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Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts

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SUMMARY

The regulation of cell shape, fibronectin mRNA level, secretion and assembly by substratum surface topography was investigated in early passage human gingival fibroblasts cultured on titanium-coated smooth or V-shaped grooved substrata produced by micromachining. Cells on grooved surfaces were significantly elongated and orientated along the grooves of the substratum, while cell height, measured using confocal scanning laser microscopy, was ~1.5-fold greater than that of cells on smooth surfaces. Northern hybridization analysis revealed that on a per cell basis the grooved surface increased the amounts of fibronectin mRNA/cell ~3.5-fold at 16 hours, ~1.9-fold at 40 hours and ~2.2-fold at 90 hours, while the mRNA levels of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPD) were constant. The amounts of secreted fibronectin on the grooved surface were increased ~2-fold for all time points. The stability of fibronectin mRNA was also altered by substratum surface topography. The half-life of fibronectin mRNA on smooth surfaces was estimated to be ~5 hours, but on the grooved surfaces the half-life of fibronectin mRNA showed a two-phase response: a rapid 60% reduction in the first half-life ($t_{\frac{1}{2}}$ ~2 hours) and a 2.4-fold increase in the second half-life ($t_{\frac{1}{2}}$ ~12 hours) relative to that observed on the smooth surface. The GAPD mRNA half-lives were essentially unaffected by the surface topography of the substrata. The grooved surface was also found to alter the amount of fibronectin assembled into the extracellular matrix, producing a ~2-fold increase in the cultures at all time points. It thus appears that substratum surface topography alters cell shape and modulates fibronectin at the transcriptional and post-transcriptional levels, as well as the amount of fibronectin assembled into extracellular matrix. Micromachining, which has the ability to precisely control surface topography over a wide range of dimensions and shapes, appears to be a useful technique in investigating the relationship between cell shape and function.

Key words: surface topography, cell shape, fibronectin, implant material

INTRODUCTION

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. Little is known about the mechanism whereby surface topography exerts its effects, although several studies have focused on the role of cytoskeletal elements such as microfilament bundles (Dunn and Heath, 1976; Ben-Ze'ev, 1986), focal contacts (Ohara and Buck, 1979) and microtubules (Oakley and Brunette, 1993), as all these structures are observed to align with topographic features such as grooves.

Cell shape can be markedly influenced by surface topography of substrata (Oakley and Brunette, 1993; Dunn and Brown 1986; Curtis and Clark, 1990), as illustrated by cells becoming polarized and elongated in the direction of the grooves on grooved substrata. Cell shape can also regulate cell growth (Watt, 1987; Folkman and Moscona, 1978), cytoskeleton gene expression (Ben Ze'ev, 1984), collagenase and stromelysin gene expression (Werb et al., 1989), extracellular matrix metabolism (Watt, 1986; McDonald, 1989) and cell differentiation. Ben Ze'ev (1984), Watt (1987) and Watt et al. (1988) have suggested that the architectural features of eukaryotic cells, which determine cell shape and contacts, are important for cell growth, gene expression and differentiation.

In view of the effects that 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 (FN), as FN is one of the major molecules mediating cell attachment (Yamada, 1989). Moreover, FN has been found to be co-distributed with FN receptors and microfilament bundles at focal contacts (Burridge et al., 1988), and it would be expected that FN 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 FN mRNA level, FN mRNA half-life, and the secretion and assembly of FN in the extracellular matrix.

MATERIALS AND METHODS

Cell culture

Fibroblasts from outgrowths of adult human gingiva were isolated and subcultured as described by Brunette et al. (1976). In brief, cells or explants were cultured on tissue culture plastic dishes (Falcon, Cockeysville, MD) in α-minimal essential medium (MEM) supplemented with antibiotics and 15% (v/v) fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Fibroblasts were removed and suspended using a trypsin solution (0.25% (w/v) trypsin (Worthington TPCK, McLean, VA) and 0.1% (w/v) glucose dissolved in citrate-saline, pH 7.8), and seeded at a cell population density of 1×10^4 cells/cm^2.

Grooved culture substrata were prepared by micromachining using the methods described by Camporese et al. (1981), which were originally developed for the fabrication of high quality photomasks for solar cells and subsequently applied to cell culture (Brunette, 1983, 1986a,b). In these experiments the etching process produced silicon wafers with V-shaped grooves 3 μm in depth and 6-10 μm repeat spacing. Smooth silicon wafers were also fabricated for control studies. The grooved and smooth silicon wafers were then evaporatively coated with 50 nm commercially pure titanium, a material widely used for dental and orthopaedic implants, cleaned by ultrasonication for 20 minutes in 7× Cleaning Solution (Flow Lab., McLean, VA), washed 20 times with distilled water, and finally treated with radio frequency glow discharge to clean and sterilize the surfaces (Baier et al., 1988; Jansen et al., 1991) before culture.

In each of three experiments, fibroblasts (6-10th passage) were plated on 55 mm diameter silicon wafers coated with titanium. The smooth and grooved wafers were placed in plastic tissue culture dishes in MEM containing 5% (v/v) fetal bovine serum (FBS), a concentration of serum determined to be optimal for fibronectin expression in vitro (see below), at a cell density of 1.2×10^4 cells/cm^2. Before plating, cell subcultures were cultured for 48 hours in flasks in MEM with 5% (v/v) FBS to adapt them to the conditions that would be used in the subsequent experiments. Cells plated on grooved and smooth titanium surfaces were incubated for 16 hours (~60% confluent), 40 hours (~90% confluent) and 90 hours (confluent). Cell numbers were determined by electronic cell counting (Coulter Electronics, Inc., FL).

Effect of serum concentration

The influence of serum concentration in the culture medium on the expression of the fibronectin gene was examined before the experiments on the effects of surface topography were performed. Human gingival fibroblasts (6-10th passage) were cultured in triplicate for 24 hours or 48 hours on tissue culture dishes at an initial density of 1.3×10^4 cells/cm^2 in MEM with various concentrations of FBS (0.3%, 5% or 15% (v/v)). Cells were equilibrated for 24 hours in flasks with a corresponding concentration of FBS 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 below.

Electron and confocal scanning laser 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, NY), and examined with a Cambridge Stereoscan (Cambridge, UK). In addition, 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 medium of 50% (v/v) glycerol in PBS, and kept at 4°C until being measured. 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.

Isotopic labeling, protein and fibronectin analysis

Secreted proteins were labeled by continuous incubation with 10 μCi/ml of L-[^{35}S]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. Labeled matrix proteins bound to the cell layers were extracted using the method of Yamada and Akiyama (1984). In brief, cell monolayers were rinsed four times with 3 ml Hanks' 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 extract was centrifuged for 15 minutes at 25,000 g to remove cells and other particles. The cell pellets were fixed, sectioned and viewed under a transmission electron microscope to confirm the integrity of cell membranes. Total secreted proteins were estimated at 16, 40 and 90 hours by liquid scintillation counting of the [^{35}S]methionine-labeled media proteins and extractable matrix 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. FN was identified in the secreted proteins after purification by miniaffinity columns of gelatin-Sepharose (Pharmacia, Uppsala) as described by Overall et al. (1989). Material 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 FN was recovered after elution with 4× electrophoresis sample buffer (8.0 M urea, 80 mg/ml SDS, in Tris buffer, pH 6.8). Aliquots (10 μl) of the purified media

and matrix FN were analyzed by SDS-polyacrylamide minislab gels as described below. SDS-polyacrylamide gel electrophoresis was performed 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 minutes. FN bands on fluorographs were quantitated using an image digitized 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 data from scanning media and extractable matrix FN were analyzed separately. 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).

Total cellular fibronectin extraction and immunoblotting analysis

Total cell layer bound FN was also extracted with SDS plus DTT in a separate experiment according to McKeown-Longo and Mosher (1983), modified as described below. Cultures in duplicate on either smooth or grooved surfaces were washed 3 times in Hanks' balanced salt solution (HBSS), and cell layers were extracted in 4% SDS in 0.02 M Tris buffer (pH 8) containing 65 mM DTT, 2 mM PMSF, 2 mM EDTA, 2 mM *N*-ethylmaleimide for 30 minutes at room temperature with gentle rotation at 1 rpm. The extract was centrifuged for 15 minutes at 25,000 ***g***. Aliquots (10 μl) of the extraction were fractioned by discontinuous SDS-PAGE with 8% acrylamide as described above. After electrophoretic transfer to Immobilon filters, the blots were treated with blocking buffer containing 2% bovine serum albumin (Sigma) and 0.25% Tween-20 in PBS, pH 7.4, for 1 hour and then incubated with anti-human FN mouse antiserum (1:3,000, Boehringer Mannheim) at 4°C for 16 hours, followed by incubation with anti-mouse IgG conjugated to biotin (1:5,000, Dako, Carpintaria, CA). Bound antibody was visualized with streptavidin-biotinylated horseradish peroxidase complex (Amersham, IL) and enhanced chemiluminescence (ECL) method (Amersham, IL). Prestained protein standards (14-200 kDa) were from Gibco BRL Co. (Gathebury, MA). The FN bands of ~250 kDa in size were scanned by an image digitized optical scanner (Apple Computer Co., Cupatino, CA.) and analyzed with computer software (Image 1.4, NIH).

Total RNA extraction and mRNA half-life determination

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 on either smooth or grooved surfaces 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-isoamylalcohol (49:1, v/v) 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, vacum 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. To estimate the half-life of fibronectin mRNA, cells were cultured in triplicate as described above on either grooved or smooth titanium surfaces, and after 40 hour incubation (~90% confluent) 60 μM 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, Sigma), a specific RNA polymerase II inhibitor, was added to the cultures (Overall et al., 1991). The total RNA was then extracted as described above from triplicate cultures at each time point (0, 1.5, 3, 6, 16, and 24 hours) and analyzed after slot-blotting, as well as northern blotting, and hybridization with FN and glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA probes (see below). The data were collected only up to 24 hours, because the inhibitor may also reduce the expression of house-keeping genes and proteins involved in mRNA stability.

Northern hybridization and slot-blot analysis

Aliquots of extracted cell RNA (5 μg) were prepared in a 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). In the mRNA half-life experiments, aliquots (5 μg) of the RNA sample were spotted onto Hybond-N nylon membrane using a HSI-PR 600 slot-blot apparatus (Hoefer Sci. Inc. CA), while aliquots (5 μg) of the same sample RNA were also analyzed by northern hybridization to monitor the specificity of the cDNA probes used in the slot hybridization. Following 3 minutes of UV cross-linkage, the blots were prehybridized at 62°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}P]dCTP-labeled FN or GAPD cDNA probes at a concentration of ~3.0×10^7 cpm/300 cm^2 in 15 ml of prehybridization solution. After hybridization, the blots were washed in 1× SSC (standard saline citrate), 5 mg/ml SDS at room temperature for 10 minutes, followed by a change of the same solution at 62°C for 30 minutes, and then for 20 minutes with two changes of 0.1× SSC, 0.5 mg/ml SDS at 62°C. Blots were autoradiographed at −70°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 bands of ~7.9 kb in size and GAPD mRNA bands of ~1.2 kb in size were then scanned by an image digitized optical scanner (Apple Computer Co., Cupertino, CA.) and analyzed by computer software (Image 1.4, NIH). The data were normalized for the amount of RNA loaded as determined from photographs of the ethidium bromide-stained bands. The specificity of slot-blot hybridization for the mRNA half-life determination was confirmed by northern hybridization. The data were adjusted for the RNA yield from each corresponding RNA preparation to give the total yield of specific FN mRNA on a per cell basis. The data from an experiment in triplicate were expressed as the mean ± s.d.

Preparation of cDNA probes

The freeze-dried *E. coli* carrying recombinant plasmid pBR322 with the insertion of 1.3 kb *Eco*RI 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 the American Type and Culture Collection (ATCC). To enhance the yield of recombinant pBR322, 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 with 0.2 M NaOH, 1% (w/v) SDS. Phenol-chloroform (1:1, v/v) extraction and ethanol precipitation were used to purify supercoiled plasmids containing FN or GAPD 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 (BRL) with [^{32}P]dCTP (>3,000 Ci/mmol, Amersham Corp.) to a specific activity of ~1.8×10^9 cpm/μg cDNA prior to hybridization.

RESULTS

Effects of serum on cell growth and fibronectin expression

To select a condition under which the effects of surface composition and topography could be examined most sensitively, the influence of different concentrations of fetal bovine serum (FBS) on cell growth and FN gene expression was studied. Cell proliferation, total RNA yield and FN 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 (Fig. 1). Cell numbers, as determined by Coulter counting, were essentially unaltered ($1.4\text{-}1.6\times10^4$ cells/cm^2) for all FBS concentrations at 24 hours, while at 48 hours cell numbers in both the 5% and 15% FBS cultures were increased (Fig. 1). The yield of total RNA increased slightly in both 5% and 15% FBS cultures over that in 0.3% FBS. FN mRNA level as determined by northern analysis (Fig. 1) revealed that 0.3% FBS resulted in a delayed expression of FN mRNA, 15% FBS resulted in a low and progressively decreasing level of FN mRNA, whereas 5% FBS produced the most abundant FN mRNA and was, therefore, selected as the optimal concentration in culture medium for further experiments.

Effects of grooved surface on cell shape and height

The orientation and shape of cultured cells were determined after 16, 40 and 90 hours growth on smooth and micromachined grooved titanium-coated surfaces. 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. 3).

Effects of grooved surface topography on cell number, total RNA and total secreted proteins

Cell number, total RNA yield and total secreted proteins were determined in fibroblast cultures on grooved and smooth titanium surfaces after 16, 40 and 90 hours (Fig. 4). 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. No significant difference in cell number on a per area basis was noted among the cultures on these two different surfaces (~1.2×10^4, ~2.2×10^4 and ~5.3×10^4 cells/cm^2 at 16, 24 and 90 hour 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.

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), we measured the amounts of extractable extracellular matrix proteins from urea extraction (matrix proteins), as well as the amounts of proteins in the con-

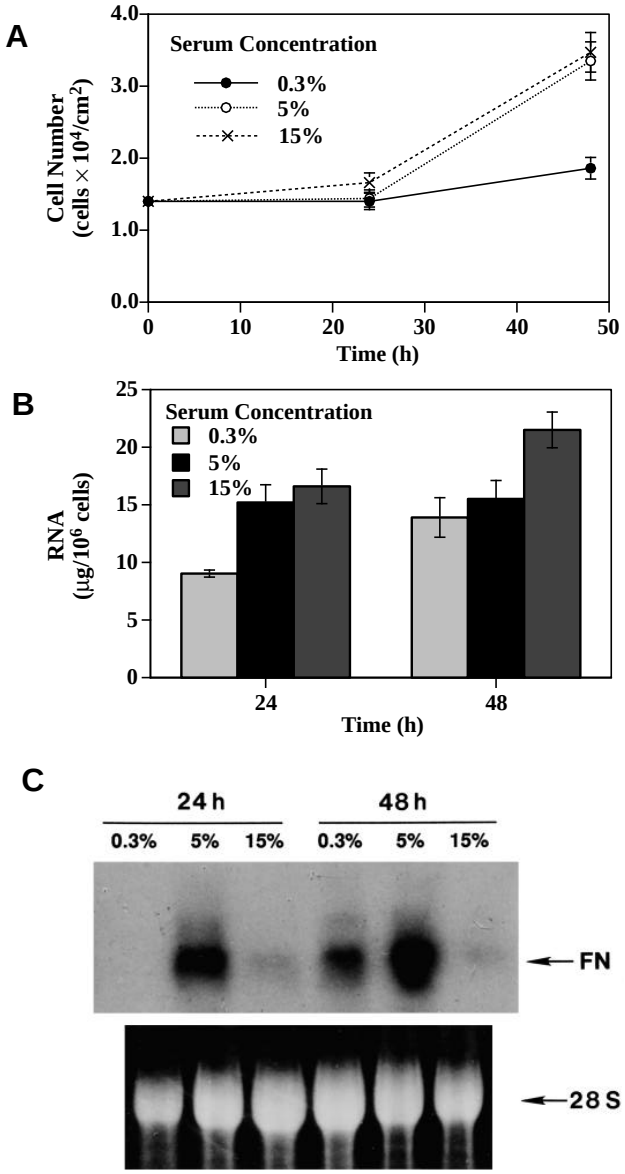


Fig. 1. The effects of serum concentration on cell growth, cellular RNA yield and fibronectin gene expression. Human gingival fibroblasts (6-10th passage) were cultured at a density of ~1.3×10^4 cells/cm^2 on plastic tissue culture dishes in 0.3%, 5% or 15% (v/v) of fetal bovine serum (FBS). Cells were equilibrated by a 24 hour incubation in medium with the three different FBS concentrations described above before plating onto the culture dishes. (A) Cell numbers were determined by electronic counting. (B) The total RNA was extracted at the indicated times, and the RNA concentration was determined from spectroscopic analysis. (C) 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 under Materials and Methods. Data from one time course experiment are presented as the mean ± s.d., n=3.

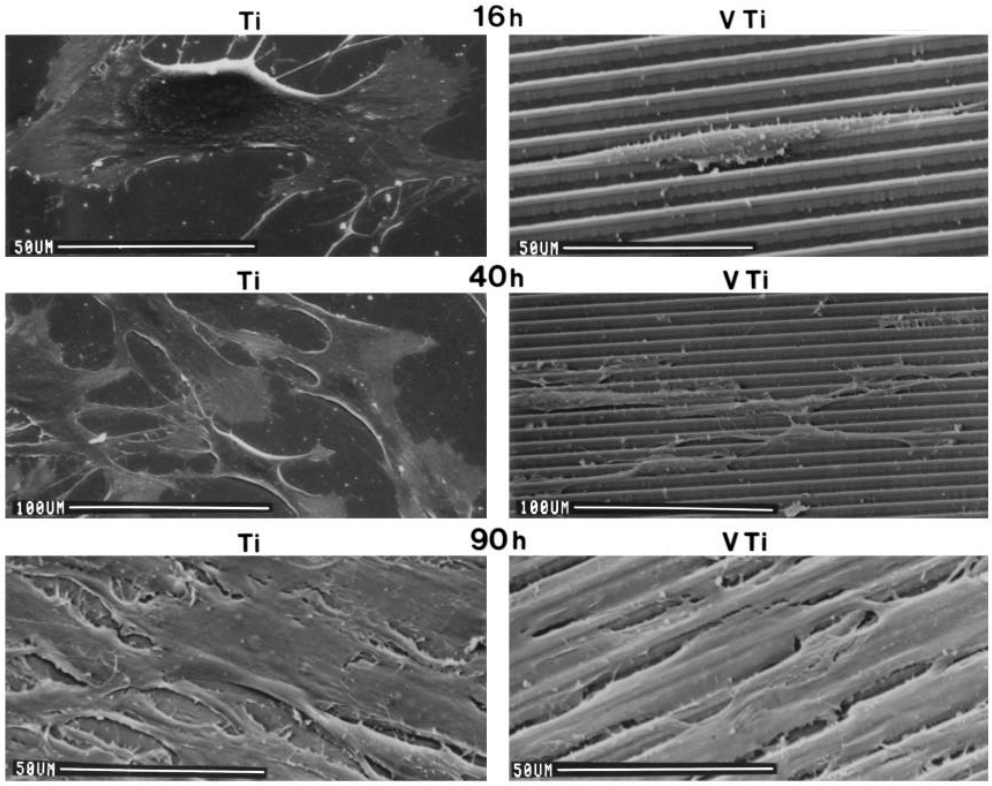


Fig. 2. Effects of a grooved surface on cell morphology. Human gingival fibroblasts were cultured (~1.2×10^4 cells/cm^2) on titanium-coated grooved surfaces of 3 μm in depth (right), and titanium-coated smooth surfaces as a control (left), in culture medium with 5% (v/v) fetal bovine serum, for the indicated times. Cells were processed for scanning electron microscopy by fixation in 2.5% (w/v) glutaraldehyde for 1 hour and in 2% (w/v) OsO_4 in PBS, pH 7.2, at 4°C for 1 hour. Samples were DC-sputtered with 15-20 nm gold and examined with a Cambridge Stereoscan.

ditioned medium (media proteins) (Fig. 4). The amounts of [^{35}S]methionine incorporation in medium collected from the grooved surface did not differ from that from the smooth surface at 16 hours, but at 40 and 90 hours the amounts in cultures on grooved surfaces were slightly reduced. Labeled proteins extracted by urea from extracellular matrix of the cultures on the grooved surface 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 surface. The total amounts of 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 these two different surfaces.

Effects of grooved surface topography on fibronectin mRNA levels

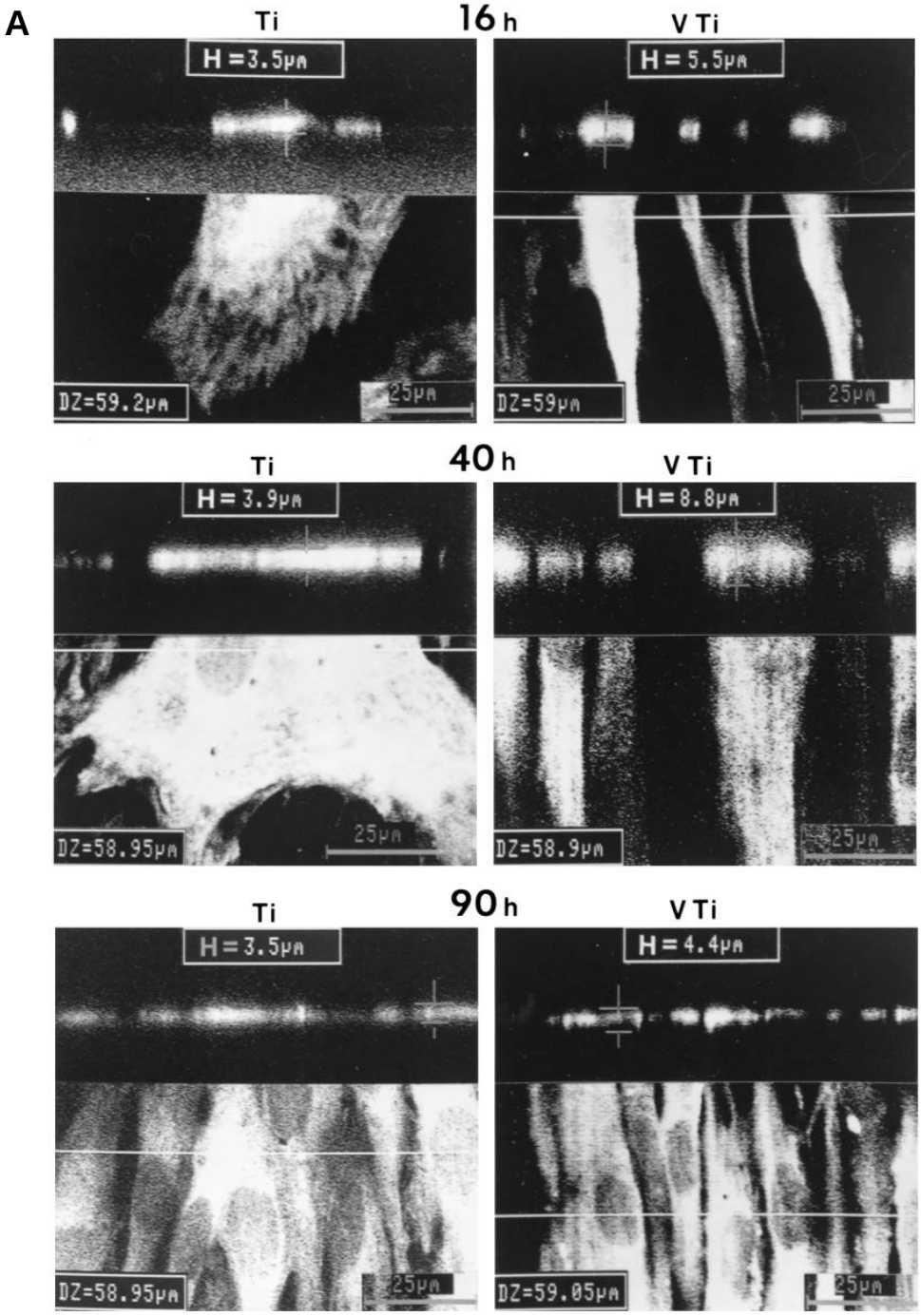
Although the levels of total cell RNA and total protein secretion on a per cell basis did not change appreciably between grooved and smooth surfaces (Fig. 4), the level of FN mRNA was notably increased in cells cultured on the grooved surface. The grooved surface increased the amounts of FN mRNA ~3.5-fold 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. 5).

Effects of grooved surface topography on fibronectin levels

To investigate the effect that substratum surface topography has on FN levels, the FN was affinity purified using gelatin-Sepharose from equal samples of conditioned medium (media FN) or cell layer extract (matrix FN). The purified FN 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. 6). The amount of media FN from the cultures on the grooved surface showed a slight increase for all time intervals over the amount observed in cultures on the smooth surface (Fig. 6). The amount of matrix FN 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 in comparison with cells cultured on the smooth surface (Fig. 6). However, the amounts of cellular bound FN extracted by SDS and DTT showed ~2-fold increases in cultures on the grooved surfaces at each time point as indicated in western blot results (Fig. 7). Nevertheless, both approaches indicated that the total secretion of FN was significantly increased in cultures on grooved surfaces, and that the increase in amounts of FN was associated with the matrix FN rather than the media FN. Total secreted FN, determined by adding the amounts of media FN and SDS-extracted cellular FN at each time point on a per cell basis, showed greater increases (~2-fold) at each time point for the cultures on grooved surfaces.

Fibronectin mRNA stability

As the level of protein synthesis is known to be affected partially by the stability of mRNA (Cleveland, 1989), we further investigated the effect of surface topography on the stability of FN mRNA. Following the addition of the RNA polymerase inhibitor, DRB, to block transcription, the decrease in the levels of FN mRNA and GAPD mRNA were measured over time (Fig. 8). In cultures on smooth titanium surfaces, the



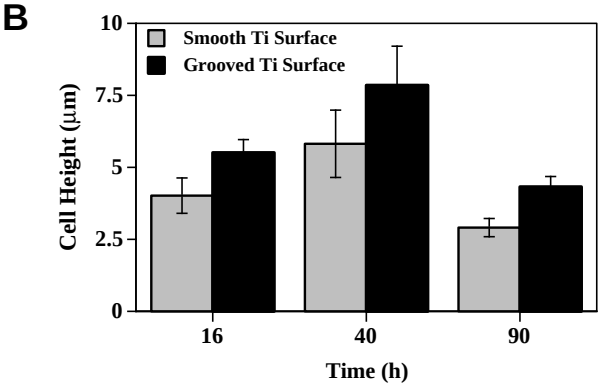


Fig. 3. The effects of a grooved surface on the height of cultured cells using confocal scanning laser microscopy. Human gingival fibroblasts were cultured (~1.2×10^4 cells/cm^2) on grooved surfaces and control smooth surfaces as described under Materials and Methods. Cells were processed for confocal scanning laser microscopy (CSLM) by fixation in 4% (w/v) formaldehyde in 0.1 M PBS, pH 7.2, at 4°C for 1 hour, stained with Gill No. 2 haematoxylin for 10 minutes, and covered with a coverslip 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 10 CSLM software so that cell height could be measured. The lower part of the micrographs shows the X-Z 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. (B) Fifty cells on grooved and smooth surfaces were measured and data are 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).

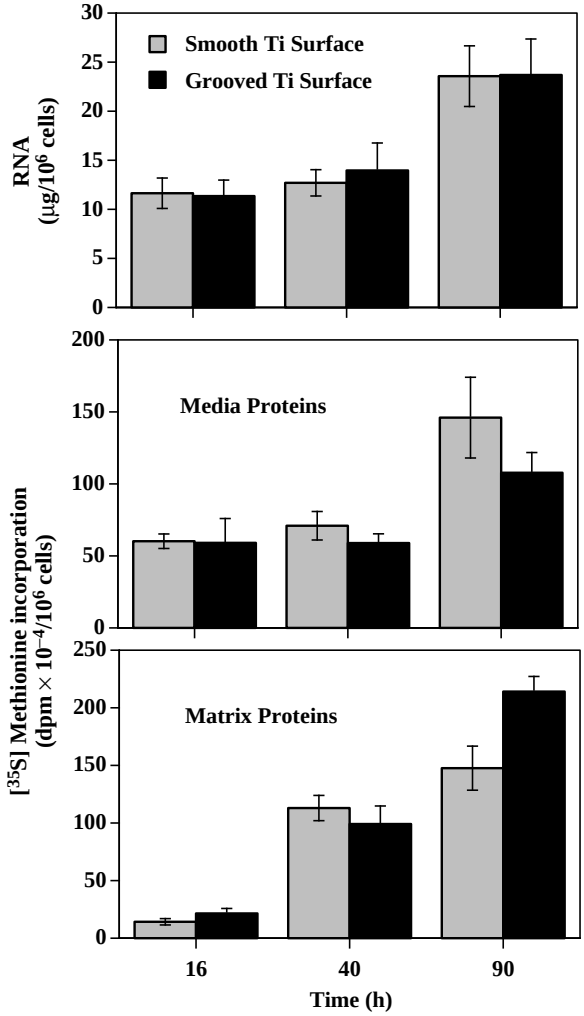


Fig. 4. Quantitation of cellular RNA yields and secreted protein synthesis in response to the grooved surface of substratum. The total RNA from human gingival fibroblasts (6-10th passage, plating density of ~1.2×10^4 cells/cm^2, n=3) was extracted at the indicated times, and the RNA concentrations were determined from spectroscopic analysis as described in Materials and Methods. [^{35}S]methionine incorporation into secreted proteins in conditioned medium (Media Proteins) and proteins extracted by urea from the extracellular matrix (Matrix Proteins) at the indicated times was determined by liquid scintillation counting after exhaustive dialysis as described in Materials and Methods. Data from one time course experiment are presented as the mean ± s.d., n=3. The difference in [^{35}S]methionine incorporation of matrix proteins between grooved and smooth surfaces was significantly different in cultures examined at 90 hours (P<0.05, t-test).

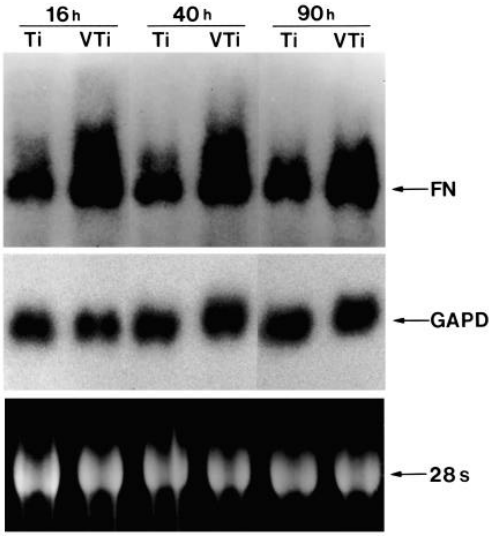


Fig. 5. Northern hybridization analysis of fibronectin gene expression of the cells cultured on grooved and smooth surfaces. Fibronectin (FN) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA levels of human gingival fibroblasts cultured on grooved titanium-coated surfaces (VTi) and smooth titanium-coated surfaces (Ti) for 16, 40, and 90 hours 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}P]dCTP-labeled fragments of human cDNA FN or GAPD as described in Materials and Methods. FN mRNA was identified at the bands of ~7.9 kb in size, and GAPD mRNA was identified at the bands of ~1.2 kb in size. Equal sample loading was confirmed by the density of 28 S ribosomal bands stained by ethidium bromide in gels before transfer. The duration of film exposure was 6 hours.

half-life of FN mRNA was ~5 hours. In cultures on grooved surfaces, the stability of FN mRNA showed a two-phase response. In the first 2 hours, the amount of FN 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. On the other hand, the half-lives of GAPD mRNA were estimated to be ~24 hours in cultures on both smooth and grooved surfaces. In addition, a three-hour delayed response of GAPD transcription to DRB inhibition was noticed in cultures on both smooth and grooved surfaces.

DISCUSSION

The topography of the substratum to which cells attach plays a fundamental role in regulating cell behaviour. In an attempt to determine the effect of substratum surface topography on cell shape and the influence of the changes in cell shape on the major extracellular adhesive proteins, we investigated the mechanism of FN regulation at the molecular level in cells cultured on a grooved substratum produced by micromachining. The striking findings of this study were that the shape of human gingival fibroblasts was significantly altered by grooved substratum topography and that FN mRNA levels increased significantly (1.9 to 3.5-fold) on the grooved surface, although the cell number and total cell RNA yield were essentially unchanged. 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 dif-

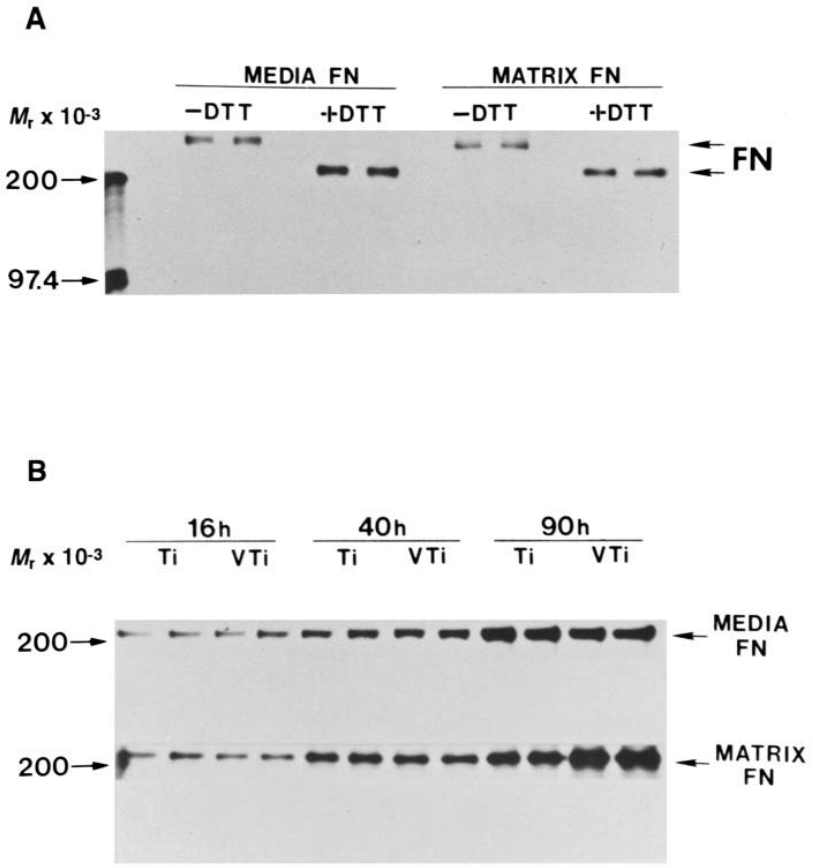


Fig. 6. SDS-PAGE analysis of gelatin-Sepharose affinity-purified fibronectin. [^{35}S]methionine-labeled fibronectin (FN), collected from extracellular matrix by urea extraction (MATRIX FN) or from conditioned cell culture medium (MEDIA FN) on the grooved titanium-coated surface (VTi) and smooth titanium-coated surface (Ti) for indicated time points, was affinity purified on gelatin-Sepharose as described in Materials and Methods. (A) Duplicate samples of the gelatin-Sepharose-bound material from 40 hours 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 consitituent polypeptide chains (230-270 kDa) of FN were demonstrated after reduction with DTT. The left lane indicated the reduced molecular mass marker proteins. (B) Effect of surface topography on the accumulation of media FN and matrix FN was analyzed by SDS-PAGE of gelatin-Sepharose affinity-purified eluents on 8% polyacrylamide gels under reduced conditions.

ferentiated 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 our experiments, human fibroblasts also showed a selective pattern of gene expression as revealed by significantly increased levels of FN mRNA level by the cells on the grooved surface, although their house-keeping gene GAPD mRNA remained essentially at the same levels as the cells on control smooth surfaces.

Since mRNA accumulation has been proven to be directly proportional to both transcription rate and half-life of the mRNA, changes in mRNA stability can affect the amount of gene products. In attempting to investigate the effect of surface topography on mRNA stability, we monitored the remaining quantity of FN mRNA after mRNA synthesis was inhibited by the RNA polymerase II inhibitor (DRB) (Chodosh et al., 1989). We found a difference in FN mRNA stability between cultures on smooth and grooved surfaces. There was a two-phase pattern of FN 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 FN mRNA stability in the second half-life period. The mechanism for this topography-dependent regulation and time-dependent divergence of FN mRNA stability by the grooved surface may be related to the relationship 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 FN mRNA stability through topography-defined

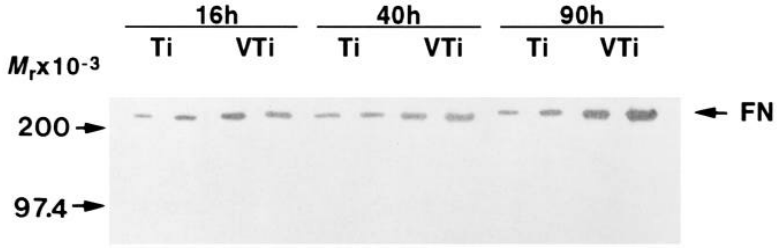


Fig. 7. Western blot analysis of cellular bound fibronectin extracted with SDS plus DTT. Cellular bound proteins were extracted with 4% SDS plus 65 mM DTT from the cultures in duplicate on either smooth (Ti) or grooved (VTi) surfaces. Aliquots (10 μl) of the extraction were fractioned on 8% polyacrylamide minigels under reduced conditions, transferred to Immobilon filter, and incubated with anti-fibronectin antiserum followed by visualization with the enhanced chemiluminescence detection system.

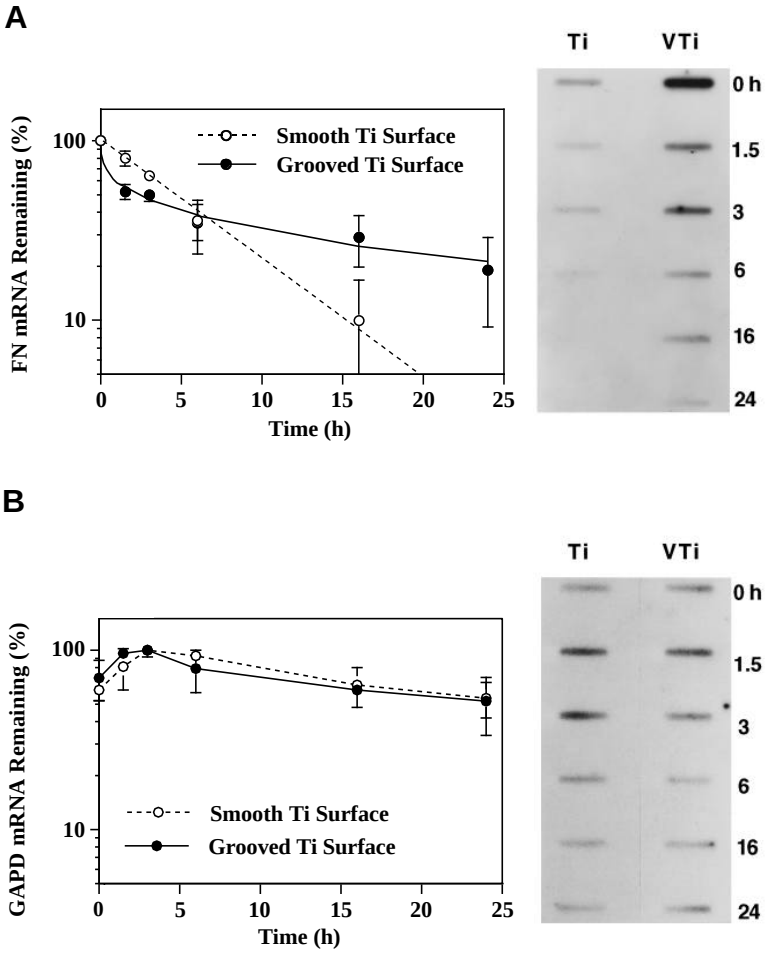


Fig. 8. Effect of surface topography on the stability of fibronectin and GAPD mRNA in human fibroblasts. Human fibroblasts were cultured on smooth titanium surface (Ti) or grooved titanium surface (VTi) as described in Materials and Methods. After 40 hours the RNA polymerase II inhibitor DRB (60 μg/ml) 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 [^{32}P]dCTP-labeled fibronectin (FN) cDNA (A) and GAPD cDNA (B). 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 or GAPD mRNA remaining at the indicated times relative to the 0 hour level. Least squares fit lines for cultures on smooth surface (-----) and grooved surface (——) were used to estimate half-life (see Results).

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 FN between the medium and extracellular matrix. In our experiments, the amounts of FN 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 FN remaining in the medium were at about the same levels for all time points. These results also provide further evidence to support the previous findings by Hanein et al. (1994) that even subtle differences in inorganic substrata can have major effects on cell adhesion. However, the molecular mechanism underlying this altered assembly of FN into the matrix is not known. Several events have been suggested to be important in the process of assembly: such as the binding of FN to its receptors (Hayashi and Yamada, 1983) or heparan sulfate proteoglycan (HSPG) (Woods et al., 1985), and FN-FN interaction (McKeown-Longo and Etzler, 1987; Allen-Hoffman and Mosher, 1987) or cross-linking (McKeown-Longo and Mosher, 1984; Peters et al., 1990). The altered distribution of FN between extracellular matrix and media observed in our experiments may be regulated through any of these processes.

Micromachining is a versatile technique that provides a flexible means to control precisely surface topography (Angell et al., 1983) and manipulate cell behavior (Brunette et al., 1983; Brunette, 1986a,b; Dunn and Brown, 1986). Thus, it has considerable potential to be used in studies on the effects of surface topography on cell shape and gene expression. Understanding the influence of cell shape on cell behaviour is relevant to understanding both naturally occuring cellular processes and the interactions of cells with implanted devices. For example, during morphogenesis cells appear to be under precise control by topographic guidance cues of various dimensions (Curtis and Clark, 1990). Typically the selection of the surfaces employed on implanted devices such as dental and orthopedic implants is driven by available materials technology and a general goal of enhancing tissue integration. The present study shows that the effects of surface topography on implant devices can involve much more than simple mechanics because the synthesis and secretion of a major adhesive protein, as well as its distribution between the cellular matrix and surrounding fluid, can be altered. It is unlikely that the effects of surface topography are restricted to just FN, and preliminary results have indicated that some enzymes involved in extracellular matrix remodelling were also affected by grooved substratum. From a biomaterials perspective a potential use of studies on micromachined surfaces will be to develop surfaces that alter cell shape in such a manner that desired cell responses at the molecular level on artificial devices can be obtained. In

this way the surfaces of implanted devices could be engineered so as to function appropriately rather than being selected on an ad hoc or empirical basis.

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