursors can be divided into two portions: a proximal part that becomes stationary and forms the initial adhesion plaque on the cytoplasmic face of the focal adhesion membrane, and a distal part, which continues to advance with the leading edge (DePasquale & Izzard, 1988). Then, talin and vinculin accumulate at the new focal adhesion site and stress fibers develop centrally through the leading lamella (Izzard, 1988; Harris, 1994; Lauffenburger and Horwitz, 1996). Talin, but not vinculin, accumulates at the distal tips of the precursors (Izzard, 1988).

After initial formation, some focal adhesions disassemble while others enlarge and nucleate associated stress fibers (Burridge et al., 1988). The association of microtubules and intermediate filaments at these sites may increase focal adhesion stability and promote nucleation of stress fibers (Geiger et al., 1984; Rinnerthaler et al., 1988; Bershadsky et al., 1987). Within mature focal adhesions, there is a slow exchange of actin and vinculin with the cytoplasmic pool of these proteins while focal adhesions remain competent to nucleate stress fibers (Burridge et al., 1988).

D. Functions of Focal Adhesion

The role of different types of cell substratum contacts in cell adhesion is still unclear although it has been speculated that close contacts and focal adhesions have mechanically distinct functions. Cell spreading has been associated with close contacts, while focal contacts provide an anchorage for microfilament bundles which are capable of contraction (Izzard and Lochner, 1976).

Focal adhesions are clearly important for anchoring stress fibers to the plasma membrane, but they probably also regulate the assembly and disassembly of the attached actin filaments (reviewed by
Burridge et al., 1988). The anchorage provided by focal adhesions permits the development of tension which promotes the formation of stress fibers which, in turn, align parallel to the tension (Kolega, 1986). Focal adhesions may also be involved in transmembrane signaling through the interaction of transmembrane receptors with extracellular matrix and cytoplasmic molecules (Bissell et al., 1982; Burridge et al., 1988). High concentrations of transmembrane integrins within focal adhesion and tyrosine kinases at the cytoplasmic face of focal adhesion have been demonstrated (Maher et al., 1985; Backerle et al., 1987).
IV. Topographic Guidance in vitro

Topographic guidance refers to the tendency of cells to be guided in their direction of locomotion by the surface topography of the substrata (Reviewed by Dunn, 1982; Brunette, 1988a,b). As the cell behavior in response to surface topography was evaluated in this thesis, the background of topographic guidance and the mechanism proposed underlying this particular cell behavior will be reviewed. The following discussion will be limited to the in vitro observations of topographic guidance on different substrata and different cell types, with an emphasis on fibroblasts, the most studied cell.

A. Topographic Guidance

Topographic guidance was one of the first phenomena observed in cell culture (Harrison, 1914) and was systematically and extensively studied by Weiss who proposed the name of contact guidance and used substrata as diverse as plasma clots, fish scales and engraved glass (Weise and Garber, 1952; Weiss and Taylor, 1956; Weiss, 1959). Detailed studies on the relationship between surface topography and cell behavior were made possible by the introduction of the use of microfabrication and, more recently, nanofabrication methods that enable precise control over topographic features to be obtained (Brunette, et al., 1983; Brunette, 1986a,b; Dunn and Brown, 1986; Wood, 1988; Clark et al., 1990, 1991; Meyle et al., 1993).

1. Artificial Substratum Topography

In many respects the most useful structures for investigating the topographical reaction of cells are those which can be fabricated to chosen dimensions out of chemically well-characterized materials. The simplest method of producing an anisotropic surface geometry is to rub the surface with a paste containing different sizes of abrasive particles (Ohara and Buck, 1979). Cliff structures, ridges and grooves can be fabricated with great precision by photolithography combined with various types of plasma or ion beam etching (Curtis and Clark, 1990). Although plasma and ion
etching techniques allow fabrication of precisely defined topographies, neither of these techniques can eliminate the differences in surface chemistry between etched and unetched surfaces (Singhvi et al., 1994). It is essential that surfaces are chemically homogenous or effects of surface topography upon cell behavior cannot be considered separately from possible chemical differences (Singhvi et al., 1994).

2. Micromachined Surface Topography

Micromachining combined with a coating technique developed by Brunette (1983, 1986a,b), employed for the fabrication of grooved substrata used in the experiments included in this thesis, avoids the problem and possibility of chemical heterogeneity (Singhvi et al., 1994). A grooved surface of a substratum can be defined by their depth and width, and by pitch (repeat) spacing and cross section. Depending on the etching and crystal orientation of the silicon wafer, grooves may have parallel, vertical ‘U’-shaped wall, ‘V’-shaped walls, or truncated ‘V’ shapes (Brunette et al., 1983; Brunette, 1986a). The spaces between the grooves can be regarded as ridges, though if the grooves are very far apart this definition becomes irrelevant (Curtis and Clark, 1990). After fabrication, all substrata can be evaporatively coated with a thin layer of titanium which results in a homogenous titanium surface that has been extensively characterized (Chehroudi, 1991). Titanium was selected because it has been commonly used as a biocompatible material for dental implants in the clinic (Branemark et al., 1977). The details about the micromachining technique and its application in the study of topographic guidance will be described in Chapter 3, Section I,D.

Micromachined surfaces have been compared with smooth surfaces for their ability to support cell attachment, and the regular geometry of micromachined surfaces enables the surface areas of the grooved surface to be calculated. More oral epithelial cells attached to surfaces with V-shaped grooves produced by micromachining than to a smooth control surface (Brunette, 1986a,b). It was found that the increase in epithelial-cell attachment on grooved surface was not simply the
3. Directed Cell Migration

Micromachined grooved surfaces can effectively direct the migration of cultured human gingival fibroblasts (Brunette, 1986; 1988a; Brunette et al., 1989). The control of cell migration by topographic cues appears to be localized to the leading lamellae and need not involve microfilament bundles (Oakley and Brunette, 1993) which traverse from the lamellae to a perinuclear location as has been suggested in one theory of contact guidance (Dunn and Heath, 1976). These studies also indicated that the various filaments segregate preferentially with time into subcellular locations defined by the substratum (Oakley and Brunette, 1993). Microtubules are the first cytoskeletal element to form and align, and they are first observed in the deepest portion of the groove. In contrast, actin-containing microfilament bundles form later and are typically first located close to the groove/ridge edge (Oakly and Brunette, 1993). The ability of grooved surfaces to control cytoskeleton distribution probably accounts for their marked effects on cell shape.

The relationship between cell shape and cell function have been studied using the cells cultured on the substrata with different chemical compositions. Changes in cell shape can regulate cell growth (Folkman and Moscona, 1978), cytoskeleton gene expression (Ben-Ze’ev, 1987), collagenase and stromelysin gene expression (Weeb et al., 1986), radiation-induced DNA unwinding (Olive and MacPhail, 1992), extracellular matrix metabolism (McDonald 1989), and differentiation (Watt et al., 1988). Surface topography can markedly alter cell shape in vitro, an effect that was noted for grooved surfaces in the early observations of Weiss and analyzed at a sophisticated mathematical level for cells on micromachined surfaces by Dunn and Brown (1986).

B. Proposed Mechanisms for Topographic Guidance

In general, moving fibroblasts in vitro are fan-shaped with sheet-like protrusions termed lamellae
that extend from the cell surface and also attach to the substrata. Time-lapse observation of locomoting cells indicates that the cells move in the direction of the largest lamella termed the leading lamella (reviewed by Harris, 1994). In practice, topographic guidance is inferred as being operative if a greater proportion of cells align with a topographic feature than would be predicted by chance. Several of the theories proposed to explain topographic guidance are reviewed below.

1. **Microexudate Hypothesis**
   
The earliest attempt to provide an explanation as to how cells detect and are influenced by topographic features was made by Weiss (1934, 1945) based initially on the guidance of cells by fibrillar materials. He suggested that the cells responded to the molecular orientation of their environment. Specifically, oriented fibrillar or ultrafibrillar aggregations of polar colloid particles or micellae are produced in the plasma medium as the results of oriented mechanical forces such as exhibited by stretching or pressing the plasma medium or stroking the medium with a hairbrush or needle (Weiss, 1934). This oriented fibrilla in turn could cause cells migrating from the explants to be oriented. Similarly, when two explants of chick embryonic tissue are placed a short distance apart onto clotted plasma, the outgrowing cells from each explant are oriented towards each other (Weiss, 1959). Weiss suggested that this "two-center" effect is the result of cells exerting tension on the fibrin fibers which in turn, produces oriented lines between the explants.

The strongest argument against this theory comes from the findings both by Curtis and Varde (1964) and Dunn and Heath (1976) which show that extensive changes in cell behavior take place at radii of curvature or at changes of angle which are most unlikely to alter the capillary spread of a microexudate. Dunn also argued that it is difficult to demonstrate guidance by oriented ultrastructures because of the difficulty in preparing a substratum which is uncontaminated by large oriented structures. Thus, the microexudate theory has won little support as an explanation for topographic guidance.
2. **Cytoskeleton-based Hypothesis**

Current theories for the mechanism of topographic guidance generally agree that cells align with the substratum topography in a manner which minimizes distortions in their cytoskeleton or attachment components (Dow et al., 1987). Microfilaments and focal adhesions have been suggested to play fundamental role in topographic guidance *in vitro*.

Dunn and Heath (1976) proposed a cytoskeletal mechanism by which cells detect the topography of a substratum. They examined the behavior of fibroblasts on cylindrical substrata of different radii of curvature, and on prism edges of increasing angle to the plane. They concluded that inflexible bundles of microfilaments or stress fibers acted as a reference standard for detecting the curvature of the substratum. Microfilament bundles are typically found between focal adhesions of the leading lamella and the nuclear regions of fibroblasts. These microfilament bundles usually subtend an angle of about 4° to a flat substratum and any changes in inclination of the substratum of more than this angle could interfere with the bundle geometry (Dunn, 1982).

The microfilament-based mechanism was extended as an explanation for topographic guidance of fibroblasts on grooved substrata by Dunn (1982). Dunn (1982) suggested that substrata with 'V'-shaped grooves could be considered as a repeating sequence of sharp convex and concave changes in inclination and that cells reacted to the convex discontinuities of grooved substrata in the same manner as they reacted to isolated convex curvatures. However, Brunette (1988b) reported that cell alignment on grooved surface preceded the formation of discrete microfilament bundles and therefore aligned microfilament bundles were an effect rather than a cause of cell alignment on grooved surfaces.

The microfilament mechanism was considered by Ohara and Buck (1979) to be inadequate to
explain how cells reacted to the multiple features offered by grooved substrata. They examined a number of cell types on various grooved substrata and observed that cells preferentially aligned along the ridges rather than within the grooves and cells tended to bridge over grooves, avoiding them altogether. Cells spanned over grooves and utilized the limited areas where the formation of focal adhesions were taking place for attachment on the ridges due to the stiffness of cells which prevents them from attaching to the substrata within a groove (Ohara and Buck, 1979). Thus, Ohara and Buck (1979) proposed that linear focal adhesions governed alignment of cells on grooved substrata with narrow pitch.

Dunn and Brown (1986) combined the microfilament mechanism with the focal adhesion mechanism of Ohara and Buck (1979) and suggested that focal adhesions could form in grooves but the direction in which cells could exert traction on them was restricted by the walls of the groove. Because the microfilament bundles associated with the focal adhesions would not be easily bent, focal adhesions at a shallow angle could not exert a force for traction if the focal adhesion was within the groove and the direction of the force was perpendicular to the axis of the groove. Thus, expansion of cell margins and cell locomotion would be restricted in directions other than those parallel with the grooves (Dunn and Brown, 1986).

The role of microtubules (MT) in topographic guidance has been investigated in several studies. Brunette (1986a,b) suggested that for cells aligned with grooves, alignment of cytoskeletal filaments reflected the orientation of the cell as a whole. Dunn and Brown (1986) reported that, although cells were oriented with the groove pattern, MTs only appeared occasionally to be influenced by the grooves. A recent study by Svitkina et al., (1995) indicated that the pattern of microfilament bundles in cells on cylinders depends upon the integrity of MTs. When MTs are disrupted by colcemid, cell shape becomes polygonal and less elongated. Thus, it is suggested that MTs in fibroblasts spread on cylinders promote cell elongation and shift the longitudinal and
transverse orientation of microfilament bundles to a monodirectional one and with the dominance of longitudinal bundle alignment (Svitkina et al., 1995).

3. Stochastic Hypothesis

Brunette (1986a, 1988a) suggested based on his studies that cells do not respond to the substratum as an “all or nothing affair”. Instead, cells react probabilistically to topographical features. The topographical feature could reduce the probability of a cell making a successful protrusion and adhesion in a given direction (Brunette, 1986a,b, 1988a; Clark et al., 1987), so that protrusions and adhesions made in other directions may be favored (Brunette, 1988a; Clark et al., 1987). Time-lapse observations also suggested that topographic guidance of cells results from interactions between the grooves and the cell’s leading edge rather than from interactions between the substratum and mechanically-stiff linear cytoskeletal elements (Brunette, 1986a,b, 1988b). When fibroblasts migrate from one set of grooves to another groove intersecting the first at right angles, cells change their alignment and direction of migration. The leading lamellae of these cells become oriented to the second set of grooves before the cell body is reoriented (Brunette, 1988b). Thus, it is suggested that the flexible lamellae may also contain the machinery responsible for topographic guidance on grooves (Brunette, 1988b).

Given these disputed considerations, it seems that a single hypothesis of a simple cytoskeletal elements cannot sufficiently explain the mechanism of topographic guidance, although hypotheses for topographic guidance are often presented as discrete cytoskeletal mechanisms.
V. Fibronectin

In this thesis, fibronectin was selected as a molecule to be studied for its regulation by the surface chemistry and topography of substrata, because of its important role in the cell attachment and matrix assembly at the implant-tissue interface. Fibronectin is one of the major adhesive proteins with a remarkably wide variety of functional activities. Besides binding to the cell surface in mediating adhesive interactions, it binds specifically to a number of biologically important macromolecules, including collagen, fibrin, and glycosaminoglycans. These interactions appear to be mediated by a series of different structural and functional domains arranged in a modular organization along the polypeptide chain of fibronectin. This section will briefly review the structure and functional activities of fibronectin.

A. Structure

The basic unit of fibronectin is a disulfide-bonded dimer of two similar, but not identical, polypeptide chains. Each chain has a molecular weight of approximately 250,000. The dimer is secreted and can be further organized into fibrils in the extracellular matrix or can remain soluble in the circulation. In general, fibronectin subunits isolated from plasma have a lower molecular weight than fibronectin isolated from cell culture, resulting in the terms “plasma fibronectin” and “cellular fibronectin”.

The major part of the fibronectin sequence can be organized into three types of homologous repeats (reviewed by Petersen et al., 1989). A type I repeat is about 45 amino acid residues long, a type II repeat is 60 residues long, and a type III repeat is about 90 residues. Twelve type I, two type II, and seventeen type III repeats have been found. The two chains of fibronectin are linked to each other by two disulfide bonds formed by Cys2427 in one chain and Cys2431 in another chain, both located near the carboxy-terminal ends (Petersen et al., 1983).
The many isoforms of fibronectin are all encoded by only one gene (Kornblihtt et al., 1983; Tamkun et al., 1984). The fibronectin gene spans 50 kilobases with at least 48 exons and over 20 introns, whereas its mRNA is about 7.9 kilobases (Kornblihtt et al., 1983). The variants of fibronectin polypeptides arise by differential splicing of the primary transcript and by different post-translational modifications. The longest known variant of the fibronectin subunit is 2446 amino acids long.

B. Functional Activities

Fibronectin has been implicated in an impressive number of biological activities. These putative functions can be understood at the molecular level in terms of the binding of specific parts of the fibronectin molecule to specific ligands. There can be more than one binding site for each ligand on fibronectin itself.

1. Cell-Binding Sites

The major cell-binding site crucial for fibronectin binding to fibroblast and certain other cell types has been identified as the cell receptor recognition sequence Arg1584-Gly-Asp-Ser (RGDS) located at type III-11 repeat unit in the 75-kDa “cell binding fragment” (Pierschbacher et al., 1982). The importance of the RGDS adhesive recognition sequence for the biological function of fibronectin can be seen most clearly in mutants generated using recombinant DNA methods. A point mutation substituting a Glu for Asp (D) in the RGDS sequence (RGES vs RGDS) or omitting the serine residue to produce the peptides RGD resulted in a nearly complete loss of adhesive activity (Yamada and Kennedy, 1987; Obara et al., 1988).

The cell-binding domain of fibronectin contains a “second site” region necessary for interaction with cells. This accessory binding site was identified by a major drop in the estimated binding affinity of fibronectin as the 75-kDa “cell binding fragment” is reduced in size to the 11.5-kDa
fragment in which the RGDS sequence remains (Akiyama and Yamada, 1985; Akiyama et al., 1985). Deletion mutagenesis studies have localized a peptide sequence crucial for full adhesive activity to an area more than two type III homology repeats (>20-30 kDa) away from the RGDS sequence (Obara et al., 1988). This “second” site appears to function synergistically with the major site (RGDS), since mutational inactivation of either of these sites produces a >95% loss of adhesive activity measured in either cell-spreading or cell attachment assays (Obara et al., 1988).

2. Collagen-Binding Site
Intact fibronectin binds readily to collagen type I, II, III, and V (Engvall et al., 1978; Jilek and Hormann, 1978). The smallest collagen-binding fragments obtained to date from intact fibronectin are 30-40 kDa in size, containing the second type II repeat and the following type I repeat. The functional site appears to be present in a 14 amino acid region at the junction of type II and type I units (Owens and Baralle, 1986).

After fibronectin has bound to collagen by means of its collagen-binding domain, the two molecules can be cross-linked by transglutaminase (Mosher et al., 1979). The covalent linkage is formed between a lysine on collagen and a glutamine residue of fibronectin. This glutamine is located in the adjacent “amino-terminal transglutaminase cross-linking domain” rather than within the collagen-binding domain itself (Mosher et al., 1980). This covalent cross-linking to a second site would stabilize the binding of fibronectin to collagen.

3. Fibrin-Binding Sites
Fibronectin binds to fibrin and with less avidity to fibrinogen. Fibronectin appears to bind to fibrin via three separate domains. Binding at the amino-terminal 30-kDa domain occurs with the highest apparent affinity (Hormann and Seidl, 1980; Hayashi and Yamada, 1983). A second binding site
exists close to the carboxyl-terminal of both \( \alpha \) and \( \beta \) subunits of fibronectin (Hayashi and Yamada, 1983). A third binding site is immediately adjacent to the collagen-binding domain (Seidl and Hormann, 1983).

These fibrin-binding sites all display weak affinity at 37°C (Grinnell et al., 1980b). Covalent cross-linking of fibrin to fibronectin occurs in a reaction mediated by transglutaminase (Mosher, 1975), which may be important for stabilizing this relatively weak interaction at physiological temperatures (Grinnell et al., 1980b).

4. Glycosaminoglycan-Binding Sites
Fibronectin also binds to the closely related sulfated glycosaminoglycans heparin and heparan sulfate (Stathakis and Mosesson, 1977; Yamada et al., 1980). The interaction with heparan sulfate in vivo would be with the complete proteoglycan form containing this glycosaminoglycan (Culp et al., 1979). There are at least two separate heparin-binding domains on each monomer subunit of fibronectin under physiological conditions (Yamada et al., 1980). Studies have suggested that, for at least some cell types, the heparin-binding domains of fibronectin can potentiate cell adhesion to the classical cell-binding domain, suggesting the existence of cooperativity between these two types of domains to obtain maximal strength of cell adhesion to fibronectin (Laterra et al., 1983a,b; Beyth and Culp, 1984; Lark et al., 1985; Izzard et al., 1986).

5. Fibronectin-Fibronectin Association
Fibronectin is present on the cell surface in dramatic fibrillar patterns, which are probably noncovalently associated fibronectin dimers and multimers (Yamagate et al., 1986). The mechanisms of fibronectin-fibronectin interactions are not yet fully understood. Domain-domain interactions in fibrillogenesis could be electrostatic, since some domains are highly basic whereas
other are moderately acidic; interactions of alternating charged domains with domains of the opposite net charge could lead to self-association and the formation of polymers (Hormann, 1982; Rocco, et al., 1983). Of particular interest is the binding of heterologous domains: the amino-terminal heparin-binding domain can bind directly to the carboxy-terminal heparin-binding domain (Homandberg and Erickson, 1986). These homologous and heterologous interactions may account for the intramolecular interactions involved in monomer-monomer association to form dimeric structures (Robinson and Hermans, 1984).

6. **Function of Fibronectin in Wound Healing**

Fibronectin plays an important role in wound healing, functioning to promote clot formation, development of granulation tissue, and re-epithelialization (reviewed by Oliver et al., 1992; Clark, 1990). The first stage in wound healing is inflammation, which involves rapid deposition and polymerization of fibrin (Dvorak et al., 1988). Plasma fibronectin binds and covalently crosslinks with fibrin to form a fibrous clot structure that promotes migration and attachment of leukocytes and fibroblasts (Postlethwaite et al., 1981; Dvorak et al., 1988), and macrophage recruitment (Clark, 1988). Fibroblasts subsequently migrate into the wound area and produce additional fibronectin, which is assembled into a well-ordered, disulfide cross-linked provisional matrix (Grinnell et al., 1981; Kurkinen et al., 1980). During matrix assembly, fibronectin is able to specifically interact with other matrix proteins and macromolecules by means of multiple, distinct binding domains (Hynes, 1985). As healing progresses, collagen content increases, the fibrils are organized into fiber bundles, and the provisional matrix of fibronectin diminishes (Grinnell et al., 1981; Kurkinen et al., 1980). Re-epithelialization is accomplished as epidermal cells migrate over the provisional matrix produced by granulation tissue and re-establish the basement membrane. Fibronectin is important for this migration because expression of the fibronectin receptor is evident in the migrating keratinocytes (Takashima et al., 1988; Clark, 1990).
In the clinic, surgical placement of dental implants may create a condition similar to the wound healing process (Chehroudi et al., 1990). Thus, fibronectin may play an important role in facilitating the cell attachment to the implant surface and matrix assembly at the implant-tissue interface.

C. Regulation of Fibronectin Expression

The cellular production of fibronectin is influenced by biological processes such as embryonic development (Duband and Thiery, 1982; Norton and Hynes, 1987), wound healing (Kurkinen et al., 1988; Oliver et al., 1992), and neoplastic transformation (reviewed by Vaheri et al., 1989; Chandler et al., 1994). The specific regulators which operate in each of these physiological situations still remain unclear. The lack of functional substitutes for fibronectin, the diversity of structure, the multitude of potential regulators, as well as a role in many different biological processes suggest complexity in the mechanisms controlling fibronectin gene expression and regulatory pathways. In this section, a brief review is made of some molecules that have been found to modify fibronectin expression.

Altered expression of fibronectin has been a frequent consequence of fibroblast transformation (reviewed by Vaheri et al., 1989). For example, loss of cell surface fibronectin is a hallmark of many oncogenically transformed cells (Hynes, 1990) and has been correlated with some of the key features of neoplastic transformation, in particular, alterations in cell morphology, adhesion, migration, and metastasis (Yamada et al., 1978; Chen et al., 1979; Neri and Nicholson, 1981; DiRenzo et al., 1985). Phenotypic reversion of these changes can be accomplished, at least in part, by restoring fibronectin levels (Yamada et al., 1976; Ali et al., 1977).

The effects of oncogenesis on fibronectin expression have been extensively studied. Transformation associated reduction of fibronectin biosynthesis has been reported for chicken,
rodent, and human fibroblasts and at different points in the fibronectin biosynthetic pathway. In chick embryo fibroblasts, fibronectin mRNA levels are reduced in v-src transformation as a result of a specific decrease in the rate of fibronectin gene transcription (Tyagi et al., 1983, 1985). A recent study (Gu and Oliver, 1995) on rat fibroblasts indicates that the distal portion of the fibronectin promoter contains a v-src-sensitive element(s) which mediates a decrease in the rate of fibronectin transcription. In a study on the Ha-ras transformed human osteosarcoma cell line, the down-regulation of fibronectin expression is demonstrated at a novel nuclear posttranscriptional level (Chandler et al., 1994). Decreased fibronectin content in transformed fibroblasts can also result from poor incorporation into the extracellular matrix, probably due to the reduced content of the major fibronectin receptor (Plantefaber and Hynes, 1989). Increased fibronectin degradation has also been reported to occur in neoplastic transformation (Olden and Yamada, 1977; Chen and Chen, 1987).

Transforming growth factor-beta (TGF-β) can also stimulate fibronectin gene expression in bronchial epithelial cells (Beckmann et al., 1992). This finding is confirmed by a study that a reported inhibitor of nuclear poly(ADP)-ribosyl transferase, 3-AB, can antagonize the ability of TGF-β to induce fibronectin secretion and cognate steady state mRNA levels (Beckmann et al., 1992). Similar inductive effects of TGF-β are observed on mesenchymal cells both in the presence and in the absence of changes in mRNA stability (Penttinen et al., 1988). Other stimulatory molecules can also regulate fibronectin expression. For example, gallium, an antiresorption drug used in the treatment of metabolic bone disorders, may increase steady-state mRNA levels for fibronectin in primary rat calvarial osteoblasts and nontransformed human dermal fibroblasts (Bockman et al., 1993).
An ordered sequence of differential expression of fibronectin has been associated with the *in vitro* progression of hamster fibroblasts from low passage to high passage (senescence) (Choi et al., 1992; Rasoamanantena et al., 1994). As the cells reached midhigh passage, fibronectin mRNA levels increased. As the cells reached the end of their *in vitro* proliferative life span, the level of fibronectin mRNA declined (Choi et al., 1992). A similar overlapping cascade pattern of up-regulation of fibronectin genes is also seen during the wound healing process (Oliver et al., 1992).
VI. Matrix Metalloproteinase 2 (72-kDa Gelatinase)

One of the matrix metalloproteinases (MMPs), MMP-2, was studied in this thesis for its regulation by the substratum surface chemistry and topography. Thus, its structure, function and regulation are briefly reviewed in this section.

MMPs are a family of proteolytic enzymes that mediate the degradation of extracellular matrix macromolecules, including interstitial and basement membrane collagens, fibronectin, laminin, and proteoglycan core proteins (reviewed by Birkedal-Hansen, 1993; Birkedal-Hansen et al., 1993). The enzymes are secreted or released in latent form and become activated in the pericellular environment by disruption of a Zn++-cysteine bond which blocks the reactivity of the active site (Birkedal-Hansen, 1993). The following review will only focus on MMP-2.

Matrix metalloproteinase 2 (MMP-2), also known as 72-kDa gelatinase, is probably the most widely distributed of all MMPs and has been identified in skin fibroblasts (Seltzer et al., 1981), keratinocytes (Salo et al., 1991), chondrocytes (Lefebvre et al., 1991), endothelial cells (Kalebic et al., 1983), monocytes (Garbisa et al., 1986), osteoblasts (Overall and Sodek, 1987), and in a number of other normal and transformed cells (Birkedal-Hansen, 1993; Birkedal-Hansen et al., 1993).

A. Structure

The MMP family members, including interstitial collagens, stromelysins and gelatinases (reviewed by Birkedal-Hansen et al., 1993), may be regarded as derivatives of the five-domain modular structure characteristic of collagenases formed by addition or deletion of domains. These five domains include NH2-terminal signal peptide, propeptide, catalytic domain, hinge region and COOH-terminal hemopexin domain. The catalytic domain contains the catalytic machinery
including the Zn\(^{2+}\)-binding site. The MMP-2 (72-kDa gelatinase) differs from most other MMPs in that it has a fibronectin-like domain inserted into the catalytic domain without disrupting the cysteine-zinc complex (Van Wart and Birkedal-Hansen, 1990). In fact, the only two domains that all MMPs have in common are the propeptide domain that contains the key cysteine residue and the catalytic domain that contains the zinc-binding site (Birkedal-Hansen et al., 1993).

**B. Functional Activities**

The molecular disassembly of intact solid phase substrata such as natural collagen fibrils, proteoglycan aggregates, and basement membranes is considerably more complex than the cleavage of isolated soluble or reaggregated molecules exposed to a single enzyme, and these processes are still incompletely understood (Birkedal-Hansen et al., 1993).

1. **Substrate Specificity**

The degradation of the extracellular matrix involves a complex cascade activation by several related MMPs. These degradation processes have been reviewed by Overall et al. (1989) and Birkedal-Hansen et al. (1993). Activated MMP-1, collagenase, can cleave the native triple helical region of interstitial collagens into characteristic 3/4- and 1/4-collagen degradation fragments (Sakai and Gross, 1967). The denatured \(\alpha\)-chains of collagen can be further degraded by MMP-2, 72-kDa gelatinase (Murphy et al., 1985; Nakano and Scott, 1986). MMP-3, stromelysin, is widely active on fibronectin, proteoglycan core protein, laminin and gelatin (Chin et al., 1985; Okada et al., 1986).

2. **Activation of the Enzyme Precursor**

The MMPs are secreted in a latent form and can be activated \textit{in situ} by a variety of seemingly disparate means (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). Examples of
these activation methods include treatment with proteases (Stricklin et al., 1983), conformational perturbants such as sodium dodecyl sulfate (Birkedal-Hansen and Taylor, 1982), heavy metals such as Au compounds (Lindy et al., 1986), oxidants such as NaOCl (Weiss et al., 1985), and sulfhydrylalkylatine agents such as N-ethylmaleimide (Murphy et al., 1980).

All these methods used to activate the MMPs actually result from the common basis that the Cys\textsuperscript{73} residue in the propeptide domain is dispositioned (Springman et al., 1990). This residue is not freely accessible in the latent enzyme, but it is exposed or modified by the activation treatments. It has been proposed that Cys\textsuperscript{73}-Zn\textsuperscript{2+} bond links the unpaired propeptide Cys residue to the active site Zn\textsuperscript{2+} and displaces the H\textsubscript{2}O molecule, which is necessary for catalysis (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). Disruption of this putative Cys\textsuperscript{73}-Zn\textsuperscript{2+} bond in its “docked” position may be achieved by the chemical and physical means described above. Accordingly, when Cys\textsuperscript{73} is “on” the zinc, the activity of the enzyme is “off.” Thus, the dissociation of Cys\textsuperscript{73} from the zinc atom is viewed as the “switch” that leads to activation.

C. Regulation of MMP-2 Expression and Activity

The MMP-2 seems to be widely expressed by most cell types. The cellular control of this proteolytic enzyme is exerted at several levels including its synthesis and secretion, activation, and inhibition by the stoichiometric complexing of the specific matrix metalloproteinase inhibitor 2 (TIMP-2) to the activated enzyme (Cawston et al., 1981; Welgus et al., 1981; Birkedal-Hansen et al., 1993).

1. Transcriptional Regulation of MMP-2 Expression

The remodelling of connective tissue can be controlled through the regulation of MMP expression at transcriptional and post-transcriptional levels (Overall et a., 1989a, 1991; Overall and Sodek,
Regulation of MMP-2 expression by transforming growth factor-β1 (TGF-β1) has been intensively studied. TGF-β has been found to promote connective tissue formation by stimulating the synthesis of extracellular matrix components (Sporn et al., 1983; Wrana et al., 1991), by suppressing overall proteolytic activity through reduced proteinase synthesis (Chiang and Nilsen-Hamilton, 1986; Kerr et al., 1988; Overall et al., 1989a), and by increasing proteinase inhibitor expression (Edwards et al., 197; Overall et al., 1989a,b). However, it has been found that TGF-β1 moderately increases (between 1.5-2.2-fold) the level of MMP-2 mRNA expression and protein synthesis secreted by human fibroblasts and rat bone cells (Overall et al., 1989a,b; Overall et al., 1991). TGF-β1 may also induce the increase in the stability of MMP-2 mRNA, which would further augment the elevated levels of MMP-2 mRNA produced by increased MMP-2 transcription (Overall et al., 1991).

The lectin concanavalin (ConA) has been reported to increase MMP-2 expression through increased transcription, and also induce activation of MMP-2, but to a lesser extent (Overall and Sodek, 1990). However, ConA does not increase the stability of MMP-2 mRNA (Overall and Sodek, 1990).

Tetracyclines have been used both systemically and orally in the treatment of various infections. Beside their antimicrobial property, the doxycycline and chemically modified tetracyclines have direct inhibitory effects on MMP-2, and through decreased transcription of MMP-2 mRNA (Uitto et al., 1994).
Induction or stimulation of MMP expression may also occur in response to signals or events that are physical rather than chemical in nature (Birkedal-Hansen et al., 1993). Cell shape changes often, but not invariably, induce MMP expression (Aggeler et al., 1984; Werb et al., 1986), probably due to the reorganization of polymerized actin rather than cell rounding per se (Unemori and Werb, 1986). Because cell-shape changes are often dictated by cell-substrata adhesion, several studies have suggested that substrata alone can modulate MMP expression. For example, engagement or cross-linking of integrin receptors by monoclonal antibodies or fibronectin fragments results in increased transcription of MMP-1 (collagenase) and MMP-3 (stromelysin) genes (Werb et al., 1989). However, there has not been any report regarding the association between MMP-2 expression and cell-shape changes as a result of topographic guidance. As grooved surfaces can alter cell shape, the effect of grooved substrata on MMP-2 expression was investigated in this thesis.

2. Activation of MMP Precursors

Proteolytic activity of MMPs is also dependent on the activation of their latent precursors. Although the biological activation of MMP is still incompletely understood, studies have suggested that the latency of the virgin enzyme is maintained by a putative Cys$_{\text{73}}$-Zn$^{2+}$ bond that links the unpaired propeptide Cys$_{\text{73}}$ residue to the active site Zn$^{2+}$ and displaces the H$_2$O molecule, which is necessary for catalysis (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). Organomercurials, metal ions, thiol reagents, and oxidants may interact directly with the propeptide Cys-residue to disrupt this putative Cys-Zn$^{2+}$ bond, resulting in conformational change of the enzyme (Birkedal-Hansen et al., 1993). Chaotropic agents (NaSCN) and detergents (1 to 2% SDS) can induce polypeptide chain conformational changes that also open the Cys-Zn$^{2+}$ bond (Birkedal-Hansen et al., 1993).
Proteolytic enzymes can also cleave a portion of the MMP propeptide so that the switch of enzyme opens. Once stabilized in the open form, the enzyme catalyzes several autolytic cleavages to generate the fully activated enzyme (Grant et al., 1987; Nagasa et al., 1990; Suzuki et al., 1990). For example, exogenous proteinases, such as trypsin and chymotrypsin, attack a short basic sequence exposed on the surface of MMP. This initial cleavage is sufficient to allow an autolytic cleavage 5 to 8 residues upstream of the Cys$^{73}$ residue (Nagase et al., 1990; Suzuki et al., 1990). The final mature form of the enzyme is produced autolytically by intermolecular trimming of 14 to 18 residues including the unpaired Cys$^{73}$ residue (Nagase et al., 1990; Stricklin et al., 1983; Grant et al., 1987). Organomercurial activation of MMP-2 results in autocleavage, which removes a Mr 8-kDa peptide by hydrolysis at the Tyr85 - Asn86 bond, eight residues downstream from the Cys$^{73}$ (Stetler-Stevenson et al., 1989).

3. Inhibition of MMP-2 Activity
The proteolytic activity of MMP-2, like other MMPs, can be inhibited by a number of inhibitors, the most extensively characterized is the tissue inhibitor of MMPs (TIMPs). Two members of the TIMP family, TIMP (Carmichael et al., 1986) and TIMP-2 (Stetler-Stevenson et al., 1989), have been characterized. TIMPs appear to be distributed widely in tissue and fluids and expressed by many different normal and transformed cell types (reviewed by Birkedal-Hansen et al., 1993). TIMPs specifically block the activity of all MMPs by forming an essentially irreversible 1:1 stoichiometric complex with the activated MMPs (Welgus et al., 1979, 1985; Sellers et al., 1979; Cawston et al., 1983). In addition to the inhibition of active MMP-2, TIMP-2 can also form noncovalent complexes with latent MMP-2 in a stoichiometric manner (Goldberg et al., 1989; Stetler-Stevenson et al., 1989). The function and physiologic significance of TIMP-2 bound to latent gelatinase is not clear.
VII. Intracellular mRNA Distribution in vitro

Messenger RNA localization serves as a means of spatially controlling macromolecular assembly reactions. Several types of cytoskeletal proteins, such as vimentin (Isaacs and Fulton, 1987) and muscle myosin (Issacs et al., 1989), self-assemble rapidly after translation, which necessitates restricting their synthesis to regions where filaments are required (Cripe et al., 1993). Actin mRNA is also concentrated in the lamellaepodia of motile cells (Lawrence and Singer, 1986), where actin is synthesized locally to drive filament formation. However, there has not been any report regarding the intracellular distribution of mRNAs coding for extracellular matrix proteins.

As the distribution of fibronectin may influence the focal adhesions and cell attachment of cultured fibroblasts, it would be interesting to study the effects of substratum surface topography on localization of fibronectin mRNA. Such studies are demanding and time-consuming, requiring a special technique to double-label the fibronectin mRNA and cytoskeletal proteins. Although there have been some reports introducing the methods for double-labeling mRNA and cytosolic proteins, a method for simultaneously detecting mRNA and delicate microtubules was not available. In the period of my thesis work, I attempted to establish a protocol for this approach. Thus, some background about microtubules, mRNA translocation and the proposed mechanisms underlying the mRNA distribution related to the future approaches are briefly reviewed.

A. Microtubules

1. Structure

MTs contain a hollow core 15 nm in diameter surrounded by a ring constructed of longitudinally arranged protofilaments. The main building block of the protofilament is tubulin, a dimer of approximately 110,000 molecular weight composed of nonidentical polypeptide chains designated α- and β-tubulin (Luduena et al., 1992). Each αβ-dimer binds 2 molecules of GTP, one per
monomer, forming a “αβ-2GTP” complex (Mandelkow and Mandelkow, 1995).

The globular αβ tubulin dimers are assembled into protofilaments in a “head to tail” pattern. The polarity of the protofilaments is such that α-tubulin points to the plus or fast-growing end and β-tubulin points to the minus or slow-growing end (Luduena et al., 1992).

2. Interactions with other Intracellular Components

Interphase MTs are nucleated and organized from a microtubule-organizing center (MTOC) or centrosome. The minus end of each MT is sequestered within the MTOC, and the plus end of MT extends into cytoplasm towards the plasma membrane. The effect of the MTOC appears to be the inhibition of tubulin polymerization from the minus end, the end that is sequestered at the MTOC. As a result, intracellular MTs in fibroblasts have only one free end from which to grow or disassemble (Cassimeris 1993).

MTs are observed to interact with cytoplasmic actin MFs to form end-to-side contacts (Schliwa and van Blerkom, 1981) and MTs can bind and interact directly with actin MFs (Holifield and Heath, 1992). Thus, these two systems may be physically linked and functionally coordinated (Lees-Miller et al., 1992; Langford, 1995).

MTs also direct and potentiate the formation of cell-substrata contacts (Geiger et al., 1984; Rinnerthaler et al., 1988) and subsequently, the formation of tension between stabilized attachment sites directs formation of actin MF bundles (Greenspan and Folkman, 1977; Kolega, 1986). Cell spreading subsequently continues along the axis of tension (Kolega, 1986). MTs may interact with microtubule-associated proteins (MAPs) such as kinesin (Hoyt, 1994) and dynein (Endow,
1991), and other cellular components such as the Golgi (Kreis, 1990) and the elements of endoplasmic reticulum (Dabora and Sheetz, 1988).

Kinesin is a cytoplasmic motor which can track along single MT protofilaments and move towards the plus ends (Hoyt, 1994). Kinesin has been associated with the movement of secretory vesicles between the Golgi region and the endoplasmic reticulum (Lippincott-Schwartz et al., 1995). Kinesin has been recently identified as a mediator of polarization (Rodionov et al., 1993). In instances in which cytoplasmic MTs have been disrupted by agents such as colchicine, kinesin appears to co-distribute with some stress fibers in the absence of MT (Okuhara et al., 1989). Dynein is a cytoplasmic motor generally associated with transport of Golgi towards the minus ends of MTs (Endow, 1991; Vale, 1987).

Intracellular transport of vesicles and organelles between different cell components is believed to be mediated by the dynein and kinesin motor families along MTs (Vale, 1987), as well as by the actin-based myosin motor and actin filament transport (Titus, 1993; Cheney and Mooseker, 1992). Therefore, some functions of the actin and MT systems are closely interrelated functionally and even partially redundant, and both interactions may be mediated by MAPs and associated motor proteins (reviewed by Fath and Burgess, 1994; Hoyt, 1994; Langford, 1995).

B. Proposed Mechanisms for the Intracellular mRNA Distribution in vitro

Messenger RNA localization has been most extensively studied in Drosophila embryogenesis, where its role is to establish protein gradients that give rise to the embryonic body plan (reviewed by St. Johnston et al., 1992). It has been noticed that different mRNAs can be localized under several distinct mechanisms which include spatial control of mRNA stability, anchoring to localized binding sites, and directed transport (reviewed by St. Johnston, 1995; Wilhelm and Vale, 1993). The following section describes briefly these distinct mechanisms underlying the
localization of different mRNAs which have been studied. In contrast to a number of studies that
have been done on the localization of mRNAs encoding cytosolic proteins; very little is understood
about the intracellular distribution of mRNAs coding for extracellular matrix proteins. Thus, the
review will be limited to the information based on the available studies on cytosolic protein models.

1. Spatial Control of mRNA Stability

A simple but efficient way to localize mRNA is to stabilize the mRNA that is in the correct position
and to degrade the unlocalized transcripts (reviewed by St. Johnston, 1995). hsp83 mRNA in the
Drosophila embryo seems to be localized under this mechanism. When the egg is laid hsp83
transcripts are present throughout the cytoplasm, but by the time that the pole cells form, the
mRNA has been degraded everywhere except in the pole plasm (Ding et al., 1993). This localized
stabilization is probably due to some unidentified protecting factors in this particular area (St.
Johnston, 1995).

2. Anchoring of Localized mRNAs

Another way that mRNAs can become concentrated in a particular region of a cell without being
actively transported is if they are sequestered by localized binding sites (reviewed by St. Johnston,
1995). Some element of the cytoskeleton is almost certainly involved in anchoring mRNA, since
localized mRNAs, in contrast to other RNAs, are not solubilized by the detergent, Triton X-100
(Yisraeli et al., 1990). Actin filaments have been considered as the most likely components
involved in this anchoring system, since the study on Vgl transcripts indicates that these mRNAs
become dispersed after cytochalasin treatment (Yisraeli et al., 1990), which disrupts actin filament
organization (see Section VIII, D).

The anchoring of mRNAs to the cytoskeleton presents another opportunity for the cell to regulate
mRNA distribution. For instance, the localized Vgl transcripts in Xenopus oocytes are found
initially in the detergent-insoluble cytoskeletal fraction, but then become detergent soluble at the
time of oocyte maturation (Melton, 1987). In contrast, another transcript, XCAT-2, in the
Xenopus oocytes remains in the detergent-insoluble fraction throughout oogenesis (Mosquera et
al., 1993). Thus, cytoskeletal anchoring of different localized mRNAs can be controlled
independent of one another (Wilhelm and Vale, 1993).

3. Directed Transport

The mechanism that is most commonly invoked to explain mRNA localization is directed active
transport along the cytoskeleton (reviewed by St. Johnston, 1995; Wilhelm and Vale, 1993). In
living cells, particles containing fluorescently labeled membrane-binding protein (MBP) mRNA
moved unidirectionally from the cell body through the long processes to the membranous sheets
where the endogenous transcript is normally found. It is also observed that MBP mRNAs are in
close proximity to microtubule bundles, suggesting that these polymers serve as the tracks for
mRNA translocation (Ainger et al., 1993). The localization of Vg1 mRNA to the vegetal pole of
the Xenopus oocyte may also involve active transport along microtubule bundles, as the
microtubule depolymerization reagents can inhibit the translocation of this mRNA (Yisraeli et al.,
1990).

A number of microtubule force-generating proteins such as kinesin and dynein have been identified
to serve as motors for mRNA transport (Endow and Titus, 1992; Goldstein, 1991; Vale, 1992).
The clue as to what type of motor might be involved comes from ascertaining the direction of
mRNA movement with respect to the polarity of the microtubules. The studies on the transcripts
of the Drosophila oocyte indicated that the posterior pole mRNA (oskar) may be moved by plus-
end directed motor (e.g., kinesin) to the plus ends of microtubules which project toward the
posterior pole, whereas the anterior pole mRNA (bicoid) may be translocated by minus-end
directed motors (e.g., cytoplasmic dynein) to the minus-ends of microtubules which are clustered
at the anterior end of the oocyte (Pokrywka and Stephenson, 1991). Actin and myosin may also participate in the translocation of some mRNAs. In fibroblasts, actin mRNA localization is inhibited by the actin depolymerizing agents (Sundell and Singer, 1991).

These findings raise the possibility that mRNAs might be capable of moving along actin and microtubule filaments, similar to neuronal vesicles which have been found to translocate on both microtubules and actin filaments (Lawrence and Singer, 1986). Microtubule-depolymerizing drugs such as colchicine disrupt the localization of tau mRNA to the proximal region of the axons and of MAP2 mRNA to the dendrites of cultured neurons. The localization of β-actin mRNA in cultured fibroblasts is disrupted by cytochalasin D (Sundell and Singer, 1991).

4. Summary
In summary, mRNA localization plays an important role in directing specific proteins to their correct sites within a cell. However, the molecular mechanisms responsible for these different localization pathways are still largely obscure, and the intracellular distribution of mRNAs coding for secreted proteins has virtually not been investigated. Most examples of localized mRNAs encoding cytosolic proteins are likely to share several common features that the site of localization will be determined by the preexisting polarity of the cell, and this will most often depend on the organization of the cytoskeleton, either directly, in the case of active transport, or indirectly, when localization is mediated by localization sites or stability factors (St. Johnston, 1995). The development of a method for double-labeling fibronectin mRNA and microtubules in this thesis work should provide a possible means for investigating the effects of substratum topography on intracellular mRNA distribution.
CHAPTER 2

STATEMENT OF THE PROBLEM

With the introduction of various and novel surfaces of dental and medical implants during the past 15-20 years, scientists and clinicians are confronted with a mass of sometimes conflicting results about implant biocompatibility as assessed mainly by histological methods. The mechanisms underlying the tissue-implant interface reaction are largely unknown. There seems to be a gap between the sophisticated characterization of physical and chemical properties of implant materials and the poorly demonstrated biological relevance of these properties. At the outset of this thesis, I wished to adopt an approach to gain insight into the biological response to different types of the materials at a level beyond morphology and histology. I hypothesized that the surface chemistry and microtopography of biomaterials could serve as signals that could regulate the behavior of attached cells at the gene expression level.

As the tissue reaction induced by biomaterials is predominant in the cells closely attached to the surface of biomaterials and is referred to as interface reaction (Andrade, 1973), an in vivo measurement for individual molecules at the mRNA level on these target cells at a cell population level would be extremely difficult. Other variables of in vivo studies, such as the design of device and the surgical procedure of implantation, could cloud the effects generated by biomaterial surface properties. In this thesis, therefore, an in vitro approach was used to study monolayers of human gingival fibroblasts attached onto substrata with different surface chemistries (titanium vs. tissue culture plastic) or topographies (smooth vs. grooved surfaces).

To test my hypothesis, two molecules, fibronectin and 72-kDa gelatinase (MMP-2), were selected for study. Fibronectin is one of the major adhesive proteins associated with cell attachment. MMP-2 is one of the enzymes associated with tissue remodelling. The study of phenotypic regulation of fibronectin and MMP-2 in the cells cultured on different substrata was designed to
dissect several aspects of the expression pathway of these molecules including mRNA level, mRNA half-life, protein secretion, and their activities.

To test the hypothesis that subtle changes in substratum surface chemistry and microtopography alter specific phenotypic behaviors of the attached cells, it was necessary to measure more general aspects as well, such as changes in cell number, total cellular RNA and protein levels, and housekeeping gene expression in response to the designated substrata.

The effects of surface topography on cellular behavior were also determined by measuring cell shape, height, and orientation on the grooved surface. As cell shape and cytoskeletal organization can be altered by surface topography, it was of interest to examine the spatial relationship between the mRNA and the cytoskeleton. Thus, a novel technique for double labeling of cytoskeleton and mRNA was developed in this thesis to facilitate future studies that could explore the relationship of topographic guidance and intracellular fibronectin mRNA distribution.

I expected when I started this study that these experiments would provide some evidence to support the view that biomaterial surface chemical and topographic determinants can selectively and sensitively influence the specific molecules which are involved in cellular reactions at the cell-biomaterial interface.
CHAPTER 3
MATERIALS and METHODS

I. Fibroblast Cell Culture
A. Starting Cultures from Gingival Explants

Fibroblasts were isolated from human gingival explants using the method described by Brunette et al., (1976). Clinically normal gingival tissue obtained from two young (<35 years), healthy patients was collected in a sterile culture tube containing 10 ml sterile medium [Alpha Minimal Essential Medium (MEM) (Terry Fox Labs, Vancouver, B.C.) supplemented with antibiotics [100 μg/ml penicillin G (Sigma, St. Louis, Missouri), 50 μg/ml gentamycin (Sigma), 3 μg/ml amphotericin B (Fungizone, Gibco)] and 15% fetal bovine serum (Calf Supreme, Gibco)]. Immediately after excision of the tissue, the following procedures were performed using sterile techniques. No difference was noted between these two sources of tissue in subsequent assays.

The tissue was rinsed in fresh medium and placed in a 60 mm dish. The epithelium covering the tissue was mainly removed by excising. The remaining connective tissue was minced using 2 sterile scalpels. The minced tissue was then collected into a tube of medium and centrifuged for 5 minutes at 1,500 rpm. The supernatant was discarded, and the pellet resuspended and centrifuged. At the same time, sterile 60 mm tissue culture dishes were prepared by placing two small dabs of sterile silicone grease about 22 mm apart on the bottom of the dishes.

The tissue pellet was mixed in 100 μl of medium. A few explants were placed in each 60 mm dish, between the spots of silicone grease using a sterile Pasteur pipette. A sterile glass coverslip was placed over the explants and pressed down onto the silicone-spots. The dish was then filled with 4.5 ml medium with 15% fetal bovine serum, and placed into a humidified incubator with 5%
CO₂ at 37°C for 7 days.

When the cells were established on the coverslip and dish, the coverslip was removed and placed upside-down into another 60 mm dish in 4.5 ml medium. Both dishes were filled with 4.5 ml medium with 15% fetal bovine serum, and placed into a humidified incubator with 5% CO₂ at 37°C. When the cells reached confluence, selective trypsinization was employed, because this primary explant culture resulted in the growth of both epithelial cells and fibroblasts. The two cell types were separated based upon the differential resistance of fibroblasts and epithelial cells to detachment by trypsin. Fibroblasts are more easily detached from the growth surface by trypsin, requiring 5 - 10 minutes to round up and detach. Epithelial cells require longer (15 - 20 minutes) to detach (Brunette et al., 1976). After removal of the medium in the 60 mm dishes, 2 ml of trypsin solution [0.25% typsin (Gibco, Grand Island, New York) and 0.1% glucose dissolved in citrate saline (pH 7.8)] were added to each dish, rinsed and discarded. Another 2 ml trypsin were added for 5 - 10 minutes to round up the cells. The trypsinized cell suspension was added to tubes containing 4 ml medium and centrifuged. The pellet was resuspended in medium and transferred to tissue culture flasks containing 20 ml medium.

B. Established Fibroblast Cultures

Fibroblasts between the 6th and 10th passages were used for the studies in this thesis. Cells were removed from the tissue culture flask using a trypsin solution as described above, centrifuged and resuspended in medium. To determine the population density (cell/ml) of the suspended cell solution, 0.5 ml of cell suspension was added into a cuvette containing 9.5 ml isoton (Isoton II, Coulter Electronics of Canada Limited, Surrey, B.C.), and counted by a Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida). Counts were repeated three times. The Coulter Counter Coincidence Correction Chart was used to correct the values of any counts exceeding 10,000.
C. Culture Conditions

1. Substratum-Dependent Cultures

To determine the effects of substrata surface chemistry and topography on the cultured fibroblasts, fibroblasts (6th - 10th passage) were plated on different substrata at the population density of \(1.2 \times 10^4\) cells/cm\(^2\) in MEM with 5% (v/v) fetal bovine serum, a concentration of serum determined to be optimal for fibronectin expression \textit{in vitro} (see Section C-2 below). Smooth and grooved titanium-coated substrata (prepared as described in Section D below) were used to study the effects of substratum topography on cell behavior. To study the effects of substratum surface chemistry on cell behavior, cultures on smooth titanium substrata were compared with cultures on tissue culture plastic dishes. Before plating, cell subcultures were cultured for 48 hours in flasks in MEM with 5% (v/v) fetal bovine serum to adapt them to the condition that would be used in the subsequent experiments. Cells plated on these substrata were incubated for various times to achieve various final cell population densities: 16 hours (~60% confluent), 40 hours (~90% confluent) and 90 hours (confluent).

2. Serum Concentration-Dependent Cultures

The influence of serum concentration in the culture medium on the expression of fibronectin gene was examined before the study on the effects of surface chemistry and topography were performed. To determine the effects of serum concentration on fibronectin gene expression, fibroblasts were plated at a density of \(~1.3 \times 10^4\) cells/cm\(^2\) on tissue culture plastic dishes with 0.3%, 5% or 15% (v/v) fetal bovine serum for 24 and 48 hours. Cells were equilibrated by a 24 hour incubation in medium with these three different fetal bovine serum concentrations before plating onto the culture dishes. Cell numbers were determined by electronic counting. Total cell RNA was extracted and quantitated as described below. Aliquots of extracted total RNA (5 μg) were fractionated on 1.2% (w/v) agarose gels, blotted onto nylon membranes and probed for fibronectin mRNA as described in Section III below.
D. Micromachined Substrata

1. Micromachining

Micromachining is a technique, typically employing wafers of single-crystal silicon and chemical etching, used to produce a variety of three-dimensional shapes including pits, holes, trenches, walls, pyramids, hemispheres, etc. (Angell et al., 1983). The technique for the substrata used in this thesis was originally developed by Camporese et al. (1981), Department of Electronic Engineering, University of British Columbia, for the fabrication of photomasks for solar cells. The micromachined grooved substrata for this study were produced as described previously by Brunette (1986a,b). The micromachined wafers were produced in the laboratory of the Center for Advanced Technology in Microelectronics, Department of Electrical Engineering, University of British Columbia, directed by Dr. N. Jaeger. The production of substrata includes the following steps (Oakley, 1995).

a. Cleaning: n-type (1-0-0) silicon wafers (Virginia Semiconductor Inc., Fredricksburg, Virginia) were used for these studies. The wafers were 5 cm in diameter, 200-350 μm in thickness, and had polished front surfaces and bright-etched rear surfaces. The following steps were accomplished to thoroughly clean the silicon wafers:

i. 10 minutes in a solution of H₂O (300 ml), 10% H₂O₂ (60 ml), and 5% NH₄OH (60 ml) at 75 - 85°C followed by 10 minutes rinse in distilled water

ii. 10 minutes in a solution of H₂O (300 ml), HCl (60 ml) and 10% H₂O₂ (60 ml) followed by 10 minutes rinse in distilled water

iii. 4 minutes immersion in isopropyl alcohol followed by blow drying in filtered nitrogen

b. Oxidation: To grow a 0.6-μm-thick silicon dioxide layer on both surfaces of the wafer, the
wafers were treated with wet oxygen at 1150°C for 2 hours in a furnace (two-inch tube furnace [No. 7], Fairchild Semiconductor Corporation, USA).

c. Photolithography: The pattern of grooves was produced on a photomask by a computerized optical pattern generator and the pattern was produced in chromium-gold. The front surface of the silicon wafer was coated with negative photoresist (Microposit S-1400 Series, Sipley, Newton, Massachusetts) and the photomask was positioned onto the silicon wafer and exposed with a 320 nm wavelength UV light through the photomask. The exposed wafer was then developed and baked at 160°C. Precise alignment of the photomask on the wafer was required to assure the predictable etching for the grooves with precise depth and spacing.

d. Oxide patterning: The oxide layers that were unprotected from the UV-light exposure were removed by buffered HF. Then the remaining developed photoresist was removed in microstrip solvent (Microstrip 2001, Olin Hunt Specialty Inc., West Paterson, NJ). This procedure produced a silicon wafer whose front surface was patterned with oxide layer. For example, wafers used for grooved surfaces had strips of silicon oxide.

e. Final etching and groove pattern: The wafers were then etched in 19% potassium hydroxide solution at 80°C. The potassium hydroxide etches away the silicon, leaving the oxide layer intact. The etch rate is about 1.4 µm/minute for the silicon wafers used. The shape of the grooves was dictated by the crystal orientation of the silicon wafer. The depth of grooves was controlled by the time of etching. The desired repeat spacing (comprising one groove and one ridge between the grooves) was incorporated in the design of the master pattern.

The experiments described in this thesis employed a groove pattern consisting of a series of 3 µm
deep grooves with a 6-μm pitch. All of the grooves had a truncated V-shape in cross-section and the walls of the grooves formed an angle of 45 degrees with the top of the ridges (Fig. 1)

2. **Titanium Coating**

Both micromachined grooved wafers and unmicromachined smooth wafers were evaporatively coated with 50 nm of titanium. The smooth wafers were used in these studies as control surfaces. The titanium-coating protocol was developed by Brunette (1986a,b) in order to eliminate possible heterogeneities in surface chemistry and surface energy between etched and unetched regions. Therefore, the micromachined substrata used (1986a,b) were chemically homogeneous (Singhvi et al., 1994).

3. **Final Preparation of Substrata**

For the experiments of mRNA level, mRNA half life, protein level and activity, the smooth and grooved wafers 3.5 cm in diameter were used. The size of the wafer just fitted into 3.5 cm tissue culture plastic dish. Thus, the cell cultures on these micromachined grooved and smooth surfaces could be conducted within the culture dishes.

For the experiments on mRNA/protein double-labeling, smaller pieces of smooth substrata ranging 1 cm by 0.5 cm with the shape just fitting into the 0.5 ml eppendorf tube (Fisher, Pittsburg, PA) were prepared. With this set up, cells on substrata were placed in the Eppendorf tubes, one substrate for each tube. Thus, the following in situ hybridization and immunohisto-chemical reactions could be effectively carried in the Eppendorf tube containing 100 μl reaction solution.

a. **Washing**: The titanium-coated substrata were cleaned by ultrasonication for 10 minutes in a detergent formulated for tissue culture (7X, ICN Biomedicals, Inc., Costa Mesa, CA) followed by
thorough rinsing and ultrasonication in filtered deionized water and air dried.

b. **Radio-frequency glow-discharge treatment:** Finally, clean and dried smooth and grooved titanium substrata were treated with 3-minute argon-gas radio-frequency glow-discharge (RFGD) (Baier and Meyer, 1988). In the RFGD treatment, plasma clouds of argon gas bombard the surface of the substrata and ash away organic contaminants to render the substrata sterile, highly hydrophilic and very receptive for cell adhesion (Doundoulakis, 1987; Gombotz and Hoffman, 1987).
Fig. 1. Schematic drawing of titanium-coated micromachined silicon wafer with 3-μm-deep "V" grooves and 6-μm-pitch in cross section. The walls of the grooves form an angle of 45 degrees with the top of the ridges. To obtain the designed substrate, a computer-generated pattern was produced on a photomask and transferred onto silicon wafers by photolithography. Anisotropic etching produced a series of grooves of designed depth and spacing in silicon wafers that were then evaporatively coated with 50 nm of titanium, a biocompatible material suitable for cell culture. Titanium-coated smooth silicon wafers were used as control surfaces. Both substrata were sterilized by argon-gas glow-discharge treatment prior to seeding with the cell suspension.
II. Examination of Cell Shape and Orientation

The cells cultured on smooth and grooved substrata were examined for their morphology and orientation with scanning electron microscopy. The height of the cells cultured on these conditioned substrata were measured by confocal laser scanning microscopy. The protocols for these experiments are described below.

A. Scanning Electron Microscopy

Cells cultured on grooved and smooth titanium surfaces were processed for scanning electron microscopy by 1 hour fixation in 2.5% (w/v) glutaraldehyde and 1 hour post-fixation in 2% OsO$_4$ (w/v) in PBS, pH 7.2, at 4°C. Samples were DC-sputtered with 15-20 nm gold in an Edwards coating unit (Gibco, Grand Island, N.Y.), and examined with a Cambridge Stereoscan (Cambridge, UK).

B. Confocal Laser Scanning Microscopy

Cell height was measured using confocal scanning laser microscopy (CSLM) (Zeiss, Oberkochen, Germany). Cells were fixed for 30 minutes in freshly made 4% (w/v) formaldehyde in 0.1 M PBS, pH 7.2, at room temperature, which resulted in cell shrinkage of less than 5% (Berod et al., 1981), and then stained with haematoxylin for 10 minutes, mounted with media of 50% (v/v) glycerol in PBS, and kept at 4°C until measurement.

The haematoxylin stained cells were observed using an excitation wave length of 488 nm (Argon laser). A continuous series of optical sections were taken at 0.5-μm intervals through the cell, stacked, and finally the Z section planes were taken at the center of cell nuclei. Cell height was measured from the bottom of the surface immediately below the center of the nucleus to the top of the cell. Fifty cells were measured for each group.
III. Measurement of mRNA Level

Cells cultured on duplicate wafers were collected as one sample to allow enough cells to be available for mRNA measurement. Triplicates of such samples were used for each examination. The protocol for total RNA preparation, Northern blotting, probe preparation and hybridization are described separately below.

A. Total RNA Preparation

Cellular RNA was prepared by guanidinium thiocyanate (GT) according to Glisin et al. (1974) and Ullrich et al. (1977), modified as described below. Cultures in triplicate at different time points were washed 3 times in cold PBS before the cells were lysed by 3 ml of guanidinium thiocyanate buffer (GT buffer, containing 4 M guanidinium thiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl, 70 mM β-mercaptoethanol, and 5 mM vanadyl-ribonucleoside complex (VRC, BRL Inc.) as a RNase inhibitor).

Cell lysates were immediately transferred into a polypropylene centrifuge tube. After brief vigorous vortexing, 0.3 ml 2 M sodium acetate, pH 4.1, 3 ml of RNase-free water-saturated phenol and 0.6 ml of chloroform-isoamyl alcohol were added separately with vortexing between each addition. After incubation on ice for 15 minutes, RNA was collected from the upper aqueous phase after centrifugation at 12,000 g for 20 minutes at 4°C, and the aqueous phase was precipitated overnight at -20°C in 1 volume of ice-cold isopropanol. The precipitated RNA pellet was rinsed with 80% cold ethanol, vacuum dried, and dissolved in 100 μl RNase-free water. The total RNA yields were determined for each sample by spectroscopic analysis of 1/10th of the final sample volume.

B. Northern Blotting

Aliquots of extracted cell RNA (5 μg) were prepared in loading solution containing 2.2 M
formaldehyde and ethidium bromide (40 μg/ml), incubated at 65°C for 15 minutes, chilled on ice, then fractionated on 1.2% (w/v) agarose gels containing final concentrations of 2.2 M formaldehyde and 20 mM 3-N-morpholinolpropanesulfonic acid (MOPS, pH 7.0), and transferred onto Hybond-N nylon membrane (0.45 μm pore size, Amersham) using a Posiblot Pressure Blotter (Stratagene, CA). The transferred RNA was cross-linked to the membrane by 3 minutes of UV exposure.

C. Preparation of cDNA Probes

Three types of cDNA probes were applied in this thesis: fibronectin, 72-kDa gelatinase (MMP-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPD). The freeze-dried E. coli carrying recombinant plasmid pBR322 with the insertion of 1.3-kb EcoRI fragment derived from the 3’end of the human FN cDNA (Clone FN771), originally contributed by Bernard et al. (1985), and E. coli carrying recombinant plasmid pBR322 with the insertion of 1.2-kb Pst I fragment derived from human GAPD cDNA (Clone pHcGAP), originally contributed by Tso et al. (1983) were obtained from American Type and Culture Collection (ATCC). The full-length human 72-kDa gelatinase cDNA fragments were kindly provided by Drs Huhtala and Tryggvason, Department of Biochemistry, University of Oulu, Finland.

To enhance the yield of recombinant pBR 322, the replication of plasmid was selectively amplified by incubating the partially grown bacterial cultures in chloramphenicol at a final concentration of 170 μg/ml (Frenkel and Bremer, 1986). Harvested cells were lysed by 0.2 N NaOH, 1% (w/v) SDS. Phenol-chloroform extraction and ethanol precipitation were used to purify supercoiled plasmids containing cDNA inserts. Plasmids were cleaved by restriction digestion using Eco RI (FN) or Pst I (GAPD). The cDNA inserts were separated from the vectors after fractionation on 1% (w/v) agarose gel.
The excised cDNAs were collected by precipitation on glass fines and labeled by random priming with $[^{32}\text{P}]\text{dCTP} (>3,000 \text{ Ci/mmol, Amersham Corp.})$ to a specific activity of $\sim 1.8 \times 10^9 \text{ cpm/\mu g cDNA}$ prior to hybridization.

**D. Nucleic Acid Hybridization**

First, the blots were prehybridized at $62^\circ \text{C}$ for 2 hours in 5\% (w/v) SDS, 50 mM PIPES, 0.1 M sodium chloride, 50 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA), pH 7.0. Hybridization was performed for 18 hours with $[^{32}\text{P}]\text{dCTP}$-labeled FN, MMP-2 or GAPD cDNA probes at a concentration of $\sim 3.0 \times 10^7 \text{ cpm/300 cm}^2$ in 15 ml of prehybridization solution.

After hybridization, the blots were washed in 1 X SSC (standard saline citrate), 5 mg/ml SDS at room temperature for 10 minutes, followed by a change of the same solution at $62^\circ \text{C}$ for 30 minutes, and then for 20 minutes with two changes of 0.1 X SSC, 0.5 mg/ml SDS at $62^\circ \text{C}$.

Blots were autoradiographed at $-70^\circ \text{C}$ using double emulsion Cronex 4 x-ray film (Dupont) with two thulium Quanta Detail intensifying screens (Dupont) (Kircos et al., 1989). The FN mRNA band of $\sim 7.9 \text{ kb}$ in size, MMP-2 mRNA band of 3.1 kb in size, and GAPD mRNA band of $\sim 1.2 \text{ kb}$ in size were then scanned by an image digitized optical scanner (Apple Computer Co., Cupatino, CA.) and analyzed by computer software (Image 1.4, NIH). The data were normalized for the amount of total RNA loaded as determined from photographs of the ethidium bromide-stained bands. The data were then adjusted for the RNA yield from each corresponding RNA preparation to give the total yield of specific mRNA on a per cell basis. The data from an experiment in triplicate was expressed as the mean ± S.D.
IV. Measurement of mRNA Stability

A. Culture Conditions

To determine the effects of surface topography on the intracellular distribution of fibronectin mRNA in cultured fibroblasts, cells were cultured in triplicate at the population densities of 1.2 x 10^4 cells/cm^2 in MEM with 5% (v/v) fetal bovine serum on either grooved or smooth titanium-coated surfaces as well as tissue culture plastic controls, at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 40 hour incubation (~90% confluent), 60 μm 5,6-dichloro-1-B-D-ribofuranosyl benzimidazole (DRB, Sigma) was added to the cultures (Overall et al., 1991). DRB is a specific RNA polymerase II inhibitor, which has been shown to selectively inhibit the RNA synthesis by enhancing the premature termination of transcription by RNA polymerase II (Fraser et al., 1979; Tweeten and Molloy, 1981). The cells were cultured for up to 24 hours. The total RNA was extracted (see Section III-A) at each time point (0, 1.5, 3, 6, 16, and 24 hours).

B. Slot Blotting and Hybridization

The aliquots (5μg) of the RNA sample were spotted onto Hybond-N nylon membrane using a HSI-PR 600 slot apparatus (Hoefer Sci. Inc. CA), while aliquots (5 μg) of the same sample RNA were also analyzed by Northern hybridization to monitor slot hybridization. The Northern hybridization was performed using the same protocol as described in Section III-B,D.

Following 3 minutes of UV cross-linkage, the blots were prehybridized and hybridized under the same conditions set for Northern hybridization as described in Section III-D. The blots were scanned by an optical scanner, and band density and area were measured with NIH Image 1.4 with appropriate softwear (Apple Computer Co., CA). The data were adjusted for the RNA yield from each corresponding RNA preparation by the measurement of 28 S and 18 S RNA bands on
Northern blots. Such an adjustment gave the total yield and remaining amounts of specific mRNA on a per cell basis after the RNA synthesis was inhibited by DRB. The data from the experiments in triplicate were expressed as the mean ± s.d.

V. Measurements of Protein Synthesis and Enzyme Activity

In this thesis, the effects of chemistry and topography of titanium on the secretion of fibronectin and MMP-2 in the culture medium were studied, as well as the gelatinolytic activity of MMP-2. The materials and methods used in these experiments are described below.

A. Isotopic Labeling

In the cell cultures on the grooved and smooth titanium-coated surfaces and the tissue culture plastic dishes, secreted proteins were labeled by continuous incubation with 10 μCi/ml of L-[35S]methionine (1100 Ci/mmol, ICN Radiochemicals, Irving, CA). For 40-hour cultures, fresh labeling medium was replaced at 16 hours, and for 90-hour cultures fresh labeling medium was replaced at 16 hours and 40 hours. Labeled medium (3 ml) was harvested at 16 hours, 40 hours and 90 hours.

B. Total Matrix Protein Extraction

Labeled matrix proteins bound to the cell layers were extracted with urea using the method of Yamada and Akiyama (1984). In brief, cell monolayers were rinsed four times with 3 ml Hank’s balanced salt solution, to which freshly prepared proteinase inhibitor, 2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma), was added. After incubation for 1 hour at 37°C in 3 ml of serum-free MEM containing 2 mM PMSF with gentle rotation at 1 rpm, the cultures were rinsed with 3 ml serum-free MEM containing 2 mM PMSF. Labeled matrix proteins were then extracted by incubation for 2 hours at 37°C with rotation at 1 rpm in 1.5 ml freshly made 2 M
urea in serum-free MEM containing 2 mM PMSF. The extracts were centrifuged for 15 minutes at 25,000 g to remove cells and other particles. The cell pellets were fixed, sectioned and viewed under transmission electron microscope to confirm the integrity of cell membranes.

C. Liquid Scintillation Counting
Total secreted proteins either in the culture medium or extracted from the cell layer were estimated at 16, 40, and 90 hours by liquid scintillation counting of the $[^{35}S]$ methionine-labeled proteins after exhaustive dialysis against 0.15 M NaCl, 10 mM Tris-Cl, pH 7.0. Data were then expressed on a per cell basis.

D. Fibronectin and MMP-2 Purifications
Secreted fibronectin and MMP-2 were identified after purification by miniaffinity columns of gelatin-Sepharose (Pharmacia, Uppsala) as described by Overall et al. (1989). Material loosely bound to the gelatin-Sepharose column was eluted first with 1.0 M NaCl in 50 mM Tris-HCl, pH 7.2 buffer containing 5 mM CaCl$_2$, 0.5 μg/ml Brij 35. After thorough washing of the column with the same buffer, the more avidly bound proteins were recovered after elution with 4 x electrophoresis sample buffer (8.0 M urea, 80 mg/ml SDS, in Tris buffer, pH 6.8).

E. SDS-Polyacrylamide Gel Electrophoresis
Total secreted proteins in the culture media and extracted from cell layers, as well as purified fibronectin and MMP-2, were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described below. Aliquots (10 μl) of the samples were analyzed in the presence of 1.0 mg/ml SDS using separating gels containing 8% acrylamide and stacking gels containing 4% acrylamide. Samples were electrophoresed either with or without reduction with 65 mM DTT and heating at 56°C for 20 min. The protein bands on fluorographs were quantitated using an optical scanner.
(Apple Computer Co.) and image analysis computer software (Image 1.4, NIH, Bethesda) after exposure of dried 2,5-diphenyloxazol impregnated gels at -70°C to Cronex 4 x-ray film (Dupont, Wilmington, DE) for various times selected to be in the linear range of the densitometric response. The following proteins were electrophoresed under reduced conditions as relative molecular mass marker proteins: myosin (200 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

F. Gelatin Enzymography

Gelatinolytic activity of secreted MMP-2 in the culture medium was assayed by gelatin-substrate enzymography using a SDS-polyacrylamide gel electrophoresis system by adding gelatin (1 mg/ml) substrate to the gel (G-6650, Sigma, St. Louis, MO) (O'Grady et al., 1984). Conditioned medium samples were mixed with nonreducing sample buffer and loaded onto 7.5% gels. After incubation for approximately 18 hours at 37°C in a buffer of 50 mM Tris, 0.02% NaN₃, 5 mM CaCl₂, and 1 µM ZnCl₂ (pH 7.5), the gelatinolytic reaction was stopped by a 3-min wash in 15% (v/v) acetic acid. Gelatinolytic activity was detected as cleared bands against the staining of 0.1% Coomassie Brilliant blue.
VI. **Novel Technique of Fibronectin mRNA/Microtubule Double-Labeling**

A novel double-labeling system was developed to protect both the antigenicity of targeting cytoskeletal proteins for antibody labeling and the integrity of targeting mRNA for riboprobe labeling in the same cell. This technique will be used in the future studies to determine the relationship between the distribution of fibronectin mRNA and the organization of cytoskeleton, as well as the influence of substratum surface topography to this relationship.

**A. Sample Preparation**

Human gingival fibroblasts (6-10th passage) were plated at a density of \( \sim 1.2 \times 10^4 \) cells/cm\(^2\) for 6 or 24 hours on the smooth titanium surfaces prepared as described in Chapter 3, Section I-D. The titanium-coated silicon wafers were cut into small pieces to fit into the 0.5 ml Eppendorf tubes (Fisher, Pittsburg, PA) as described in Chapter 3, Section D-3. All tests were performed while the cell culture samples were kept in the Eppendorf tubes containing reaction solutions. Cell cultures were first rinsed in 37°C with cytoskeleton-stabilization (CS) buffer (Oakley and Brunette, 1993) containing 0.1 M PIPES, 1mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9, fixed for 10 min in 4% paraformaldehyde, pH 8, in 37°C phosphate-buffered saline (PBS), rinsed in CS at 37°C, followed by 3 min fixation in acetone at -20°C. After CS rinsing at room temperature, the samples were used for the following immunohistochemical labeling procedure for cytoskeletal components and *in situ* hybridization for fibronectin mRNA.

**B. Labeling of Cytoskeletal Elements**

The cell samples were first labeled for cytoskeletal elements. The cells were blocked with 1% (w/v) bovine serum albumin in PBS for 1 hour, then incubated with primary monoclonal antibody against microtubules (Sigma, Pittsburgh, PA) for 16 hrs at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse secondary antibody (Boehringer-Mannheim GmbH, Germany). After 10 min rinsing in 1% BSA/PBS and 10 min rinsing in CS, the samples
were post-fixed with 4% paraformaldehyde for 10 min in room temperature, rinsed in CS for 10 min, and in 1% BSA/PBS for 10 min. The specificity of labeling was determined by the negative control, in which the primary antibody was omitted in the labeling procedure.

C. Labeling of mRNA by in situ Hybridization

Fibronectin riboprobes, both sense and antisense, were obtained from Dr. V. J. Uitto at the University of British Columbia. Fibronectin antisense riboprobe of 377 bases in size covering RGDS cell-binding domain sequence, subcloned from ATCC fibronectin cDNA clone pFH154, was labeled with biotin. The specificity of these probes were confirmed by the hybridization of antisense probes with fibronectin mRNA on the Northern blots, while the negative control was performed using sense riboprobe on the same Northern blots.

Prior to mRNA labeling, the samples were rinsed with 1% BSA/PBS, and followed by two changes of fresh glycine solution (2 mg glycine/ml PBS/1% BSA), 3 min for the first change and 10 min for the second. The samples were further treated by fresh 0.25% acetic anhydride/0.1 M triethanolamine, pH 8, for 10 min at room temperature, and rinsed with 1% BSA/PBS for 5 min at 37°C.

Samples were then incubated in prehybridization solution (containing 10 mM Tris-HCl/pH 7.4, 0.5 mM EDTA, 0.6 M NaCl, 50% formamide, 10% dextran sulfate, 200 μg/ml sheared salmon sperm DNA, 0.02% ficoll, and 20 mM vanadyl-ribonucleoside complex) at 37°C for 1 hour. The biotin labeled antisense or sense riboprobes were used for hybridization at 37°C for 1 hour, followed by high stringency washes in SSC with 10 mM DTT for 30 min, 2x SSC with 50% formamide and 10 mM DTT for 20 min, 1x riboprobe washing solution (RWS, containing 0.1 M Tris-HCl, 0.4 M NaCl, 0.05 M EDTA) for 10 min, as well as the treatment of 0.2% (w/v) RNase
for 30 min at 37°C. After further washes in 1x and 0.2x SSC for 15 min at 37°C and 0.2x SSC for 10 min at 50°C, samples were incubated with Texas Red-conjugated streptavidin (25 μg/ml) (Molecular Probes, Inc., Eugene, OR) for 10 min at 37°C, followed by two washes in PBS with 1% BSA, 37°C, 10 min of each. Sense riboprobe was used for the negative control of hybridization in this experiment. The samples were mounted with glass coverslips using 1:1 glycerol:PBS solution containing 0.02% (w/v) sodium azide and 1,4-diazabicyclo[2.2.2]octane, stored in dark at 4°C, and examined within 48 hrs of preparation.

D. Microscopy of Doubly Labeled Cultures
The signals from double-labeled cells were examined with confocal laser scanning microscopy (CLSM, BioRad System). An argon laser (λ_{max}=488 nm) was used to scan FITC-labeled microtubules first, as FITC bleaches more rapidly than Texas-Red. Sequential 0.5 μm-optical scanning sections of the labeled cell were stacked to generate the image of microtubule distribution. A helium-neon laser (λ_{max}=543) was then used to scan Texas-Red-labeled fibronectin mRNA in the same cell. Sequential 0.5 μm-optical sections were stacked to generate the image of fibronectin mRNA distribution. The FITC image of cytoskeletal elements and Texas-Red image of fibronectin mRNA were then overlapped by the CLSM computer program, providing an image illustrating the relationship of microtubules and fibronectin mRNA in the cell.
CHAPTER 4
RESULTS

Introduction
This chapter is divided into five sections which correspond to the different questions of the study. Section I comprises the experiments to determine the regulation of cell morphology and orientation by the substratum surface topography, as well as the regulation of total cellular RNA and protein synthesis by the substratum surface chemical composition and topography. Section II presents the results of cell behavior and specific fibronectin mRNA expression in response to different concentrations of serum in cell culture, which provided the choice of serum concentration used for the experiments in this thesis. Sections III and IV comprise the experiments to determine the effects of substratum surface chemistry and topography on the mRNA level, stability of mRNA, amounts and activity of fibronectin and 72-kDa gelatinase. Section V presents the preliminary data demonstrating the novel double-labeling system, which might be used for the future studies to investigate the influence of surface topography on fibronectin mRNA distribution.

In each section, a number of figures have been included to illustrate specific features that are presented in the results. To promote the continuity of reading this chapter, the figures that pertain to each section are presented in order, together at the end of each section.
I. Behavior of Fibroblasts in Response to Substratum Surface Chemistry and Topography

A. Cell Shape and Height

The orientation and shape of cultured human fibroblasts were determined after 16-, 40-, and 90-hour growth on smooth and micromachined grooved titanium-coated surfaces (see Chapter 3, II-A,B). Under scanning electron microscopy the fibroblasts appeared elongated and orientated along the grooves of the micromachined surface, particularly in cultures which had low cell population densities, whereas on the smooth control surface cells were randomly spread with no apparent orientation (Fig. 2). Cell height was measured using confocal scanning laser microscopy and it was found that cells cultured on grooved surfaces had a greater height than did cells cultured on smooth surfaces for the same time period (Fig. 3A and B).

B. Cell Number, Total RNA and Protein Yield

Cell number, total RNA yield and total secreted proteins were determined in fibroblast cultures on grooved and smooth titanium surfaces, as well as tissue culture plastic as a control, after 16, 40 and 90 hours (see Chapter 3, III-A, VI-A,B,C).

No significant difference in cell number on a per area basis was noted among the cultures on these three different surfaces (~1.2 X 10^4, ~2.2 X 10^4 and ~5.3 X 10^4 cells/cm² at 16, 24 and 90 hours of cultures respectively). In expressing data on a per area basis, the areas of the grooved surfaces were increased by a factor of 1.28 which was calculated from the geometry of the grooved surface.

The amounts of total RNA on a per cell basis did not significantly differ between the cultures on grooved and smooth titanium surfaces at any of the time points, whereas the total RNA levels in cultures on smooth titanium surfaces showed small reductions of 7% at 16 hours, 32% at 40 hours, and 11% at 90 hours, relative to the cultures on tissue culture plastic (Fig. 4).
Since a large fraction of extracellular molecules may bind to the cell surface and be assembled into extracellular matrix shortly after being secreted into the medium (Ploetz et al., 1991; Birk et al., 1990), the amount of extracellular matrix proteins obtainable by urea extraction (matrix proteins), as well as the amounts of proteins in conditioned medium (media proteins) were measured (see Chapter 3, VI-A,B,C). The results are presented in Fig 5.

The amounts of $^{35}$S- methionine incorporated into medium proteins collected from the grooved surfaces did not differ from that from the smooth surfaces at 16 hours, but at 40 and 90 hours the amounts in cultures on grooved surfaces were slightly reduced. However, when compared with the cultures on tissue culture plastic, cultures on titanium surfaces had a slightly reduced level of total medium protein at 16 hours and slightly increased levels at 40 and 90 hours (Fig. 5).

Total labeled matrix proteins extracted by urea from the cell layer on the grooved surfaces increased ~1.5-fold in both 16 and 90 hour cultures, but were reduced slightly in 40 hour cultures, relative to the cultures on the smooth titanium surface. When compared with the cultures on tissue culture plastic, cultures on smooth titanium showed increased matrix total protein levels of 2-fold at 16 hours, and 1.7-fold at 40 hours, followed by a slight reduction at 90 hours (Fig. 5).

The amounts of total secreted proteins, estimated by adding the amounts of media protein and extractable matrix protein together, were about equal on a per cell basis in the cultures on all three different substrata. Therefore, these experiments demonstrated that the cultures on grooved surfaces produced an increased binding or assembly of secreted proteins into the cell layer in early (16 hour) and late (90 hour) cultures. Also, titanium affected the distribution of total protein in a time-dependent manner with more proteins being bound to matrix at early times and to lesser amounts at later cultures relative to cultures on tissue culture plastic, while the amounts of total
protein secretion on these two surfaces were essentially the same.

C. Summary

No difference in cell numbers was noted among the cultures on three different substrata at each of different culture times.

Cells on grooved surfaces were significantly elongated and orientated along the grooves of the substratum. The cell height was significantly greater than that of cells on smooth surfaces.

The amounts of total cellular RNA in the cultures on smooth titanium surfaces were about equal on a per cell basis to the cultures on grooved titanium surfaces, but were moderately down-regulated in comparison with the cultures on tissue culture plastic.

The secretion of total proteins were about the same on all three different substrata on a per cell basis. However, the grooved surface topography significantly increased the amounts of extracellular matrix proteins assembled into the matrix.
Fig. 2. **Effects of grooved surface on cell morphology.** Human gingival fibroblasts were cultured (~1.2 X 10^4 cells/cm^2) on titanium-coated grooved surfaces (VTi) of 3 μm in depth and 6 μm in spacing (right), and titanium-coated smooth surfaces (Ti) as a control (left), in culture medium with 5% (v/v) fetal bovine serum, for the indicated times. Cells were processed for scanning electron microscopy by fixation in 2.5% (w/v) glutaraldehyde for 1 h and in 2% (w/v) OsO_4 in PBS, pH 7.2, at 4°C for 1 h. Samples were DC-sputtered with 15-20 nm gold and examined with a Cambridge Stereoscan.
Fig. 3. **Effects of grooved surface on the height of cultured cells examined with confocal scanning laser microscopy.** Human gingival fibroblasts were cultured (~1.2 X 10^4 cells/cm^2) on grooved surfaces (VTi) and control smooth surfaces (Ti). Cells were processed for confocal scanning laser microscopy (CSLM) by fixation in 4% (w/v) formaldehyde in 0.1 M PBS, pH 7.2, at 4°C for 1 h, stained with Gill #2 haematoxylin for 10 min, and covered with a cover slip using 50% glycerol in PBS. Cells were then measured by CSLM using an argon laser (488 nm) and optical sectioning. “A”, A continuous series of optical sections of cells by CSLM were stacked and sections in the Z phase at the center of nuclei were constructed by Zeiss CSLM software so that cell height could be measured. The lower part of the micrographs shows the X-Y view of the cell up to the line of optical section. The top part of the micrographs shows the view of cell in the X-Z plane along the line of optical section.
Fig. 3. (cont.) "B", Fifty cells on grooved and smooth surfaces were measured and data is presented as the mean ± S.D.. The differences in cell height between the cells cultured on grooved and smooth surfaces were significantly different at all time points (p < 0.01, t-test).
Fig. 4. Quantitation of cellular RNA yields in response to the different surfaces of substrata. The total RNA from human gingival fibroblasts (6-10th passage, plating density of \(-1.3 \times 10^4\) cells/cm\(^2\), \(n = 3\)) was extracted from the cultures on tissue culture plastic (TCP), smooth titanium surfaces (Ti) and grooved titanium surfaces (VTi) at the indicated times, and the RNA concentrations were determined from spectroscopic analysis as described in Chapter 3, III-A.
Fig. 5. Quantitation of secreted protein synthesis in response to the different surfaces of substrata. $^{35}$S Methionine incorporation into secreted proteins in conditioned medium (Media Proteins) and proteins extracted by urea from the cell layer (Matrix Proteins) in cultures on tissue culture plastic (TCP), smooth titanium surfaces (Ti) and grooved titanium surfaces (VTi) at 16, 40 and 90 hours was determined by liquid scintillation counting after exhaustive dialysis as described in Chapter 3, VI-A, B, C. Data from one time course experiment is presented as the mean ± S.D., n = 3.
II. Effects of Serum Concentration on Cell Growth and Fibronectin mRNA Expression

To select conditions under which the effects of surface chemistry and topography on fibronectin gene expression could be examined most sensitively, the influence of different concentrations of fetal bovine serum (FBS) on cell growth and fibronectin gene expression was studied. Cell proliferation, total RNA yield and fibronectin mRNA levels were determined in cultures grown on plastic tissue culture dishes in α-MEM supplemented with 0.3%, 5%, or 15% FBS for 24 or 48 hours.

Cell numbers, as determined by Coulter counting, were essentially unaltered (1.4-1.6 X 10^4 cells/cm^2) for all FBS concentrations at 24 hours, while at 48 hours cell numbers in both the 5% and 15% FBS cultures had increased (Fig. 6).

The amount of total RNA increased slightly in both 5% and 15% FBS cultures over that in 0.3% FBS (Fig. 7). Fibronectin mRNA level as determined by Northern analysis (Fig. 8) revealed that 0.3% FBS resulted in a delayed expression of fibronectin mRNA, 15% FBS resulted in a low and progressively decreasing levels of fibronectin mRNA, whereas 5% FBS produced the most abundant fibronectin mRNA and was, therefore, selected as the optimal concentration in culture medium for the experiments in this thesis.
Fig. 6. Effects of serum concentration on cell growth. Human gingival fibroblasts (6-10th passage) were cultured at a density of ~1.3 X 10^4 cells/cm^2 on plastic tissue culture dishes in 0.3%, 5% or 15% (v/v) of fetal bovine serum (FBS). Cells were equilibrated by a 24 h incubation in medium with the three different FBS concentrations before plating onto the culture dishes. Cell numbers were determined by electronic counting.
Fig. 7. **Effects of serum concentration on cellular RNA yield.** The total RNA was extracted at the indicated times, and the RNA concentration was determined from spectroscopic analysis as described in Chapter 3, III-A.
Fig. 8. **Effects of serum concentration on fibronectin gene expression.** Northern hybridization was performed by fractioning the aliquots (5 μg) of RNA on 1.2% (w/v) agarose gels which were blotted onto nylon membrane and probed for fibronectin mRNA as described in Chapter 3, III. Data from one time course experiment is presented as the mean ± S.D., n = 3.
III. Regulation of Fibronectin Expression and Activity by Substratum Surface Chemistry and Topography

A. Fibronectin mRNA Level

Although the levels of total cellular RNA and total protein secretion on a per cell basis did not change appreciably between grooved and smooth surfaces (see Fig. 4 and Fig. 5), the level of fibronectin mRNA was notably increased in cells cultured on the grooved surface. The grooved surface increased the amounts of fibronectin mRNA ~3.5-fold at 16 hours, ~1.9-fold at 40 hours, and ~2.2-fold at 90 hours, on a per cell basis, while the mRNA levels of the house-keeping gene GAPD were essentially not altered by substratum surface topography in the cultures at each time point (Fig. 9).

Between the smooth titanium surface and tissue culture plastic, two surfaces with the same type of topography but different chemical composition, the levels of fibronectin mRNA were notably altered, while the level of the house-keeping gene GAPD essentially was not altered in these two surfaces at any time point (Fig. 9). Relative to tissue culture plastic, there was a 44% reduction of fibronectin mRNA level at 16 hours, increasing to a 2.93-fold increase of fibronectin mRNA level at 90 hours in the cultures on smooth titanium surfaces. Since the total RNA was reduced (7-32%) in the cultures on titanium (see Fig. 4), the data for fibronectin mRNA was normalized for the yield of total cell RNA for each corresponding RNA preparation by measuring the amounts of fibronectin mRNA proportional to the yield of total RNA at each time point. The data were also normalized for the loading of total RNA onto the Northern blotting gels by measuring the Northern signals proportional to the measurement of 18S rRNA bands on each loading. On a per cell basis, cultures on smooth titanium surface revealed a more pronounced increase with time relative to cultures on tissue culture plastic, with a 58% reduction in fibronectin mRNA expression at 16 hours, a 28% reduction at 40 hours, and up to a 2.6-fold increase at 90 hours.
B. Fibronectin mRNA Half-Life

As the levels of protein synthesis are known to be affected partially by the stability of mRNA (Cleveland, 1989), the effects of surface topography and chemistry on the stability of fibronectin mRNA were further investigated. Following the addition of the RNA polymerase inhibitor, DRB, to block transcription (see Chapter 3, IV), the decrease in the levels of fibronectin mRNA (Fig. 10) and GAPD mRNA (Fig. 11) was measured over time.

In cultures on tissue culture plastic, the half-life of fibronectin mRNA was estimated to be ~7 h, while the half-life of fibronectin mRNA was ~5 hours in cultures on smooth titanium surfaces showing a ~30% reduction of mRNA stability (Fig. 10). In cultures on grooved surfaces, the stability of fibronectin mRNA showed a two-phase response. In the first 2 hours, the amount of fibronectin mRNA decreased rapidly by 50%, exhibiting a 60% reduction in stability relative to that observed in cultures on smooth titanium surfaces. After this first half-life period of 2 hours, fibronectin mRNA remained relatively steady with a second half-life estimated to be ~12 hours, showing a 2.4-fold increase compared with the second half-life phase of FN mRNA of cultures on smooth surfaces which was estimated to be ~5 hours (Fig. 10).

The half-lives of GAPD mRNA were estimated to be ~24 hours in cultures on all three different surfaces (Fig. 11). In addition, a three-hour delayed response of GAPD transcription to DRB inhibition was noticed in cultures on each of these different surfaces. The mechanism underlying this delayed response was not studied in this thesis.

C. Secreted Fibronectin Level

To investigate the effects that substrata surface chemistry and topography have on secreted fibronectin levels, fibronectin was affinity purified using gelatin-Sepharose from equal aliquots of
conditioned medium (media FN) or cell layer extract (matrix FN) as described in Chapter 3, VI-D. The purified fibronectin was identified by a characteristic electrophoretic mobility shift from ~500-kDa, when electrophoresed under nonreduced conditions, to 230 - 270-kDa constituent polypeptide chains, when electrophoresed under reduced conditions (Overall et al., 1991) (Fig. 12a).

Because of the large number of samples from the cultures on three different surfaces at different time points, the SDS-PAGE experiments were divided into two groups to meet the capacity of the 15-well gel apparatus. Accordingly, the results are presented in two groups below: tissue culture plastic vs. smooth titanium (Fig. 12b), and smooth titanium vs. grooved titanium (Fig. 12c).

In comparison to the cultures on tissue culture plastic, the amounts of media fibronectin from the cultures on smooth titanium increased 3.5-fold at 16 hours and 1.4-fold at 90 hours, but at 40 hours were about the same level (Fig. 12b). The amounts of matrix fibronectin extractable from the matrix of cells on smooth titanium, however, revealed a large increase of ~100-fold at 16 hours which declined to a 28-fold increase at 40 hours, and a further decline to a 36% decrease at 90 hours relative to the cells on tissue culture plastic (Fig. 12b). The pattern of decreasing amounts of fibronectin in the matrix was opposite to the pattern of increasing levels of fibronectin mRNA (see Fig. 9). Total secreted fibronectin in the cultures on smooth titanium, as determined by adding the amounts of media and matrix fibronectin together at each culture time points, showed a similar pattern to the matrix fibronectin, from a 22.6-fold increase at 16 hours declining to 2.6-fold increase at 40 hours and finally to a slight reduction (10%) at 90 hours relative to the cultures on tissue culture plastic.

When comparing the smooth with the grooved titanium surfaces, the amounts of media fibronectin from the cultures on the grooved surface showed a slight increase for all time intervals (Fig. 12c).
The amounts of matrix fibronectin extracted by urea indicated a 50% reduction at 16 hours, a slight increase at 40 hours and a 2-fold increase at 90 hours in cultures on the grooved surface relative to the cells cultured on smooth surface (Fig. 12c). This result indicated that the total secretion of fibronectin increased with time in culture on grooved surfaces, and that the increase in amounts of fibronectin was associated with the matrix fibronectin rather than the media fibronectin. Compared with the cultures on smooth surfaces, total secreted fibronectin, determined by adding the amounts of media fibronectin and extracted cellular fibronectin at each time point on a per cell basis, showed greater increases (~2-fold) at late cultures on grooved surfaces.

D. Summary

The grooved topography selectively up-regulated the mRNA level, mRNA stability, protein secretion, and matrix assembly activity of fibronectin, relative to a smooth surface.

The effects of surface chemistry on the regulation of fibronectin appeared to be time-dependent: cultures on smooth titanium surfaces showed increasing fibronectin mRNA levels, but decreasing fibronectin levels with the time of culture relative to the cultures on tissue culture plastic. The apparent discrepancy between the increasing fibronectin mRNA levels and decreased proteins levels on titanium surfaces could be explained by the significant reduction in fibronectin mRNA stability of cells on the titanium surfaces.

The expression of the house-keeping gene GAPD was essentially unchanged in the cultures on all three different substrata.
Fig. 9. *Northern hybridization analysis of fibronectin and GAPD gene expression of cells cultured on different substrata.* Fibronectin (FN) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA levels of human gingival fibroblasts cultured on grooved titanium-coated surfaces (VTi), smooth titanium-coated surfaces (Ti) and tissue culture plastic (TCP) for 16, 40, and 90 h were determined by Northern hybridization (n = 3). Total cellular RNA was fractioned on 1.2% (w/v) agarose, 2.2 M formaldehyde gels, transferred onto a nylon membrane, and hybridized with \([^{32}\text{P}]\text{dCTP-labeled FN or GAPD cDNA probes as described Chapter 3, III-C. FN mRNA was identified at the bands of }\sim 7.9 \text{ kb in size, and GAPD mRNA was identified at the bands of }\sim 1.2 \text{ kb in size. Equal sample loading was confirmed by the density of 28S ribosomal bands stained by ethidium bromide in gels before transfer. The duration of film exposure was 6 h.}*)
Fig. 10. **Regulation of fibronectin mRNA half-life in response to different substrata.** Human fibroblasts were cultured on tissue culture plastic (TCP), smooth titanium surface (Ti) or grooved titanium surface (VTi) as described in Chapter 3, IV-A. After 40 h the RNA polymerase II inhibitor DRB (60 μg/mL) was added and the cultures were incubated for the indicated additional times. Total cell RNA was then extracted. Aliquots (5 μg) were analyzed by slot-blot hybridization with [32P]dCTP-labeled fibronectin (FN) cDNA probe. Autoradiographs of the slot-blot were quantitated by laser densitometry and the results presented as a semilogarithmic plot of the mean ± S.D. (n = 3) of FN mRNA remaining at the indicated times relative to the 0 h level. Least squares fit lines were used to estimate half-life.
Fig. 11. Regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA half-life in response to different substrata. Human fibroblasts were cultured on tissue culture plastic (TCP), smooth titanium surface (Ti) or grooved titanium surface (VTi) as described in Chapter 3, IV-A. After 40 h the RNA polymerase II inhibitor DRB (60 µg/mL) was added and the cultures were incubated for the indicated additional times. Total cell RNA was then extracted. Aliquots (5 µg) were analyzed by slot-blot hybridization with [32P]dCTP-labeled cDNA GAPD cDNA probe. Autoradiographs of the slot-blot were quantitated by laser densitometry and the results were presented as a semilogarithmic plot of the mean ± S.D. (n = 3) of GAPD mRNA remaining at the indicated times relative to the 0 h level.
Fig. 12. **SDS-PAGE analysis of gelatin-Sepharose affinity-purified fibronectin.**

[35S]Methionine-labeled fibronectin (FN), collected from extracellular matrix by urea extraction (Matrix FN) or from conditioned cell culture medium (Media FN), in cells cultured on the grooved titanium-coated surface (VTi), smooth titanium-coated surface (Ti) and tissue culture plastic (TCP) for indicated time points, was affinity purified on gelatin-Sepharose as described Chapter 3, VI-D.

A. Duplicate samples of the gelatin-Sepharose bound material from 40 h cultures were electrophoresed under reduced (+DTT) and non-reduced (-DTT) conditions on 8% polyacrylamide gels and processed for fluorography. FN was identified in the eluents as a ~550 kDa protein when electrophoresed under nonreduced conditions. The constituent polypeptide chains (230-270 kDa) of FN were demonstrated after reduction with DTT. The left lane indicated the reduced molecular weight marker proteins. (continuing)
Fig. 12 (cont.). B. Effects of surface chemistry (TCP vs. Ti) on the accumulation of "Media FN" and "Matrix FN" was analyzed in duplicate by SDS-PAGE of aliquots (10 μl) of affinity purified eluents on 8% polyacrylamide gels under reduced conditions. C. Effects of surface topography (Ti vs. VTi) on the accumulation of "Media FN" and "Matrix FN" was analyzed by SDS-PAGE of gelatin-Sepharose affinity purified eluants on 8% polyacrylamide gels under reduced conditions.
IV. Regulation of MMP-2 Expression and Activity by Substratum Surface Chemistry and Topography

A. MMP-2 mRNA Level

MMP-2 mRNA levels were evaluated by Northern analysis of cells grown on tissue culture plastic (TCP) controls, smooth titanium (Ti) and grooved titanium substrata (VTi) as described in Chapter 3, I. The Northern images were scanned to quantitate differences among the groups. MMP-2 mRNA levels were found to be altered, although differently by both substratum composition and topography (Fig. 13).

In cells on smooth Ti surfaces, MMP-2 mRNA levels were significantly lower relative to those on TCP at each of the three time-points. At 16 hours, the levels were ~ 34% less, at 40 hours ~ 55% less and at 90 hours ~ 45% less than the TCP control (Fig. 13).

Similarly on VTi surfaces, cells showed lower MMP-2 mRNA levels being 84% less at 16 hours, and 49% less at 40 hours than on TCP. At 90 hours VTi levels approached those of the TCP control (Fig. 13).

The effect of titanium could also be distinguished from the effect of surface topography by comparing Ti to VTi. At 16 hours, the cells on grooves showed a 75% lower level of MMP-2 mRNA compared to cells on a smooth Ti surface. By 40 hours, however, the relative expression levels had reversed with the cells on grooves showing 37% greater MMP-2 mRNA levels than the smooth. This trend continued with the cells on grooves showing a 73% greater level of MMP-2 mRNA than the smooth at 90 hours (Fig. 13).

The difference in MMP-2 mRNA levels were significant between TCP and Ti at 16 hours
(P<0.01), 40 hours (P<0.01), and 90 hours (P<0.05) by t-test. Differences were significant between TCP and VTi at 16 hours (P<0.05), and 40 hours (P<0.05). Differences were significant between Ti and VTi at 16 hours (P<0.01) and 90 hours (P<0.05).

B. MMP-2 mRNA Stability
To test whether the differences seen in MMP-2 mRNA levels were accompanied by changes in message stability, the mRNA half-life was evaluated. Following the addition of the RNA polymerase inhibitor, DRB, to block transcription as described in Chapter 3, IV-A, the decrease in the levels of MMP-2 mRNA was measured over time (Fig. 14A). Optical scans of the slotblot Northernns were plotted to determine the MMP-2 half-life on the three substrata (Fig 14B). In control cultures on TCP, the half-life of MMP-2 mRNA showed a two-phase response. In the first 2.5 hours, the amount of MMP-2 mRNA decreased rapidly by 50%, followed by significantly increased mRNA stability in the second phase. In Ti cultures, the stability of MMP-2 was ~5 hours. In VTi cultures, the first half-life of MMP-2 mRNA was estimated to be 13 hours, a 5 fold increase over the TCP control and a 2.5 fold increase over the Ti cultures (Fig. 14).

C. MMP-2 Level
To investigate the effects of substrata surface composition and topography on MMP levels, the enzymes were affinity-purified using gelatin-Sepharose from equal aliquots of conditioned medium as described in Chapter 3, VI-D. The gelatin-Sepharose column purified enzyme from media was identified as MMP-2 (Fig. 15a) by a characteristic mobility shift from 66kDa when electrophoresed under nonreduced conditions, to 72 kDa when electrophoresed under reduced conditions (Overall et al., 1991). The 66 kDa nonreduced form was also the major gelatinolytic band observed in enzymography (Fig. 16).
The effects of Ti and VTi substrata on MMP-2 levels were determined by measuring the purified 72 kDa bands of MMP-2 in duplicate samples in reduced form as shown in Fig. 15B. The amounts of purified MMP-2 from the culture medium on Ti were ~1.5-fold higher at 40 hours and by ~2-fold at 90 hours, while at 16 hours amounts showed about the same level relative to the cultures on TCP. On VTi surfaces, the purified MMP-2 showed a slightly increased level at 16 hours and slightly decreased level at 90 hours over that on Ti.

E. MMP-2 Proteolytic Activity

Gelatinolytic activity of secreted MMP-2 in the culture medium was assayed by gelatin-substrate enzymography. The Coomassie Brilliant blue stained gels showed the 66 kDa major gelatinolytic bands and the 59 kDa minor bands without significant difference in gelatinolytic activity amount three different surfaces tested at each time point (Fig. 16).

E. Summary

The study on MMP-2 was limited to determine how mRNA levels and secretion in the culture medium were influenced by surface chemistry and topography. The results from these experiments indicated that substratum surface chemistry and topography can significantly alter the MMP-2 mRNA levels, mRNA stability and secretion of MMP-2 in normal fibroblasts.
Fig. 13. Northern hybridization analysis of MMP-2 gene expression of human gingival fibroblasts cultured on different substrata. Cells were cultured on tissue culture plastic (TCP), smooth titanium (Ti) and grooved titanium (VTi) surfaces for 16, 40 and 90 h. Total cellular RNA was fractioned on 1.2% (w/v) agarose, 2.2 M formaldehyde gels, transferred onto a nylon membrane, and hybridized with [32P]dCTP-labeled fragments of human MMP-2 cDNA as described in Chapter 3, III-D. MMP-2 mRNA was identified as a band at 3.1 kb.
Fig. 14. Regulation of MMP-2 mRNA half-life in response to different substrata. A. Cells were cultured on tissue culture plastic (TCP), smooth titanium (Ti) and grooved titanium (VTi) surfaces. After 40 h the RNA polymerase II inhibitor DRB (60 μg/ml) was added and the cultures were incubated for the indicated additional times. Total cell RNA was then extracted. Aliquots (5μg) were analyzed by slot-blot hybridization with [32P]dCTP-labeled MMP-2 cDNA probe. B. Autoradiographs of the slot-blots were quantitated by laser densitometry and the results were presented as a semilogarithmic plot of the mean ± S.D. (n = 3) of MMP-2 mRNA remaining at the indicated times relative to the 0 h level.
Fig. 15. SDS-PAGE analysis of gelatin-Sepharose affinity purified MMP-2. 
$^{[35}S] \text{methionine-labeled MMP-2 was collected from conditioned cell culture medium of human gingival fibroblasts cultured on tissue culture plastic (TCP), smooth titanium (Ti) and grooved titanium (VTi) surfaces for 16, 40 and 90 hours. A. Duplicate samples of the gelatin-Sepharose-bound material from 40 h cultures were electrophoresed under reduced (+DTT) and non-reduced (-DTT) conditions on 8% polyacrylamide gels and processed for fluorography. MMP-2 was identified as a band that shifted from 65 kDa (-DTT) to 72 kDa (+DTT). (continuing)
Fig. 15 (Cont.). B. Effects of surface chemistry and topography on the accumulation of MMP 2 were analyzed by SDS-PAGE of gelatin-Sepharose affinity-purified elutions on 8% polyacrylamide gels under reduced conditions.
Fig. 16. **Gelatin enzymography.** Human gingival fibroblasts were cultured on tissue culture plastic (TCP), smooth titanium (Ti) and grooved titanium (VTi) surfaces for 16, 40 and 90 h. Under nonreducing conditions, aliquots (3 μl) of the conditioned medium were electrophoresed on gelatin (1 mg/ml) substrate, 7.5% cross-linked polyacrylamide minislab gels and assayed for gelatinase activity by enzymography (18 hour incubation) as described in Chapter 3, VI-F. The fluorography of gel indicated that a major 66 kDa gelatinolytic band and a minor 59 kDa gelatinolytic band were observed.
V. Cytoskeleton/mRNA Double-Labeling System Examined with Confocal Laser Scanning Microscopy

A novel technique to label the cytoskeletal elements and intracellular mRNA in the same cell was developed in this thesis. The double-labeled microtubules and fibronectin mRNA were examined with confocal laser scanning microscopy (CLSM) (Fig. 17). An argon laser ($\lambda_{\text{max}}=488$ nm) was used to scan FITC-labeled microtubules first. Sequential 0.5 μm-optical scanning sections of human gingival fibroblasts cultured on smooth substrate were stacked to generate the image of microtubules distribution (Fig. 17a). A helium-neon laser ($\lambda_{\text{max}}=543$) was then used to scan Texas-Red-labeled fibronectin mRNA in the same cell. Sequential 0.5 μm-optical sections were stacked to generate the image of fibronectin mRNA distribution (Fig. 17b). The FITC image of microtubules and Texas-Red image of fibronectin mRNA were then overlapped by the CLSM computer program, providing the image with a relationship of microtubules and fibronectin mRNA in the cell. In this combined image (Fig. 17c), the Texas-Red signal appears yellow-orange as noted in previous reports of protein/protein double-labeling system (Gaietta, et al., 1994). Microtubules were densely packed and closely reflected the overall shape of the cell as a whole. Fibronectin mRNA was observed to accumulate around the nucleus (Fig. 17C). This finding may reflect the location of the endoplasmic reticulum in the cells. In the future, it would be of interest to investigate the mRNA distribution in cells cultured on grooved substrata, in light of altered morphology of the endoplasmic reticulum of cells cultured on grooved substrata (Brunette, 1986a).

The specificity of double-labeling was confirmed by several negative controls. The omission of antisense riboprobes or the use of sense riboprobes in the step of in situ hybridization resulted in absence of fibronectin mRNA. The omission of microtubules antibody in the step of immunolabeling resulted in absence of cytoskeletal staining (data not shown).
Fig. 17. Cytoskeletal protein and mRNA double-labeling system examined with CLSM. The specificity of cytoskeletal protein and mRNA double-labeling system was determined with confocal laser scanning microscopy (CLSM). 

a, An argon laser (λ_{max}=488 nm) was used to scan FITC-labeled microtubules first. Sequential 0.5 μm-optical scanning sections of human gingival fibroblast cultured on smooth substrate for 24 hours were stacked to generate the green image of microtubules distribution. 

b, A helium-neon laser (λ_{max}=543) was then used to scan Texas-Red-labeled fibronectin mRNA in the same cell. Sequential 0.5 μm-optical sections were stacked to generate the red image of fibronectin mRNA distribution. 

c, The FITC image of microtubules and Texas-Red image of fibronectin mRNA were then overlapped by the CLSM computer program, providing the image demonstrating a relationship of microtubules and fibronectin mRNA in the cell. In this combined image, the Texas-Red signal appears yellow-orange when overlapped with an FITC signal.
CHAPTER 5
DISCUSSION

This chapter is divided into four sections based on the experiments conducted and the significance of the findings in this thesis.

The initial purpose of the experiments in this thesis was to test the hypothesis that surface topography would affect cell behavior at the level of gene expression and protein secretion. I hypothesized that surface topography of substrata would not only alter the shape and orientation of the cells as reported previously, but also selectively regulate the expression of individual molecules secreted by the cells attached to the substrata. Fibronectin was the first molecule selected for this study, because it has been well characterized as one of the major adhesive proteins involved in cell attachment. Originally I planned to use only titanium-coated smooth and grooved substrata for the experiments. The later inclusion of tissue culture plastic in the study as a control substratum resulted in my observing a difference in gene expression and activity of the molecules between the smooth titanium and plastic surfaces, thus, indicating that surface chemistry also affected these cellular behavior.

The application of molecular biology techniques in this thesis allowed the determination of effects of surface chemistry and topography at the transcriptional and post-transcriptional levels. However, it should be emphasized that the study in this thesis was limited to determining the levels of specific mRNA and proteins, and the half-life of the mRNA. The molecules selected to study are only representatives of extracellular proteins. The inclusion of MMP-2 regulation by the surface chemistry and topography in this study, chosen as an example of a major enzyme involved in tissue remodelling, provided additional information to support the hypothesis.
The novel technique of cytoskeleton/mRNA double-labeling developed in this thesis provides a new approach to the future assessment of topographic effects on the intracellular distribution of mRNA encoding the matrix proteins, as well as the study of mRNA regulations in response to other environmental conditions of living cells.

I. Behavior of Fibroblasts On Smooth and Grooved Substrata
Fibroblasts on grooved substrata appeared significantly elongated and oriented along the grooves of the substratum, which is in agreement with the findings by Brunette (1986a). Fibroblasts in this experiment also exhibited increased height, similar to the behavior reported for epithelial cells on grooved surfaces (Hong and Brunette, 1987). Contrary to the observations on grooved titanium surface, cells on smooth titanium surface were randomly oriented and flat. Thus, micromachining provides an effective means to control cell morphology and orientation (Brunette et al., 1983; Brunette, 1986a, b; Dunn and Brown, 1986).

Some cell processes were unaffected by surface topography. The secretion of total proteins was found at about the same level in the cultures on all three different substrata, tissue culture plastic, smooth titanium and grooved titanium surfaces. No significant differences in cell numbers were noted among the cultures on these three different substrata. It should be noted that these studies were done at the population level and there may have been differences between cells in these heterogeneous populations that were not detected here. Similarly, changes in cell size, such as occurs during the cell cycle, might have some influence on the biochemical parameters studied in this thesis, but this possibility was not investigated and could be the subject of future more detailed work on the mechanism on these effects. The levels of the housekeeping gene GAPD expression were not discernibly affected by topography. These findings indicate that effects of substratum chemistry and topography on cell behaviours involve more subtle changes to the cells without necessarily altering the proliferation and general house-keeping protein metabolism of the cells.
Therefore, an approach at the molecular level to investigating the effects of surface chemistry and topography on the expression of specific genes would be required.
II. Regulation of Fibronectin Expression by Substratum Surface Topography and Chemistry

A. Grooved Surface Topography Alters Cell Shape and Fibronectin Expression

The reaction of cells to the topography of the substratum to which they are attached was one of the first phenomena observed in tissue culture (Harrison, 1914) and subsequent studies have shown that surface topography is an important factor in controlling the shape, orientation and adhesion of mammalian cells (Brunette, 1986a; Curtis and Clark, 1990; Dunn and Brown, 1986). Curtis and Clark (1990) have noted that all cells, in vivo or in vitro, excluding those that grow in suspension, must contend with some substratum topography and stressed the importance of reactions of cells to topographic cues in diverse processes in vivo including morphogenesis, cell invasion, repair, and regeneration.

In view of the effects surface topography has on cell shape and the relationship between cell shape and gene expression it is somewhat surprising that relatively little work has been done to delineate the effects of surface topography on the processes involved in the synthesis and secretion of proteins by cells cultured on surfaces with differing topographies. One protein that is of particular interest in determining the relationship between cells and their substratum is fibronectin, as fibronectin is one of the major molecules mediating cell attachment (Yamada, 1989). Moreover, fibronectin has been found to be co-distributed with fibronectin receptors and microfilament bundles at focal contacts (Burridge et al., 1988), and it would be expected that fibronectin might be affected by substrata which alter the distribution of cytoskeletal elements.

In this study grooved surfaces with precisely defined topographies were produced by micromachining, a process originally developed for the fabrication of microelectronic components. Cells cultured on these surfaces were found to have an altered cell shape being taller and more
elongated than cells cultured on smooth surfaces. The grooved surface topography also altered fibronectin mRNA level, fibronectin mRNA half-life, and the secretion and assembly of fibronectin in the extracellular matrix.

The mechanism by which gene activity responds to the surface topography remains, however, largely unclear. Previous studies have indicated that selective patterns of gene expression could be produced by altered cell shape on different substrata. For example, chondrocytes lose their differentiated phenotype of type II collagen expression in the transition from the round shape of cells in anchorage-independent cultures to the flattened morphology of anchorage-dependent cultures (West et al., 1979; von der Mark, 1980), and adopt a complex collagen phenotype consisting predominately of type I collagen (Benya and Shaffer, 1982). In my experiments, human fibroblasts also showed a selective pattern of gene expression as revealed by significantly increased levels of fibronectin mRNA level by the cells on the grooved titanium surface, although their house-keeping gene GAPD mRNA remained essentially at the same level as the cells on control smooth titanium surfaces.

Since mRNA accumulation has been found to be directly proportional both to transcription rate and half-life of the mRNA, changes in mRNA stability can affect the amount of gene product. In attempting to investigate the effect of surface topography on mRNA stability, I monitored the remaining quantity of fibronectin mRNA after mRNA synthesis was inhibited by the RNA polymerase II inhibitor (DRB) (Chodosh et al., 1989). It was found that fibronectin mRNA stability was different between cultures on smooth (~ 5 hours) and grooved titanium surfaces. There was a two-phase pattern of fibronectin mRNA half-life in the cultures on grooved surfaces: an early rapid decrease in the first half-life period followed by a prolonged increase in fibronectin mRNA stability in the second half-life period estimated to be ~ 12 hours.
The mechanism for this topography-dependent regulation and time-dependent divergence of fibronectin mRNA stability by the grooved surface may be related to the association of mRNA to the cytoskeleton. In the processing of mRNA after transcription, many mRNAs appear to be bound to the cytoskeleton prior to and during translation but afterwards are freed from the framework to become soluble and susceptible to degradation, and the rate of polymerization of the cytoskeletal components is thought to influence mRNA half-life and the rate of translation (Fulton et al., 1980; Cervera et al., 1981). In particular, studies of the spatial organization of mRNA have indicated both that microtubules are involved in the translocation of mRNA to the areas where active protein synthesis is occurring, and that actin microfilaments are important for the anchoring of the mRNA (Yisraeli et al., 1990). Therefore, it is possible that a grooved substratum may regulate fibronectin mRNA stability through topography-defined cytoskeletal organization. Since culturing cells on grooved surfaces has been shown to affect the spatial distribution of microtubules as early as 20 minutes after seeding, and of actin microfilament bundles about 40 minutes after seeding (Oakley and Brunette, 1993), the effects of grooved surface on mRNA stability could well be apparent in the early stages of cultures.

Cultures on grooved surfaces also affected the distribution of fibronectin between the medium and extracellular matrix. In my experiments, the amounts of fibronectin assembled into the extracellular matrix in the cultures on grooved surfaces were estimated to be ~2-fold higher than that on smooth surfaces, while the amounts of free fibronectin remaining in the medium increased at about the same level for all time points. The molecular mechanism underlying this altered assembly of fibronectin into the matrix is not known. Several events have been suggested to be important in the process of assembly pathway: such as the binding of fibronectin to its receptors (Hayashi and Yamada, 1983) or heparin sulfate proteoglycan (HSPG) (Wood et al., 1985), and fibronectin-fibronectin interaction (McKeown-Longo and Etzler, 1987; Allen-Hoffman and Mosher, 1987) or cross-linking (McKeown-Longo and Mosher, 1984; Peter et al., 1990).
altered distribution of fibronectin between extracellular matrix and media observed in my experiments may be regulated through any of these processes.

B. Surface Chemistry Alters Fibronectin Expression

Most studies on the biologic reactions at the interface zone between titanium implants and host tissue have focused on histological evaluations (Donely and Gillette, 1991). Of particular importance in understanding the biomaterial-tissue interface reaction is to establish a profile of the molecules directly involved in cell adhesion to the biomaterials. The study of cell responses at the molecular level to biomaterials with different surface chemical compositions may provide sensitive indicators of cell behavior at tissue-biomaterial interfaces. The profile of gene activities regulated by the surface chemistry of biomaterials could be a valuable approach for determining the molecular biocompatibility of biomaterials in addition to the conventional clinical and histological evaluations.

Fibronectin has been the topic of extensive investigation in many areas of cell biology, but has received relatively little attention for its possible role in mediating cell attachment to titanium. In this thesis, the experiments compared human gingival fibroblasts cultured on titanium with those cultured on tissue culture plastic dishes in tests of the total cell RNA yield, fibronectin mRNA level, fibronectin mRNA half-life, and the secretion and assembly of fibronectin into the extracellular matrix. The results indicated that titanium altered fibronectin gene expression at molecular levels and matrix-assembly activity as well.

The mechanism by which gene activity responds to the titanium substratum are, however, largely unclear. As proposed by McDonald (1989) and Juliano and Haskill (1993) cell differentiation and gene expression might be regulated by a signalling system involving the association of membrane-mediated receptors with extracellular adhesive proteins which subsequently alter the cytoskeletal
organization of the cells. Several lines of evidence indicated that selective patterns of gene expression can be produced by cell adhesion to different substrata such as fibronectin-coated plastic culture dishes (Eierman et al., 1989; Sporn et al., 1990). In my experiments, human fibroblasts showed a selective pattern of gene expression as revealed by a reduced level of fibronectin mRNA in the cells on titanium substratum in the early cultures, then by significantly increased level of fibronectin mRNA at late cultures on a per cell basis, while the house-keeping gene GAPD mRNA levels were essentially unchanged and total cell RNA was only slightly reduced relative to the controls on tissue culture plastic. The fibronectin synthesis in the cells on titanium, however, was remarkably higher in early cultures and declined in the late cultures. The altered fibronectin mRNA stability exhibited by cells on a titanium substratum was found to be likely the basis for this discrepancy between the amounts of fibronectin mRNA level and the amounts of secreted fibronectin.

In attempting to investigate the different effects between titanium and tissue culture plastic on mRNA stability, I also monitored the remaining amounts of fibronectin mRNA after mRNA synthesis was inhibited by RNA polymerase II inhibitor. The half-life of fibronectin mRNA was reduced by 30% in cultures on titanium (~5 hours) at 40 hours, relative to that on tissue culture plastic (~7 hours). The mechanism for this change in mRNA stability is unknown, however, the changes in polymerization of cytoskeletal components may play a role. Several studies have indicated that fibronectin may interact through its membrane receptors with the cytoskeleton which in turn, may bind mRNA and alter the mRNA stability (McDonald, 1989; Yisraeli et al., 1990).

In this study, fibronectin assembly into the extracellular matrix was increased 100-fold at 16 hours on titanium surfaces relative to tissue culture plastic, and then declined to a 28-fold increase at 40 hours and finally to a reduction of 36% at 90 hours. This large shift of fibronectin assembly activity may be related to the rate of fibronectin adsorption onto the titanium, which in turn may be
influenced by the surface energy of the titanium substratum. The titanium substratum used in my experiments was radio-frequency glow-discharge treated for 3 minutes shortly prior to use, a treatment that results in a high surface energy upon exposure to air (Baier, 1991). Glow discharge for various times from 1 to 10 minutes has been reported to significantly decrease the wetting angles of titanium surface to less than 20° (Smart et al., 1992). In contrast, the tissue culture plastic dishes are thought to be glow discharge treated by the manufacturer and have wetting angle about 28° (Grinnell, 1987). Fibronectin has been reported to desorb more readily from wettable surfaces than from less wettable ones (Grinnell, 1987), possibly on account of such factors as competition from other proteins in the medium (Grinnell, 1987; Slack et al., 1987; Haas and Culp, 1982), cellular shear forces generated at sites of cell adhesion (Brash, 1987), and increased plasminogen adsorption and activation (Kasemo, 1983). The remarkable reduction of fibronectin assembly from an initially higher amount observed in my experiments on titanium surfaces might initiate the changes in cytoskeletal organization (Slack et al., 1987) which then down regulate fibronectin mRNA stability and lead to a reduced amount of fibronectin secretion.

An interesting finding in the experiments was that changes in fibronectin gene activity and gene product distribution by titanium were time-dependent. The mechanism underlying this pattern of regulation has not been demonstrated. The known dynamic properties of titanium in the biological environment might be speculated to influence this gene activity. The chemical properties of titanium are mainly determined by the oxide layer composed primarily of TiO₂ (Kasemo, 1983; Healy and Ducheyne, 1989). Previous studies have suggested that the time-dependent growth of the TiO₂ oxide layer on the titanium surface increases logarithmically up to 7 weeks after titanium is immersed in physiologic solutions (Healy and Ducheyne, 1989). Although the oxide layer is chemically inert, it has a high dielectric constant and is negatively charged (Toth et al., 1985). The outermost surface atoms of the oxide with its unsaturated chemical bonds (Kasemo, 1988) might
indirectly initiate the reaction of attached cells through alterations in the adsorbed macromolecular carpet. Healy and Ducheyne (1992) have developed a hierarchal model describing the hydration and preferential adsorption of serum components. Their results demonstrate that titanium surfaces absorb many types of molecules that might be expected to alter cell behavior. Only one type of titanium surface was examined in this thesis and it should be pointed out that commercially available implants vary in the techniques used to prepare clean, and sterilize their surfaces. Keller and co-workers (1989, 1990) have demonstrated that significant surface alterations resulted from sterilization treatments and that surface prepared in different ways differed dramatically in their ability to support cell attachment. It seems reasonable to expect that such treatments would also affect gene activities just as this study found that fibronectin gene activity was altered by differences between tissue culture plastic and titanium surfaces.
III. Regulation of Expression of MMP-2 by Substratum Surface Topography and Chemistry

It would be expected that surgical implantation would invoke tissue remodelling responses as healing occurs around artificial devices. Among the many aspects involved in tissue remodelling, extracellular MMPs would be crucial in extracellular matrix degradation prior to the synthesis of new tissue. The remodelling of connective tissue can be controlled through the regulation of MMP expression at transcriptional and post-transcriptional levels (Overall et al., 1989a, 1991; Overall and Sodek, 1990).

Induction or stimulation of MMP expression may occur in response to signals or events that are chemical or physical in nature (Birkedal-Hansen et al., 1993). It has been found that TGF-β1 and concanavalin A can moderately increases the level of MMP-2 mRNA expression by human fibroblasts and rat bone cells (Overall et al., 1989a,b; Overall et al., 1991). Tetracyclines have direct inhibitory effects on MMP-2, through decreased transcription of MMP-2 mRNA (Uitto et al., 1994). Cell shape changes often, but not invariably, induce MMP expression, such as increased transcription of MMP-1 (collagenase) and MMP-3 (stromelysin) genes (Werb et al., 1989), probably due to the reorganization of polymerized cytoskeletal elements (Unemori and Werb, 1986). It has been reported that MMP-2 mRNA stability could be increased significantly be TGF-β (Overall et al., 1991). However, there has not been any report regarding the association between MMP-2 expression and cell-shape changes as a result of topographic effects on cell shape. The effect of grooved substrata, as well as the effect of substratum surface chemistry, on MMP-2 expression was, therefore, investigated in this thesis.

My experiments indicated that both substratum surface chemistry and topography could alter the MMP-2 mRNA levels, mRNA stability and secretion in normal human fibroblasts. Since there
was no discernable cell morphologic differences between the cultures on tissue culture plastic and smooth titanium but there were significant differences in cell shape and orientation between the cells cultured on smooth and grooved titanium surfaces, more than one mechanism is suggested in the substratum regulation of MMP-2. One mechanism may involve gross cell shape changes while the other may involve more subtle changes to cell attachment sites without altering cell shape. An element common to both mechanisms may be mediated by perturbations to cytoskeletal organization through the altered distribution and configuration of focal contacts, the particular regions of cell attachment to the substrata (reviewed by Burridge et al., 1988; Juliano and Haskill, 1993). The half-life of MMP-2 mRNA reported here is significantly greater than that reported by Overall et al. (1991). This difference could be related to the low (0.2% serum concentration used by Overall et al., in which cell proliferation did not occur. The difference in mRNA half-life on the grooved versus the smooth titanium surfaces may be related to the altered distribution of cytoskeletal elements on these surfaces (Oakley and Brunette, 1993). Similarly, the temporal changes in MMP-2 mRNA levels that differed between smooth and grooved surfaces may be related to cell shape and cytoskeletal distribution altering gene activity (Ben-Ze’ev, 1987). For two other major MMPs, collagenase (MMP-1) and stromelysin (MMP-3), modulation of gene expression associated with cell shape change has been shown to be effected by signal transduction through the fibronectin receptor (Werb et al., 1989).

As it is expected that an implant would invoke tissue remodelling responses as healing occurs around the devices, the substratum chemistry- and topography-dependent regulation of MMP-2 gene expression as observed in this study indicate the possibility that biomaterial surface properties influence tissue remodelling at the cell-substratum interface. The actual effects of such regulation on MMP-2 at the cell-substratum interface may not be precisely defined by the expression and activity profile of a single matrix metaloproteinase, because the degradation of extracellular matrix involves the activities of multiple proteolytic enzymes, enzyme activators and inhibitors (reviewed
by Birkedal-Hansen, 1993). For example, in this thesis the gelatinolytic activity of MMP-2 in the conditioned medium measured by enzymographic assay was not significantly altered by surface chemistry and topography of the substrata on which the cells attached, although that the MMP-2 mRNA expression and secretion were moderately altered. The discrepancy between the altered amounts of expressed MMP-2 and their final activity could be associated with the changes in expression and activity of MMP-2 inhibitor and/or other regulators induced coincidentally by the substrata. Nevertheless, the results from this study demonstrated that the regulation of molecules responsible for tissue remodelling at tissue-biomaterial interface is evident at the molecular level.

The mechanism by which biomaterials modulate MMP-2 gene expression deserves further study. It may well be complex. The findings from this study showed that the substratum-dependent regulation occurred to some extent at the different stages of MMP-2 expression, with altered levels of mRNA, mRNA stability, and secreted enzymes. Fibroblasts do not bind directly to the substratum material but rather the cells attach to substratum through a carpet of macromolecules between the cell and the substratum (Healy and Ducheyne, 1992). Topography may alter the organization of this intervening layer, or alternately change the pattern of cell contacts and the distribution of the attachment receptors. These receptors can affect signal transduction and thus cell behavior (Hynes, 1992; Horwitz and Thiert, 1994). In this thesis, I concentrated on two molecules that would likely be of importance in tissue-implant interactions. Fibronectin is a major protein mediating cell attachment to surfaces. MMP-2, on the other hand, is involved in collagen remodelling. Its role may be of particular importance in the modification of the connective tissue capsule that forms around many types of implant (Chehroudi et al., 1992). The results in this thesis demonstrated that surface topography and chemistry can alter fibronectin and MMP-2 expression in different ways. Moreover, fibronectin can be degraded by MMP-2 in consort with MMP-3 (stromolysin) (Werb et al., 1989), so there is a functional interaction between these molecules. Thus, an alteration in surface topography (or chemistry) that has desired effects on one
process, for example, increased cell attachment because of increased level of fibronectin may lead to unanticipated effects on another process, such as the remodelling of the connective tissue capsule on account of altered levels of MMP-2. Therefore, it is unlikely that the empirical approach of simply inserting implants and observing outcomes is not likely to be a good strategy. I believe that a more effective approach would be to investigate systematically alterations in surface topography and chemistry using a broad range of molecular techniques and use the resulting information to design biomaterials that elicit desired tissue remodelling in the implant healing process.
IV. Cytoskeleton/mRNA Double-Labeling System

In order to investigate the effect of substratum surface topography on fibronectin mRNA distribution in future studies, a novel cytoskeleton/mRNA double-labeling system was developed for this experiment to protect both antigenicity of the targeted cytoskeletal proteins for antibody labeling and the integrity of targeting mRNA for riboprobe labeling in the same cell.

To achieve successful labeling, several technical steps were modified in the procedure. A cytoskeleton-stabilization (CS) buffer (Opas, 1989; Hollenbeck, 1989) was used in the protocol for initial washing of samples, which produced superior results to initial washing in PBS, particularly in preservation of microtubules. After cytoskeleton labeling by monoclonal antibodies the samples were fixed for the second time with paraformaldehyde to preserve the cell morphology and antibody binding prior to the harsh conditions involved in the in situ hybridization procedures. A chemical agent used for denaturing the covalent bonds, DTT, was removed from the standard formula of in situ hybridization cocktail in this double-labeling system, because adding DTT was found to wipe out all antibody-labeled signals. The double-labeling for both microtubules and mRNA in human fibroblasts has not been reported previously. The protocol established in this thesis may provide a powerful means to study the regulation of mRNA translocation by cytoskeletal organization and other studies of cell biology.
CHAPTER 6

CONCLUSIONS and FUTURE DIRECTIONS

I. Conclusions

At the outset of this thesis, I wished to test my hypothesis that surface chemical composition and topography of the biomaterials could serve as signals to the attached cells and to regulate at molecular level the specific molecules which are responsible for the phenotypic behavior of the cells at the biomaterials-tissue interface. In the study, smooth and micromachined grooved surfaces were used to study the topographic effects on the cellular behaviors. Smooth titanium surface compared with smooth tissue culture plastic to determine the effects of differed surface chemical compositions. A glycoprotein involved in cell attachment, fibronectin, and a matrix metalloproteinase, MMP-2, were selected as two molecules for the study. The regulation of these molecules in response to surface chemistry and topography was assessed in a dissected approach to their mRNA level, mRNA stability, mRNA distribution, end-product secretion and activity.

Specific conclusions of the experiments in this thesis have been considered at the cell population level and are limited to human gingival fibroblasts. The conclusions are summarized below:

1. Cell proliferation, house-keeping gene GAPD expression and total secreted protein levels were essentially unchanged in the fibroblasts cultured on different types of biomaterials tested in this thesis (titanium vs. tissue culture plastic), and on the same type of biomaterials but with different surface topographies tested in this thesis (smooth titanium vs. grooved titanium surfaces).

2. The grooved surface significantly altered cell shape, height and orientation, showing cellular elongation and directed migration along the grooves of the substrata.

3. Surface topography and chemistry of the substrata specifically regulated the fibronectin
expression at several levels of the fibronectin pathway. Fibronectin mRNA level, stability, secreted protein level and matrix-assembly activity were significantly increased in the cells on grooved titanium surface in comparison with the cells on the smooth titanium surface. Compared to cells on smooth tissue culture plastic, the cells on smooth titanium surface showed altered levels of fibronectin mRNA, secretion and matrix-assembly activity, as well as reduced mRNA stability.

4. Surface topography and chemistry of the substrata altered MMP-2 expression as well. The study of MMP-2 was limited to its mRNA levels and the amounts of enzyme secreted into the culture medium. The results indicated that substratum topography-induced changes in cell shape and substratum surface chemistry could specifically alter the MMP-2 mRNA levels and mRNA stability.

5. A novel technique was developed to doubly label microtubules and fibronectin mRNA. The technical modifications ensured protection of the antigenicity of cytoskeleton and the integrity of mRNA while both elements were appropriately labeled.

6. The findings of this thesis suggested that the effects of surface topography on attached cells can be much more than a simple mechanical interaction because the synthesis and secretion of specific proteins, as well as their distribution and function can be altered. It also indicated that the effect of chemical composition of biomaterials may not be limited to their cytotoxicity because the tissue may respond the subtle difference in chemical composition by changes in phenotypic behaviors rather than simply death. The results also demonstrated that the physical and chemical properties of the biomaterials could sensitively and selectively regulate specific molecules at several molecular levels. Thus, the techniques of molecular biology provide a sensitive and specific approach to the assessment of cell responses to biomaterials.
II. Future Directions

A. Determine the Effects of Substratum Topography on the Intracellular mRNA Distribution

The relationship between the distribution of mRNAs coding for extracellular matrix proteins and the organization of cytoskeletal elements in human fibroblasts has not been reported. The previous studies have indicated the associations of mRNA distribution with the organizations of microtubules and actin filaments (reviewed by St. Johnston, 1995; Wilhelm and Vale, 1993). Since the substratum surface topography could significantly alter the organization of cytoskeletal elements, such as microtubules and actin filaments (Oakley and Brunette, 1993), it would be interesting to investigate the effects of surface topography on cytoskeletal organization and the consequent distribution of mRNAs coding for the proteins responsible for the cell attachment. As the technique for double-labeling the fibronectin mRNA and microtubules in the same cell has been established in this thesis, future investigations in understanding the effects of substratum surface topography on fibronectin mRNA distribution may be practically pursued. In addition, as many chemical agents including colchicine and cytochalasins can perturb the microtubule (Dustin, 1984; Luduena and Roach, 1991) or actin (Yahara et al., 1982; Cooper, 1987) assembly, the study could be further expanded to examine the effects of substratum topography on the distribution of mRNA in the microtubule- or/and actin filament-depleted cells cultured on grooved surfaces and treated with corresponding chemical agents. My hypothesis is that the grooved surface topography may play an important role in regulating the intracellular distribution of mRNA. The approval of this hypothesis would not only provide the insight to the mechanism of molecular biocompatibility of implant materials, but also add some new information about the mechanisms of mRNA translocation.
B. Establish the Molecular Profile of Cellular Behaviors in Response to Implant Biomaterials

Based on the hypothesis tested in my thesis that the chemical and topographic determinants of biomaterials may effectively regulate the expression and activity of specific molecules in the cells attached, future studies may be directed to a concept of molecular biocompatibility of the materials used for dental and medical implant devices. In some instances, more detailed knowledge may be required to optimize device function. In this thesis, for example, I studied fibronectin using a method that detected all forms of this molecule and its messengers. However, fibronectin occurs in many forms in vivo as a result of mRNA alternative splicing and these different forms may have different functions. In the future, it could be of interest to determine if surface topography or chemistry change the distribution of mRNA types. Such studies would be an example of obtaining the profile of molecular families in response to well defined surfaces. The use of various types of implant materials to produce desired molecular profiles may prove to be a useful strategy for implant material selection and design.

Successful dental implants require early contact bone formation right onto the implant surface in order to sustain the maximal loading force in physiological condition. Both titanium and hydroxyapatite (HA) have been commonly used for endosseous implants (Branemark et al., 1974; Cook et al., 1987; Burr et al., 1993). However, how the chemistry and topography of these materials control the osteogenic response remains largely unknown. To date, in vivo observations in patients or animals have been the primary approach for screening the biocompatibility of implant materials. The findings from those in vivo studies have had limited success in elucidating the mechanism of interface reactions induced by biomaterials and, therefore, are insufficient to be used as a biologic guide for the development of osteoinductive materials by modifying their chemical compositions and topographies. If we can take an approach similar to that used in my thesis, the mechanism underlying the osteogenic activity in response to different types of biomaterials could
be dissected at the molecular level. Complemented by an increased knowledge of molecular mechanisms that are associated with and regulate expression of genes encoding phenotypic components of bone, and those that may control the progressive development and maturation of the bone cell phenotype, we can investigate osteogenic cell cultures on different types of biomaterials for the expression of major molecules involved in de novo bone formation, such as fibronectin (Reddi, 1984), osteogenin (Reddi et al., 1989), type I collagen (Stein and Lian, 1993), bone sialoprotein (Nagata, et al., 1991), osteopontin, osteocalcin (Owen, et al., 1990), and alkaline phosphatase (Stein and Lian, 1993).

I predict that the chemical and topographic signals of biomaterials may effectively regulate the activity of these molecules responsible for osteogenic cell attachment, differentiation and mineralization at the transcriptional and posttranscriptional levels. Therefore, the phenotypic behaviors of osteogenesis at the implant interface might be controlled very efficiently at the gene expression level by modifying material surface chemistry and/or topography.

Future study in this direction can be defined into several stages. In the first stage, one could use chemically and topographically well characterized standard titanium and HA-coated materials and molecular biological techniques, such as those used in my thesis, to establish an in vitro baseline profile of molecular osteogenesis with the parameters of each key molecule involved in bone formation at the interface. Using the chemical and topographic characterizations of the substrata as well as the profile of cell behavior at molecular level, the biological effects of different materials on osteogenesis and the mechanism of these effects can be precisely determined. In the second stage, if the pure titanium or HA substrata are determined to be incapable of stimulating osteogenic gene activities at all, or only up-regulate some of the osteogenic genes, or even suppress some others, we can attempt to actively induce these specific osteogenic genes by manipulating the material surface signals through the modification of surface chemistry and topography. For example, for
HA we could alter the chemical composition including Ca/P ratio and crystalline phase of HA, and the size of surface porosity. The response of gene activity to these modified surface determinants will then be re-screened for each osteogenic molecules and at each biological regulation level. A similar approach would be undertaken with titanium surfaces by altering their surface topography. In this way, the types of materials most favorable to osteogenesis can be selected. In the final stage, a group of prospective \textit{in vitro} osteoinductive materials with or without HA-coating or recombinant protein/HA coatings will be implanted in animal models for screening of their \textit{in vivo} effects on the bone formation. The long-term goal of this research would be the development of osteoinductive materials for dental implants.
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