Effects of titanium on transcriptional and post-transcriptional regulation of fibronectin in human fibroblasts

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The effects of commercially pure titanium (Ti) on the regulation of fibronectin gene expression and synthesis were investigated in early-passage human gingival fibroblasts. The fibroblasts were cultured on 50 nm Ti-coated silicon wafers treated with radio-frequency glow discharge prior to use and on Falcon tissue culture plastic (TCP) dishes as a control. Northern hybridization analysis revealed that fibroblasts cultured on Ti reduced the fibronectin mRNA level by 58% at 16 h, but increased it by 2.6-fold at 90 h, although the cell numbers and house-keeping gene GAPD mRNA levels on these two surfaces were essentially the same. The amount of total RNA was slightly less on the Ti surface. While the total [^{35}S]methionine incorporation was essentially unaltered, the amount of [^{35}S]methionine-labeled fibronectin was significantly increased in cells cultured on a Ti surface in early cultures but decreased in the late cultures. The apparent discrepancy between the increased fibronectin mRNA levels and decreased translation could be explained by a 30% reduction in fibronectin mRNA half life in cells cultured on Ti. The distribution of fibronectin between the medium and the cell layer also was altered on Ti surfaces, with a ~100-fold *increase* of fibronectin assembled in extracellular matrix at 16 h, but a 36% *reduction* at 90 h. In contrast, the amount of fibronectin recovered in the medium was essentially unchanged. The total amount of protein assembled into the extracellular matrix by cells on Ti increased 2.1-fold at 16 h but decreased by 19% in 90-h cultures. These significant changes in fibronectin gene activity and gene product distribution by cells cultured on Ti surfaces demonstrate that the surface chemistry of biomaterials can selectively regulate the cellular behavior at the molecular level and, conversely, that molecular biological techniques provide sensitive indicators of the molecular biocompatibility of implant materials. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Molecular biocompatibility, which is a largely unexplored area of biomaterial research, may be defined as the assessment of tissue reactions to biomaterials at the molecular level. Most studies on biologic behavior of the interface zone between titanium (Ti) implants and host tissue have focused on histological evaluations.[1] Of particular importance in understanding the biomaterial–tissue interface reaction is the establishment of a biologic profile of the molecules directly involved in cell adhesion to the biomaterials and differentiation in the interface zone.

Fibronectin (FN), one of the major molecules mediating cell attachment and development,[2,3] has been the topic of extensive investigation in many areas of cell biology, but it has received relatively little attention for its possible role in mediating cell attachment to Ti. On Ti surfaces, FN is deposited as tracks by migrating fibroblasts.[4] There are, however, hardly any studies aimed at evaluating the regulation of FN gene expression, synthesis, and secretion by Ti surface chemistry. In this study, we investigated the effect of commercially pure Ti substratum on FN metabolism at the molecular level. The experiments compared human gingival fibroblasts cultured on Ti with those cultured on tissue culture plastic (TCP) dishes, testing the total cell RNA yield, FN mRNA level, FN mRNA half life, and the secretion and assembly of FN into the extracellular matrix.

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MATERIALS AND METHODS

Cell culture

Fibroblasts from outgrowths of adult human gingiva were isolated and subcultured as described by Brunette

Journal of Biomedical Materials Research, Vol. 31, 209–217 (1996)

CCC 0021-9304/96/020209-09

et al.[5] In brief, explants were cultured on tissue culture plastic (TCP) 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 0.25% (w/v) trypsin solution (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 for subcultures.

In the experiments, cells were cultured on either Ti-coated substratum or on TCP dishes, which, as the most commonly employed substratum for cell culture, were used as a control. Smooth silicon wafers were evaporatively coated with 50 nm commercially pure Ti, cleaned by ultrasonication for 20 min in 7× Cleaning Solution (Flow Lab., McLean, VA), washed 20 times with double-distilled H_2O, and, finally, treated with radio-frequency glow discharge for 3 min to clean and sterilize the surfaces prior to culture, as described previously.[6,7]

In each of three experiments, fibroblasts (6–10th passage) were plated on 55-mm diameter smooth Ti-coated silicon wafers or 55-mm diameter TCP dishes (Falcon, Cockeysville, MD) in MEM containing 5% (v/v) fetal bovine serum (FBS), a concentration of serum determined to be optimal for FN expression,[8] at a cell density of 1.2×10^4 cells/cm^2. Before plating, cell subcultures were cultured for 48 h in flasks in MEM with 5% (v/v) FBS to adapt them to the conditions utilized in the subsequent experiments. Cells plated on Ti surfaces or TCP were incubated for 16 h (~60% confluent), 40 h (~90% confluent) and 90 h (confluent). Cell numbers were determined by electronic cell counting (Coulter Electronics, Inc., FL).

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) in each of three experiments. For 40-h cultures, fresh labeling medium was replaced at 16 h, and for 90-h cultures fresh labeling medium was replaced at 16 h and 40 h. Labeled medium (3 mL) was harvested at 16 h, 40 h, and 90 h. Labeled matrix proteins bound to the cell layers were extracted by a modification of the method of Yamada and Akiyama.[8,9] In brief, cell monolayers were rinsed 4 times with 3 mL Hank's balanced salt solution to which freshly prepared proteinase inhibitor, 2 m*M* phenylmethanesulfonyl fluoride (PMSF, Sigma), was added. After incubation with gentle rotation at 1 rpm for 1 h at 37°C in 3 mL of serum-free MEM containing 2 m*M* PMSF, the cultures were rinsed with 3 mL serum-free MEM containing 2 m*M* PMSF. Labeled matrix proteins were then extracted by incubation for 2 h at 37°C with rotation at 1 rpm in 1.5 mL freshly made 2*M* urea in serum-free MEM containing 2 m*M* PMSF. The extract was centrifuged for 15 min at 25,000 g to remove cells and other insoluble particles. Aliquots (10 μL) of the labeled medium or cell monolayer extracts were analyzed by SDS-polyacrylamide minislab gels as described below. Total secreted proteins were quantitated at 16, 40, and 90 h by liquid scintillation counting of the [^{35}S]methionine-labeled media proteins and extracted matrix proteins after exhaustive dialysis against 0.15*M* NaCl, 10 m*M* Tris-Cl, pH 7.0. Data were then normalized on a per cell basis. FN was identified in the secreted proteins after purification by miniaffinity columns of gelatin-Sepharose, as described by Chou et al.[8] Material bound to the gelatin–Sepharose column was eluted first with 1.0*M* NaCl in 50 m*M* Tris-HCl, pH 7.2 buffer containing 5 m*M* $CaCl_2$, 0.5 μg/mL Brij 35, and, 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

SDS-PAGE was performed in the presence of 1.0 mg/mL SDS using separating gels containing 8% acrylamide and stacking gels containing 4% acrylamide. Labeled matrix and media total proteins and purified FN were electrophoresed with reduction using 65 m*M* DTT and heating at 56°C for 20 min. FN bands on fluorographs were quantitated using an optical scanner (Apple Computer Co., Cupertino, CA) and image-analysis computer software (Image 1.4, NIH, Bethesda, MD) after exposure at −70°C of dried 2,5-diphenyloxazol-impregnated gels 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 both media and matrix FN were analyzed separately. Total FN secretion also was calculated by adding the amounts of media and matrix FN together at each culture time point. 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 RNA extraction and mRNA half life determination

Cellular RNA was prepared by guanidinium thiocyanate (GT) extraction according to Glisin et al.[10] and Ullrich et al.[11] and modified as described by Chou et al.[8] Cultures in triplicate at different time points on

either Ti or TCP were washed 3 times in cold PBS before the cells were lysed by 3 mL of guanidinium thiocyanate (GT) buffer containing 4*M* guanidinium thiocyanate, 0.025*M* sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl, 70 m*M* β-mercaptoethanol, and 5 m*M* 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 of 2*M* sodium acetate, pH 4.1, 3 mL of RNase-free water-saturated phenol, and 0.6 mL of chloroform-isoamylalcohol were added separately, with vortexing between each addition. After incubation on ice for 5 min and centrifugation at 12,000 g for 20 min at 4°C, RNA was collected from the upper aqueous phase and was precipitated overnight at −20°C in 1 volume of ice-cold isopropanol. The precipitated RNA pellet was rinsed with 85% 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. To estimate the half life of FN mRNA, cells were cultured in triplicate as described above on either Ti or TCP, and after a 40-h incubation (~90% confluent), 60 μ*M* 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, Sigma, St. Louis, MO), a specific RNA polymerase II inhibitor, was added to the cultures.[12] The total RNA was then extracted as described above from triplicate cultures at each time point (0, 1.5, 3, 6, 16, and 24 h) and analyzed after slot blotting, Northern blotting, and hybridization with FN and glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA probes, as described by Chou et al.[8]

Northern hybridization and slot-blot analysis

Aliquots of extracted cell RNA (5 μg) were prepared in a loading solution containing 5*M* of formaldehyde and ethidium bromide (40 μg/mL), incubated at 65°C for 15 min, chilled on ice, then fractionated on 1.2% (w/v) agarose gels containing final concentrations of 2.2*M* formaldehyde and 20 m*M* 3-N-morpholinopropanesulfonic acid (MOPS, pH 7.0). Following fractionation, the aliquots of extracted cell RNA were transferred onto Hybond-N nylon membrane (0.45 μm pore size, Amersham Inc. Arlington Heights, IL) using a Posiblot apparatus (Strategene Inc. La Jolla, 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., San Francisco, CA), and aliquots (5 μg) of the same sample RNA also were analyzed by Northern hybridization to monitor the specificity of the cDNA probes used in the slot hybridization. Following 3 min of UV cross linkage, the blots were prehybridized at 62°C for 2 h in 5% (w/v) SDS, 50 m*M* PIPES, 0.1*M* sodium chloride, 50 m*M* Na_2HPo_4, 50 m*M* NaH_2Po_4, and 1 m*M* ethylenediaminetetraacetic acid tetrasodium salt (EDTA), pH 7.0. Hybridization was performed for 18 h 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) and 5 mg/mL SDS at room temperature for 10 min, followed by a change of the same solution at 62°C for 30 min, and then for 20 min 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 intensified screens (Dupont),[13] scanned by a digital optical scanner, and analyzed by computer software, as described above. The data was 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. Experiments were performed in triplicate and the data expressed as the mean ± SD.

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.,[14] 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.,[15] were obtained from American Type Culture Collection (ATCC). 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.[16] Harvested cells were lysed by 0.2*N* NaOH, 1% (w/v) SDS. Phenol-chloroform extraction and ethanol precipitation were used to purify plasmid DNA containing the FN cDNA inserts. Clone inserts were excised by restriction digestion using EcoRI for FN and PstI for GAPD (BRL, Gaithersburg, MD). 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}P]dCTP (>3,000 Ci/mmol, Amersham Inc., Arlington Heights, IL) to a specific activity of ~1.8×10^9 cpm/μg cDNA prior to hybridization.

RESULTS

Effect of titanium on cell proliferation and total RNA yield

Cell numbers and total RNA yield were determined in fibroblasts cultured on Ti substrata and TCP for 16,

40, and 90 h (Fig. 1). The total cell RNA levels in cultures on Ti showed small reductions of 7% at 16 h, 32% at 40 h, and 11% at 90 h relative to cultures on TCP. No difference in cell numbers was noted between the cultures (~1.2×10^4, ~2.0×10^4, and ~5.0×10^4 cells/cm^2 at 16-, 40-, and 90-h cultures, respectively).

Effect of titanium on total protein synthesis

Since a large portion of extracellular molecules may be assembled into matrix shortly after being secreted into medium, the amounts of labeled proteins both in the extracellular matrix and in the conditioned media were measured [Fig. 2(A)]. [^{35}S]Methionine incorporation in media collected from the cultures on Ti showed a slight reduction (10%) at 16 h, and slight increases at 40 h (1.23-fold) and 90 h (1.16-fold) relative to the cultures on TCP. In contrast, labeled proteins extracted from extracellular matrix exhibited a time-dependent pattern on Ti, showing increases of 2.1-fold at 16 h, and 1.70-fold at 40 h, followed by a 19% reduction at 90 h relative to the cells on TCP. The secreted total protein, as measured by adding the amounts of protein in both medium (media protein) and extract (matrix protein) together, showed similar levels on a per cell basis in the cultures on both Ti and TCP at 16 and 90 h, but a 1.47-fold increase in cultures on Ti substratum at 40 h. The electrophoretic profiles of secreted total protein from the cell layer extraction and the conditioned media in the cultures on different substrata are also shown in Figure 2(B). These experiments indicated that while the amounts of total protein secretion were essentially unchanged, the Ti affected the distribution of total protein in a time-dependent manner, with more protein being bound in matrix at early times and lesser amounts at later cultures relative to TCP controls.

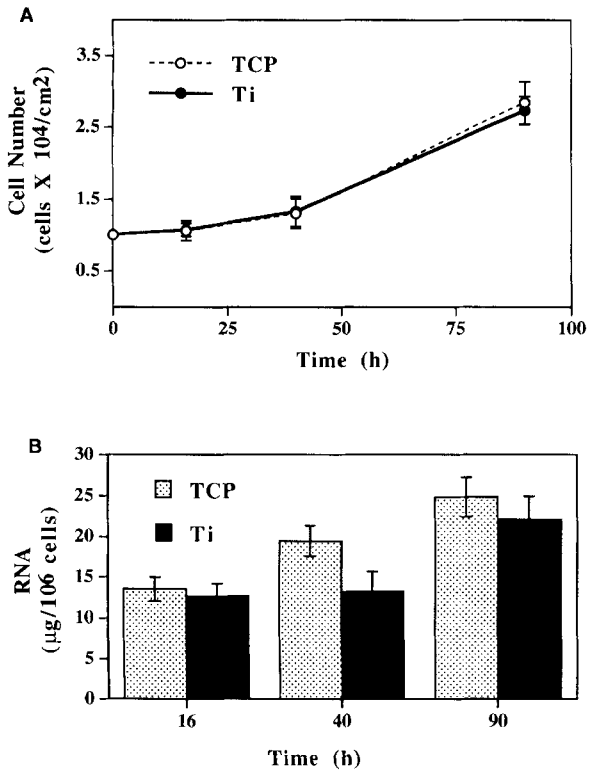

Figure 1. Effect of titanium on cell proliferation and total RNA yield. Human gingival fibroblasts (6–10th passage) were plated in triplicate for 16, 40, and 90 h with initial density of ~1.3×10^4 cells/cm^2 on titanium-coated substrata ("Ti") or Falcon tissue culture plastic (TCP) dishes as control. (A) Cell numbers were detected by Counter counting ($n = 3$, presented as the mean ± SD); (B) RNA was extracted at the indicated times, and the total RNA yields were determined from spectroscopic analysis as described in "Materials and Methods." Data from one time course experiment is presented as the mean ± SD ($n = 3$). The difference in total RNA yield at 40-h cultures was significant ($p < 0.01$, t test).

Effect of titanium on fibronectin mRNA expression

The levels of FN mRNA were notably altered on Ti surfaces while the mRNA levels of the house-keeping gene GAPD essentially were not altered by Ti surfaces at each time point (Fig. 3). Relative to TCP, there was a 44% reduction of FN mRNA level at 16 h, increasing to a 2.93-fold increase of FN mRNA level at 90 h. Since the total cell RNA was reduced (7–32%) in the cultures on Ti, the data for FN mRNA was normalized for the yield of total cell RNA for each corresponding RNA preparation. The data also were normalized for the loading of total RNA onto the Northern blotting gels. On a per cell basis, cultures on Ti revealed an increasing trend relative to that on TCP, with a 58% reduction in FN mRNA expression at 16 h, a 28% reduction at 40 h, and up to a 2.6-fold increase at 90 h.

Effect of titanium on fibronectin synthesis

To investigate the effect of Ti substratum on FN synthesis, FN was affinity purified from equal aliquots of both conditioned medium (media FN) and extracellular matrix extraction (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 the reduced condition.[8] The amounts of media FN from the cultures on Ti increased 3.5-fold at 16 h and 1.4-fold at 90 h, while at 40 h amounts showed about the same level relative to the controls (Fig. 4). The amounts of extracted matrix FN on Ti, however, revealed a large increase of ~100-fold at 16 h, which was reduced to a 28-fold increase at 40 h and further reduced to a 36% decrease at 90 h relative to the cells on TCP (Fig. 4). The pattern of decreasing amounts of FN in the matrix

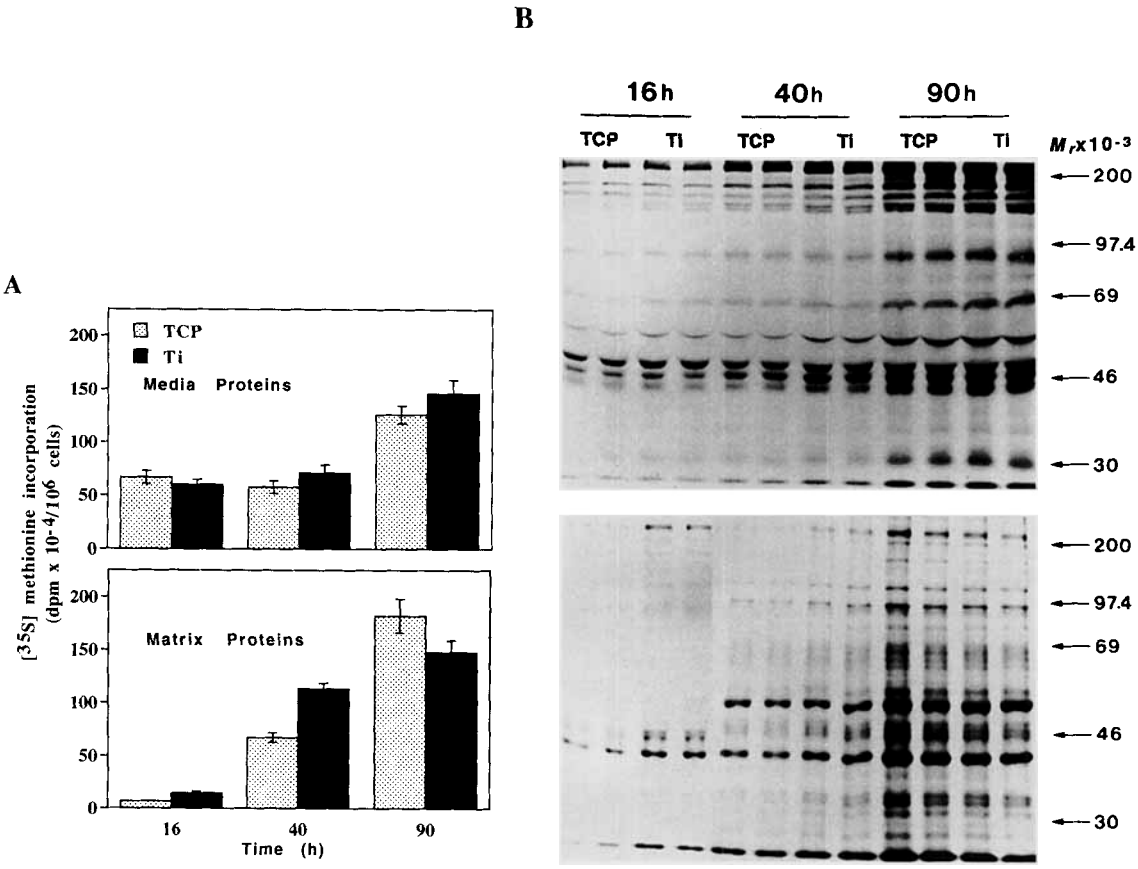

Figure 2. Analysis of secreted total proteins. (A) [^{35}S]Methionine incorporation into secreted total proteins in conditioned medium (media proteins) and extracts of extracellular matrix (matrix proteins) from the cells on titanium substrata (Ti) and tissue culture plastic dishes (TCP) at the indicated times was determined by liquid scintillation counting after exhaustive dialysis. The differences in [^{35}S]Methionine incorporation of matrix proteins between Ti substrata and the controls were significant ($p < 0.005$ at 16 and 40 h, and < 0.01 at 90 h, t-test). Data from one time course experiment is presented as the mean ± S D, $n = 3$. (B) Aliquots (10 μL) of the [^{35}S]Methionine-labeled media proteins (top gel) and matrix proteins (lower gel) were electrophoresed in duplicate under reduced condition on 8% polyacrylamide gels and processed for fluorography. For the gel of matrix proteins, the duration of film exposure for 16 h-lanes was 2.5× longer than the lanes of 40 and 90 h.

was opposite to the pattern of increasing levels of FN mRNA (Fig. 3).

Total secreted FN in the cultures on Ti, as determined by adding the amounts of media and matrix FN together at each culture time point, showed a similar pattern to the matrix FN, with a 22.6-fold increase at 16 h declining to 2.6-fold increase at 40 h and, finally, to a slight reduction (10%) at 90 h relative to the cultures on TCP.

Effect of titanium on fibronectin mRNA stability

As the amount of FN synthesis did not correlate with the levels of FN mRNA as a function of time, the effect of Ti substratum on the stability of FN mRNA was further investigated. Following the addition of the RNA polymerase inhibitor DRB to block mRNA transcription, the decrease in the level of FN mRNA and GAPD mRNA was measured over time. In the controls, the half life of FN mRNA was estimated to be ~7 h. In the cultures on Ti, the FN mRNA half life was only ~5 h, showing a ~30% reduction of mRNA stability relative to the controls [Fig. 5(A)]. The specificity of slot-blot hybridization in the mRNA stability experiment was confirmed by corresponding Northern hybridization [Fig. 5(B)]. The half lives of GAPD mRNA were estimated to be ~24 h in cultures on both TCP and Ti surfaces, although a 3-h delayed response of GAPD transcription to DRB inhibition was noticed in cultures on both surfaces [Fig. 5(C)].

DISCUSSION

The study of cell responses at the molecular level to biomaterials should provide sensitive indicators of cell behavior at tissue–biomaterial interfaces. The profile of gene activities regulated by biomaterials could be a valuable approach for determining the molecular

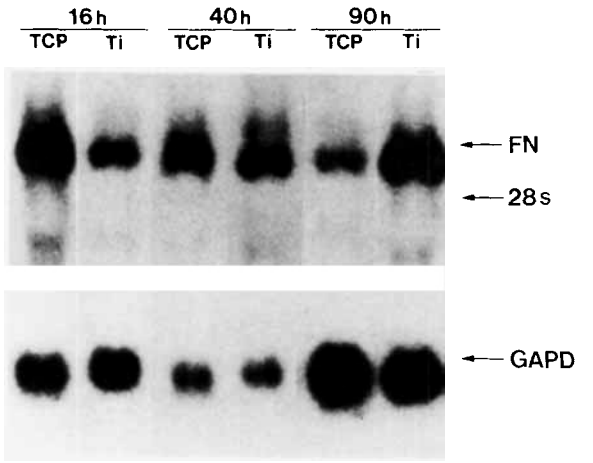

Figure 3. Northern hybridization analysis of fibronectin gene expression regulated by titanium substratum. Fibronectin (FN) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA levels in human gingival fibroblasts cultured on titanium-coated surface (Ti) and tissue culture plastic dishes (TCP) for 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 nylon membrane, and hybridized with [^{32}P]dCTP-labeled cDNA fragments of human FN or GAPD, as described in MATERIALS AND METHODS. Equal sample loading was confirmed by the amounts of 28S ribosomal bands stained by ethidium bromide in gels before transfer. The duration for film exposure was 6 h.

biocompatibility of biomaterials in conjunction with the conventional clinical and histological evaluations. This study attempted to assess the influence of Ti on the expression of genes encoding major extracellular adhesive proteins by investigating FN regulation at the molecular level in human gingival fibroblasts cultured on Ti substratum. The results indicated that Ti altered FN gene expression in both transcriptional and post-transcriptional events. The mechanism by which

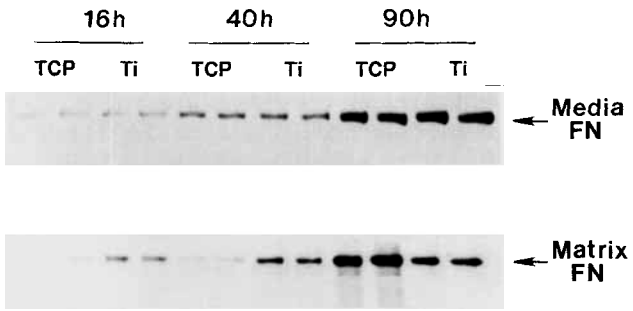

Figure 4. SDS-PAGE analysis of gelatin-Sepharose affinity purified fibronectin. [^{35}S]Methionine-labeled fibronectin (FN) from extracellular matrix (Matrix FN) of cells cultured on the titanium substrata (Ti) and on tissue culture plastic dishes (TCP) for indicated time points, and from conditioned cell-culture medium (Media FN), was affinity purified on gelatin-Sepharose. Effect of titanium on the accumulation of Media FN and Matrix FN was analyzed in duplicate by SDS-PAGE using aliquots (10 μL) of affinity-purified eluents on 8% polyacrylamide gels under reduced conditions.

gene activity responds to the Ti substratum remains, however, largely unclear. As proposed by McDonald[17] and Juliano and Haskill,[18] cell differentiation and gene expression might be regulated by a signaling system involving the association of membrane-mediated receptors with extracellular adhesive proteins that 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.[19,20] Our previous study also showed that the surface topography of substratum could selectively alter the FN gene expression and stability while the house-keeping gene activity was essentially unchanged.[8] In the present experiments, human fibroblasts showed a selective pattern of gene expression as revealed by a reduced level of FN mRNA in the cells on Ti substratum in the early cultures, then by a significantly increased level of FN 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 TCP. The FN synthesis in the cells on Ti, however, increased remarkably in early cultures and declined in the late cultures. The altered FN mRNA stability by Ti substratum was found to be the likely basis for this discrepancy between the amounts of FN mRNA level and the amounts of secreted FN.

Changes in mRNA stability may affect significantly the amount of gene expression because mRNA accumulation has been proven to be directly proportional both to the transcription rate and to the half life of the mRNA.[21] In attempting to investigate the effect of Ti on the mRNA stability, we monitored the remaining amounts of FN mRNA after mRNA synthesis was inhibited by RNA polymerase II inhibitor. The half life of FN mRNA was reduced by 30% in cultures on Ti at 40 h relative to that on TCP. 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 FN may interact through its membrane receptors with the cytoskeleton, which, in turn, may bind mRNA and alter the mRNA stability.[17,22]

In this study, FN assembly into the extracellular matrix increased 100-fold at 16 h on Ti surfaces relative to TCP declined to a 28-fold increase at 40 h, and then decreased to a reduction of 36% at 90 h. This large shift of FN assembly activity may be related to the rate of FN adsorption onto the Ti, which, in turn, may be influenced by the surface energy of the Ti substratum. The Ti substratum used in our experiments was radio-frequency glow-discharge treated for 3 min shortly prior to use, a treatment that results in a high surface energy upon exposure to air.[23] Glow discharge for various times from 1 to 10 min has been reported to decrease the wetting angles of Ti surface to less than 20°[24]

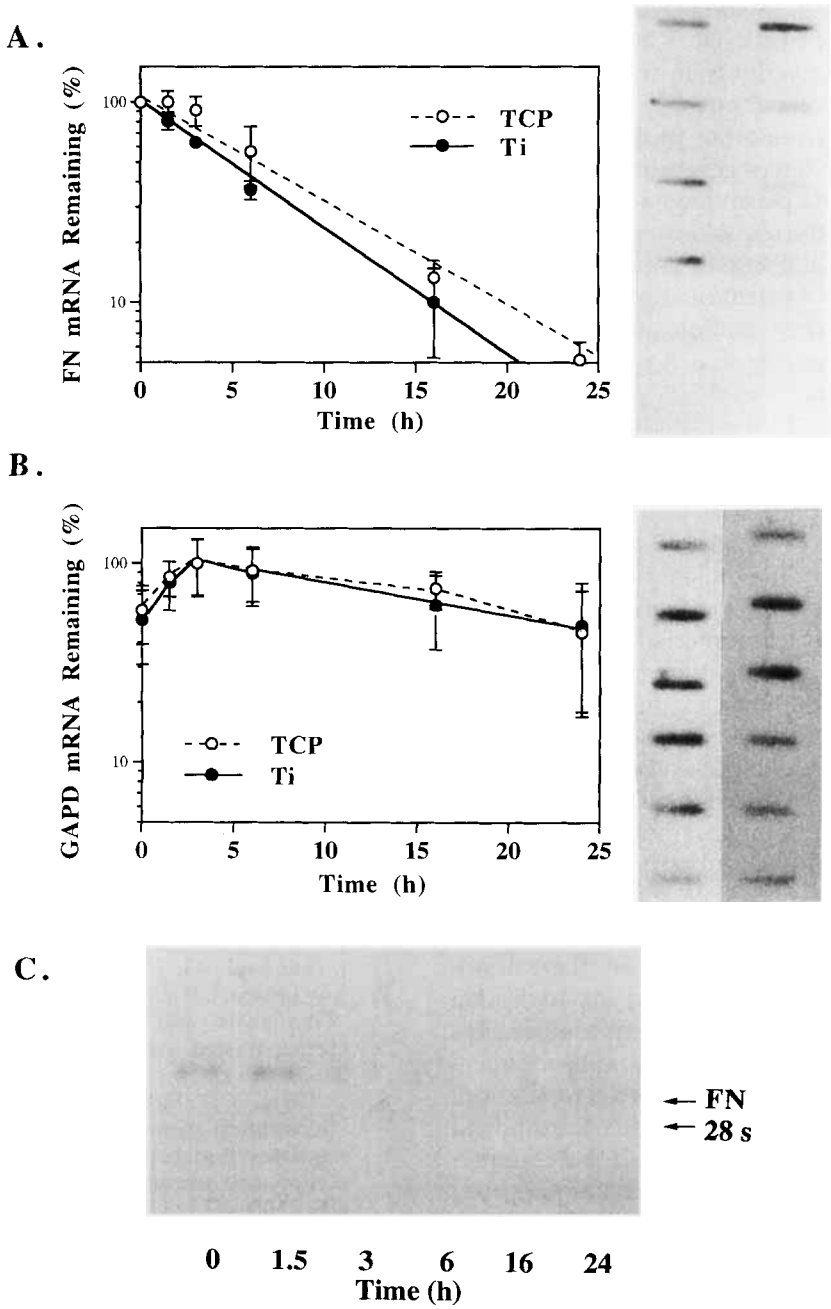

Figure 5. Effect of titanium on the stability of fibronectin mRNA in human fibroblasts. Human fibroblasts were cultured on titanium substrata (Ti) or tissue culture plastic dishes (TCP), as described in MATERIALS AND METHODS. After 40 h the RNA polymerase II inhibitor DRB (60 μg/mL) was added and the cultures were incubated for the indicated additional times. Total cell RNA was then extracted. (A) Aliquots (5 μg) were analyzed by slot-blot hybridization with [^{32}P]dCTP-labeled FN cDNA (right panel). Autoradiographs of the slot blot were quantitated by digital optical scanner and the results presented as a logarithmic plot of the mean ± SD (n = 3) of the FN mRNA remaining at the indicated times relative to the 0 h level. Least squares fit lines for cultures on titanium substrate (—) and tissue culture plastic dishes (--) were used to estimate half lives (see Results); (B) the GAPD mRNA remaining at the indicated times relative to the 0 h level was determined under the same conditions; (C) the specificity of the slot-blot hybridizations was verified by Northern hybridization of the same conditioned total RNA as used in TCP surface experimental group. Aliquots (5 μg) were fractioned on 1.2% (w/v) agarose, 2.2*M* formaldehyde gels, transferred onto nylon membrane, hybridized, and post-hybridization washed under the same conditions as used for slot-blot hybridizations.

compared to tissue culture dishes, the ones thought to be glow-discharge treated by the manufacturer and to have wetting angles of about 28°.[25] FN has been reported to desorb more readily from wettable surfaces than from less wettable ones,[25] possibly because of such factors as competition from other proteins in the medium,[25-27] cellular shear forces generated at sites of cell adhesion,[28] and increased plasminogen adsorption and activation.[29] Therefore, the remarkable reduction of FN assembly from an initially higher amount[26] observed in our experiments on Ti surfaces might initiate the changes in cytoskeletal organization, which then down regulate FN mRNA stability and lead to a reduced amount of FN secretion.

An interesting finding in the experiments was that the changes in FN gene activity and gene products distribution by Ti were time dependent. The mechanism underlying this pattern of regulation has not been demonstrated but may be related to the known dynamic properties of Ti in the biological environment. The chemical properties of Ti are mainly determined by the oxide layer composed primarily of TiO_2.[29,30] Previous studies have suggested that the time-dependent growth of the TiO_2 oxide layer on the Ti surface increases logarithmically up to 7 weeks after Ti is immersed in physiologic solutions.[30] Although the oxide layer is chemically inert, it has a high dielectric constant and is negatively charged.[31] The outermost surface atoms of the oxide with its unsaturated chemical bonds[32] might indirectly initiate the reaction of attached cells through alterations in the adsorbed macromolecular carpet. Healy and Ducheyne[33] have developed a hierarchical model describing the hydration and preferential adsorption of serum components. Their results demonstrate that Ti surfaces absorb many types of molecules that might be expected to alter cell behavior. Only one type of Ti surface was examined in this study, and it should be pointed out that commercially available implants vary in the techniques used to prepare clean and sterilized surfaces. Keller and coworkers[34,35] have demonstrated that significant surface alterations resulted from sterilization treatments and that surfaces prepared in different ways differed dramatically in their ability to support cell attachment. It seems reasonable to expect that such treatments also would affect gene activities just as this study found that fibronectin gene activity was altered by differences between TCP and Ti surfaces.

In conclusion, the significant changes in human fibroblast gene activity and gene product distribution by cells cultured on Ti surfaces observed in this study demonstrate that the surface chemistry of biomaterials can selectively regulate cellular behavior at the transcriptional and post-transcriptional levels and, conversely, that molecular biological techniques provide a sensitive and specific approach to the assessment of cell responses to biomaterials. The use of molecular profiles of cellular adhesive and osteogenic gene activities in response to various types of implant materials may prove useful as a biologic guide to implant material selection and design.

This work was supported by the Medical Research Council of Canada, MRC Fellowship Grant 5-59186 and Operative Grant 5-97617. The authors thank Dr. N. Jaeger, Center of Microelectronics, Department of Electrical Engineering, The University of British Columbia, for the preparation of titanium-coated silicon wafers.

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Received January 19, 1995
Accepted September 25, 1995