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The effect of laser-treated titanium surface on human gingival fibroblast behavior

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Abstract: Surface modification, as a means of enhancing soft tissue integration in titanium would have significant advantages including less marginal bone resorption, predictable esthetic outcome, improved soft tissue stability, and seal against bacterial leakage. The aim of this study was to evaluate the effects of laser-roughened titanium surfaces on human gingival fibroblast (HGF) viability, proliferation, and adhesion. Titanium discs were ablated with impulse laser in four different patterns. Polished and sand-blasted titanium discs were used as control groups. Specimen surface properties were determined using optical profilometry and scanning electron microscopy. HGF behavior on modified surfaces was analyzed using cell adhesion, viability, proliferation, and ELISA assays. Results suggested that modified Ti surfaces did not affect the viability of HGFs and improved adhesion was measured in laser treatment groups after 24 h. However, proliferation study showed that the adsorbance of fibroblast cells after 72 h cultured on polished titanium was higher and comparable with that of control cells. As for focal adhesion kinase (FAK), cells grown on laser modified surfaces had higher expression of FAK as compared with polished titanium. In conclusion, tested laser-treated surfaces seem to favor HGF adhesion. There were no significant differences between different laser treatment groups. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

Key Words: human gingival fibroblasts, titanium alloy, cell adhesion, cell proliferation, FAK

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INTRODUCTION

Research on dental implants and their surrounding tissues show that the success of implantation is strongly influenced by the structure and function of supracrestal peri-implant tissues.[1–3] The epithelium, functioning as a barrier that seals the implant surface from contaminants in the oral environment, has a tendency to migrate apically if not properly supported by connective tissue interface.[4] As a result, gingival recession, pocket formation, and bone resorption may occur.[5–7] Therefore, a healthy and firm implant-connective tissue attachment may, among other factors, prevent apical migration of epithelial tissue, support it and provide protection as well as blood supply for the underlying bone surface. The surface characteristics may have an effect on tissue formation by influencing the types of cells that initially attach and differentiate at the cell-implant interface.[8–10] Surface energy dictated by the surface roughness, topography, and composition of the implant play a major role in determining which proteins, growth factors, or biological agents are adsorbed onto the surface that subsequently influence cell adhesion, spreading, migration as well as implant-soft tissue interface.

Consequently, it is important to clarify the mechanisms by which cells attach to the artificial surfaces, such as titanium (Ti). It was shown that Ti surface roughening is associated with increased surface area available for cell interactions and may favor the development of some cell activities such as proliferation, total protein content, and final cellular responses such as bone-like nodule formation.[11,12] A number of surface treatment methods, including mechanical, chemical, electrochemical, electropolishing, vacuum-based, or thermal are available to create controlled implant surface roughnesses. The surfaces of prosthetic components can be easily modified by applying polishing and sandblasting. However, laser techniques have recently been developed as an alternative to change implant surface micro-topographies.[13] Dental implant surface modification techniques promoting connective tissue attachment are highly demanded.

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Cell-biomaterial adhesion involves multiple complex events, such as specific binding of membrane proteins to extracellular matrix (ECM), intracellular cytoskeleton formation and signal transduction.[14,15] In adhesion signaling, the central role is played by focal adhesion kinase (FAK)—an enzyme found in multiprotein structures linking the ECM to the cytoskeleton known as focal adhesions.[16–19] The autophosphorylation and catalytic sites of FAK are critical to this regulation of adhesion strengthening.[20] During the early stages of adhesion, FAK activates integrins to increase the number of bound integrins over time, resulting in adhesion strengthening. These findings demonstrate an important role for FAK in time-dependent generation of cell–ECM forces.[21]

The aim of this study was to evaluate the effect of Ti surface laser-treatment methods on human gingival fibroblast (HGF) attachment, viability, cell proliferation, FAK expression, and phosphorylation.

MATERIALS AND METHODS

Specimen fabrication

Grade 2 titanium alloy specimens were designed using computer-aided design (CAD) software and milled with accurate milling unit D5 (Datron, Datron Dynamics). All specimens were equal in diameter and height (ø5.2 × 2 mm^2). Titanium discs were ablated using an impulse laser (NL640, Expla, Lithuania) in four different patterns ($n = 5$): Group 1 consisted of separated holey structures (Ti-L1), Group 2—overlapping laser-ablated holey structures arranged in all directions (Ti-L2), Group 3—grid-like holey structures (Ti-L3), Group 4—overlapping laser-ablated holey structures arranged in straight rows (Ti-L4). All holey structures were the same size, about 15 μm in depth. Polished (Ti-P) titanium discs were used as a negative control group and sandblasted (Ti-S) discs as positive. Surface properties of control and modified Ti discs were determined using optical profilometry and scanning electron microscopy (SEM). Optical profilometric analysis was used to visualize surface roughness (PLμ 2300, Sensofar). Disc surface topographies were examined using a TM-1000 SEM (Hitachi, Japan).

Prior to cell culture, the specimens were cleaned with Decon90 (Decon Laboratories Limited, Great Britain), washed with tap water, sterilized in 96% ethanol with subsequent exposure of both sides to UV light for 15 min.

Cell culture

Human gingival subepithelial tissue was obtained from a healthy patient undergoing gingivectomy of the premolar region. Informed consent was acquired from the donor and the protocol was approved by the institutional ethics review board at Vilnius University (No. 158200-11-116-28). Primary cultures were obtained by culturing explants of gingiva, following an established procedure.[22,23] Briefly, the gingival tissues were cut into small pieces, cultured in separate cell-culture plates in Iscove's modified Dulbecco's medium (IMDM, Biological Industries, Israel) supplemented with 20% of fetal bovine serum (FBS, Biological Industries), 100 U/mL penicillin, 100 μg/mL streptomycin, and 12.5 U/mL nystatin (Biological Industries). Cell outgrowth from the tissue explants was observed 1–2 weeks after the beginning of the incubation. Primary cultures were subcultured twice a week using 0.25% trypsin/EDTA (Biological Industries) mixture and maintained in IMDM supplemented with 10% FBS and antibiotics. Cells between 5 and 10 passages were used in experimental work.

Immunocytochemistry

The established cell culture was monitored using primary antibodies against integrin β1, Type I collagen, fibronectin, vimentin, FSP–1 protein, CD14, CD45, and CD54 (Milipore, UK). The cells at 80–90% confluence were washed twice with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Fixed samples were washed three times with PBS. To detect integrin β1, Type I collagen, fibronectin, and vimentin, the cells were permeabilized in 0.2% Triton X-100 in PBS for 15 min at RT and washed three times with 1% bovine serum albumin (BSA) in PBS. Nonspecific binding was blocked by a 1 h treatment in 10% normal donkey serum in PBS for at least 2 h at RT. The cells were incubated with primary antibodies overnight at 4°C, washed four times with PBS, and incubated with Cy3-conjugated secondary antibody at 37°C for 1 h. Then the samples were rinsed five more times with 1% BSA in PBS and the cell nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich). Samples were placed in an antifade media and observed under a fluorescence microscope. Vimentin was visualized with secondary antibodies conjugated with horseradish peroxidase (HRP). 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) was used as a substrate.

Cell viability assay

Primary HGFs were seeded on the specimens at a density of 5×10^4 cells/mL and then cultured for 24 and 48 h. Cell viability was determined by fluorescence microscopy using two fluorescent dyes, acridine orange (AO) and ethidium bromide (EB), to analyze the mode of cell death. AO was used to characterize chromatin condensation and segmentation; ethidium bromide was used to characterize membrane integrity as described by Mercille and Massie.[24] Photomicrographs were taken with an inverted phase contrast microscope (Nikon Eclipse TS 100 F) equipped with a CCD camera (QImaging ExiBlue). Cells were categorized as follows: viable, viable apoptotic, nonviable apoptotic, necrotic, and so forth. For cell viability evaluation, two titanium discs of each surface modification type. Each experiment was repeated three times independently.

Evaluation of adhesion strength

Qualitative assay. Effects of surface topography and material on cell orientation and shape were analyzed by SEM after HGFs were cultivated on the Ti discs for 24 h. The cells were seeded at a density of 5×10^4 cells/mL. The cultured cells were incubated for 24 h at 37°C in 5% CO_2. Loosely adherent cells were removed from the experiment wells by aspiration. Wells were washed twice with a 0.1*M* PBS (pH

7.4), and the remaining bound cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) for 30 min at RT. The excess glutaraldehyde solution was removed, and the cells were rinsed three times in PBS (3 × 10 min), fixed with 2% OsO_4 (Sigma-Aldrich) for 20 min at RT. After washing three times in PBS (3 × 10 min), samples were dehydrated by immersion in ethanol solutions of progressively higher ethanol concentrations (25, 50, 75, 90, and 96%, 5 min treatment at each concentration, respectively). Subsequently, the samples were dried using a critical point dryer (K850, Quorum Technologies), then covered with a 20 nm conducting layer of gold using an ion coater (Q150R, Quorum Technologies). Cell growth was visualized using a TM-1000 (Hitachi) SEM.

Quantitative assay. HGFs were seeded on the specimens at a density of 1.5×10^4 cells/cm^2 and allowed to attach for 24 h. Detached cells were removed by shaking the samples in 600 rpm mode for 15 min (Thermomix Comfort, Eppendorf). The number of attached cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, employing a Varioskan Flash plate reader (Thermo Scientific) to measure the optical density at 570 nm. Adhesion index was calculated as a ratio of cell quantity remaining on the surface of the specimen after shaking with cell quantity before shaking for the same specimen type. Cell adhesion strength was studied using three titanium discs for each type of surface modification. Three independent assays were performed.

Cell proliferation assay

HGFs were seeded on each specimen at a density of 1.5×10^4 cells/cm^2 and cultured for 1, 2, and 3 days. MTT assay was used to register the number of living cells as well as to estimate cell proliferation. Briefly, the cell-seeded samples were transferred to a new plate and incubated with 0.2 mg/mL of MTT. After 1 h, the MTT solution was aspirated, and the formed formazan salt solubilized in 96% ethanol, absorbance of which was measured at 570 nm. To assess cell proliferation, two titanium discs of each surface modification type were used. Three independent assays were performed.

Cell-based ELISA assay

To determine the expression and phosphorylation of FAK, cells were cultured on the Ti discs in 96-well plates at a density of 1.5×10^4 cells/cm^2 one day prior to manipulation. Cells were then fixed with 4% paraformaldehyde at RT for 15 min, followed by washing with PBS containing 0.1% Triton X-100. The cells were incubated in antibody blocking buffer, followed by incubations with primary antibodies for 1 h at RT. After rinsing, HRP-conjugated antibody was added and cells were incubated for 1 h at RT. Subsequently, the plates were subjected to 0.1 mg/mL TMB, according to the manufacturer's protocol. The reaction was stopped with 2*M* sulfuric acid and absorbance was measured at 450 nm. The change in expression and phosphorylation status was calculated by dividing absorbance detected using phospho-FAK (Cell Signaling Technology) or FAK-specific (BD Bioscience, UK) antibody with that of the β-actin-specific (Milipore) antibody. For detection of the protein of interest, three titanium discs of each surface modification type were used. Three independent assays were performed.

Statistical analysis

Measurement data were expressed as a mean ± standard deviation. The effects of titanium surface topography on HGF proliferation, adhesion, FAK expression, and phosphorylation were evaluated using Kruskal–Wallis nonparametric analysis (SPSS 16.0.1 for Windows, SPSS, Chicago, IL). Statistical significance was set at $p < 0.05$.

RESULTS

Surface characterization

Ti discs used in *in vitro* experiments for surface topography and roughness analysis were characterized by SEM and optical imaging profilometer (Fig. 1). SEM images of the laser-modified titanium samples showed the characteristic surface roughness in comparison to control surface of polished Ti. Ti discs ablated with impulse laser had separated holey structures (Ti-L1), overlapping laser-ablated holey structures arranged in all directions (Ti-L2), grid like holey structures (Ti-L3), and overlapping laser-ablated holey structures arranged in straight rows (Ti-L4). Greater magnification of Ti-P sample surfaces revealed small crests, valleys, and some areas with microcracks. Most of the Ti-S surface area had irregularities with pits and spikes.

Cell characterization

Our established primary HGF cell culture was characterized by immunocytochemistry. Results revealed that HGF cells expressed two extracellular matrix molecules—fibronectin and Type I collagen that are synthesized by cultured mesenchymal stem cells as well as cell-surface molecule integrin β1 and a Type III intermediate filament vimentin, both specific for mesenchymal stem cells (Fig. 2). Expression of CD14, CD45, and CD54 markers was not observed in the studied cell population. HGFs expressed fibroblast-specific protein 1 (FSP-1).

Cell morphology and viability

The SEM analysis performed on fibroblast-seeded samples after 24 h showed typically polygonal, spindle-shaped cells, attached, and spread on all specimen surfaces (Fig. 3, left side). The apparent formation of filopodia and lamellipodia were observed. HGF cells preferentially attached to holey structures by forming bridges inside and covering the holes created by laser ablation. Some of them grew into the micropores of laser-modified Ti samples (Fig. 3, left side).

Cell viability was determined by AO/EB staining after 24 (not presented) and 48 h of culture (Fig. 3, right side). During the entire trial, >90% of cell viability was observed within all gropus. These results indicate that modified Ti surfaces do not affect the cell viability of HGFs.

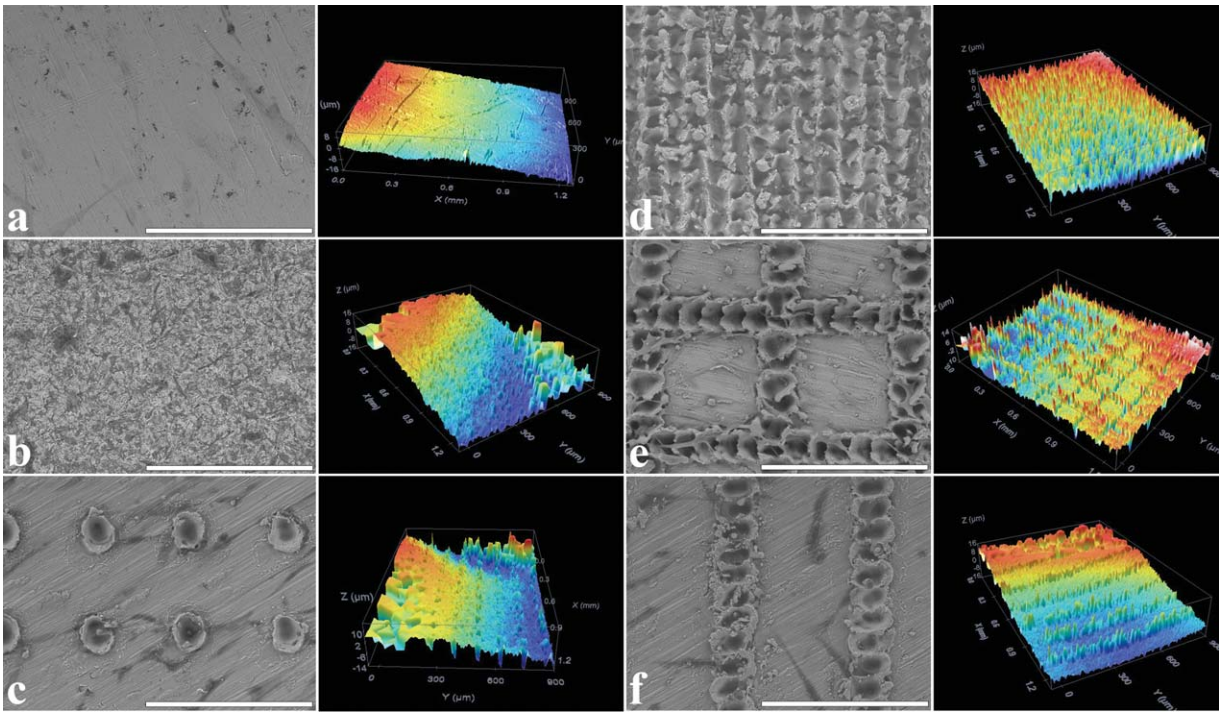

FIGURE 1. Titanium specimen surface topographies and roughnesses: (a) polished (Ti-P); (b) sand-blasted (Ti-S); (c) separated holey structures (Ti-L1); (d) overlapping laser-ablated holey structures arranged in all directions (Ti-L2); (e) grid-like holey structures (Ti-L3); (f) overlapping laser-ablated holey structures arranged in straight rows (Ti-L4). Scale bar – 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

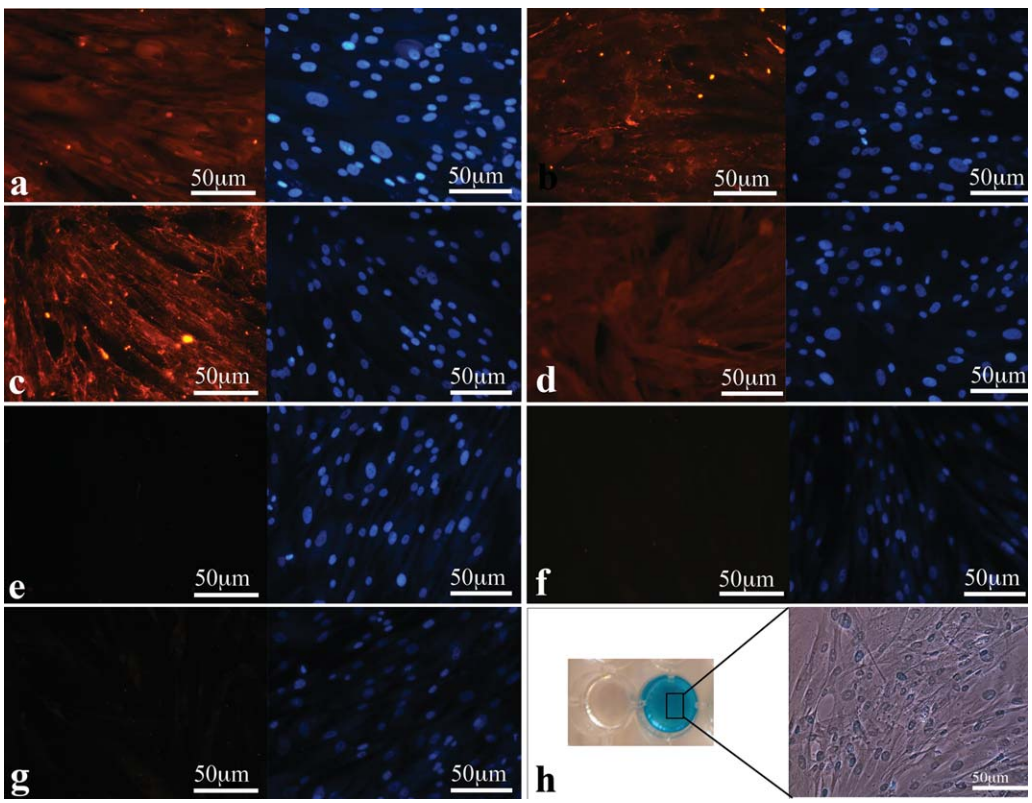

FIGURE 2. HGFs express markers specific to mesenchymal stem cells as well as fibroblasts. We determined positive expression of (a) integrin β1, (b) type I collagen, (c) fibronectin, (d) FSP-1, and (h) vimentin. Studied cell population didn't express CD14 (e), CD45 (f) and CD54 (g). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

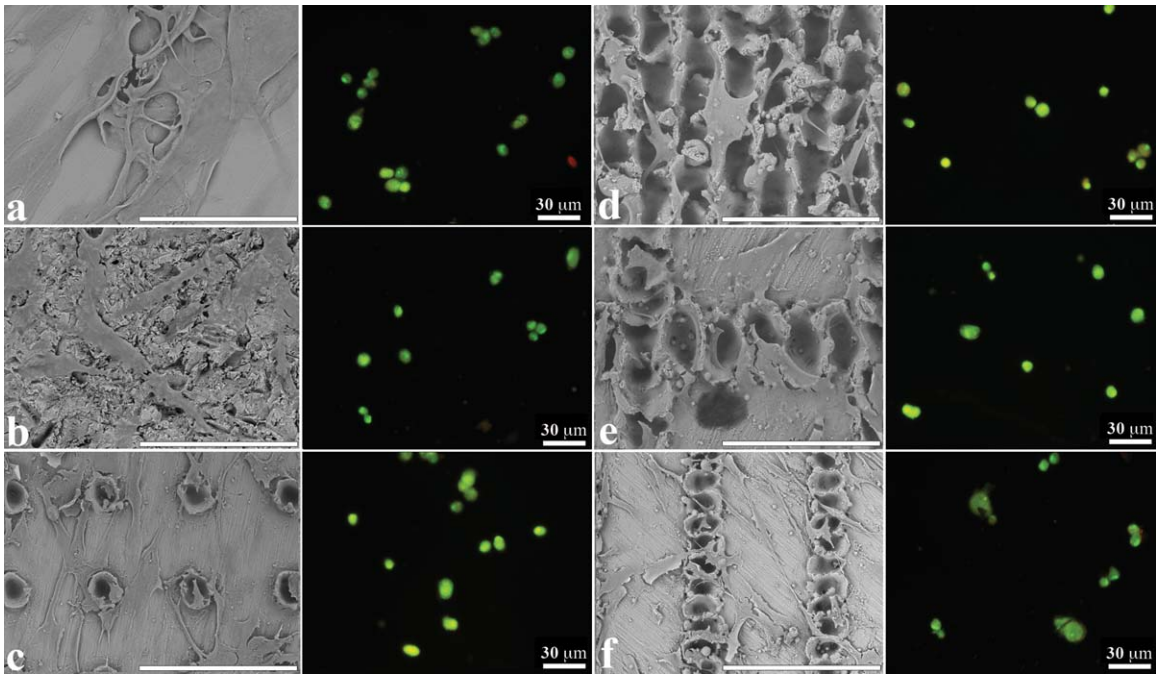

FIGURE 3. Morphology (left) and viability (right) of HGFs on structured titanium surfaces. The results suggest that cells preferentially attach to the holey structures by forming bridges inside and covering the holes created by laser. (a) Ti-P; (b) Ti-S; (c) Ti-L1; (d) Ti-L2; (e) Ti-L3; (f) Ti-L4. Scale bars in SEM images: a, b, d, e – 100 μm; c, f – 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Cell adhesion

The effect of surface topography on HGF adhesion to Ti substrata evaluated after 24 h cell maintaining in culture is presented in Figure 4. We did not observe any differences in early HGF adhesion (30, 60, 120 min postseeding) on laser-modified Ti surfaces (data not presented). The results in Figure 4 show, that cell adhesion on laser treated surfaces was stronger than on control surfaces and this difference was statistically significant ($p = 0.004$).

Cell proliferation

HGF cell proliferation was determined after 24, 48, 72 h cultivation via MTT assay (Fig. 5). Statistical differences in cell proliferation between Ti surfaces were observed for 24 ($p = 0.053$) and 72 h ($p = 0.037$) groups, indicating that modified Ti surfaces have different effects on the HGF proliferation.

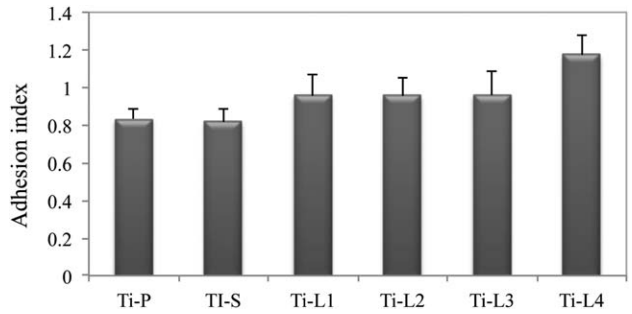

FIGURE 4. Effect of surface topography on HGF adhesion to Ti substrata after 24 h culture. Adhesion index is expressed as a ratio of cell quantity remaining on the surface of the specimen after shaking with cell quantity before shaking for the same specimen type.

Evaluation of FAK expression and phosphorylation

FAK expression and phosphorylation in HGFs grown on differently modified Ti surfaces were assessed by cell-based ELISA. A significant increase of FAK expression was observed on all modified Ti surfaces with the exception of polished Ti surfaces ($p = 0.019$) (Fig. 6). Analysis of FAK expression on laser-modified Ti surfaces did not reveal any major changes. It is interesting, that in HGFs grown on Ti-S, a significantly higher level of total and phospho-FAK protein was detected ($p = 0.01$).

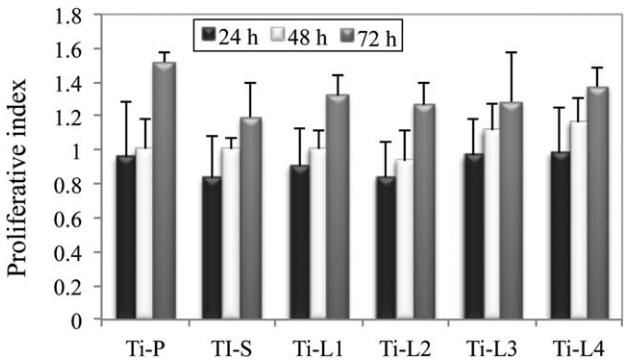

FIGURE 5. Comparison of fibroblast proliferation on Ti substrata after 24, 48, and 72 h of culture. Titanium disc surface modifications ($n = 5$): Ti-P: polished; Ti-S: sand-blasted; Ti-L1: separated holey structures; Ti-L2: overlapping laser-ablated holey structures arranged in all directions; Ti-L3: grid-like holey structures; Ti-L4: overlapping laser-ablated holey structures arranged in straight rows. Data are expressed as proliferative index, which means the ratio of viable cell number after 24, 48, or 72 h of culture to seeding cell number.

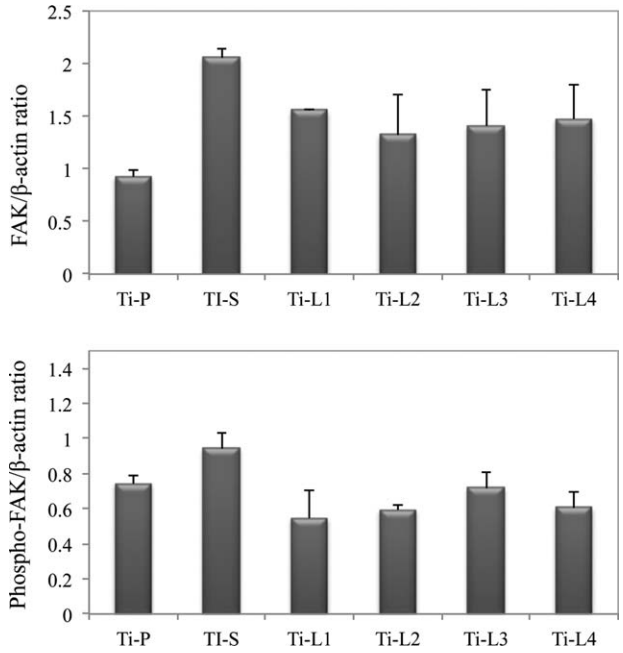

FIGURE 6. FAK expression and phosphorylation in HGFs grown on differently modified Ti surfaces.

DISSCUSION

Previous studies concluded that long-term prognosis of a dental implant depends not only on the osseointegration quality, but also on the quality of mucosal seal between gingiva and implant abutment or the implant itself.[25–27] Various studies also report that microgrooved surfaces may aid in fibroblast attachment to substratum forming a more stable connective tissue zone that inhibits downgrowth of the epithelium.[28,29] This allows the slower-growing osteoblasts and new bone tissue to reach the surface and attach to the microgrooved surface as well as to avoid possible formation of sinus tracts for bacteria that may result in significant bone resorption during healing time.

The purpose of this research was to evaluate HGF behavior on dental implants or implant abutment materials made from Ti. To determine the relationship between cell attachment and surface characteristics of roughened Ti implant materials, 6 different Ti treatments were compared for the ability to mediate HGF adhesion, proliferation as well as FAK expression and phosphorylation. For this reason, 4 differently laser-modified Ti disc groups were tested. Polished Ti and sand-blasted Ti surfaces were used as controls. Profilometric and SEM analyses of polished, sand-blasted, and laser-modified titanium surfaces revealed distinct topographical features at micron scale resolution. To obtain consistent cellular response and behavior of oral fibroblasts, only cells within the fifth and tenth passages were used in this study. Such cells are phenotypically similar to their *in vivo* equivalents. Moreover, it was shown, that fibroblasts lose their ability to synthesize matrix proteins after serial passages *in vitro*.[30] There are also data showing that different cells share different adhesion properties to certain surfaces. For instance, a fibroblast behavior differs from that of an osteoblast or an epithelial cell.[31,32] As for primary HGFs, it should be noted that there is little data on markers of these cells in the Ref. 33. Therefore, to identify their behavior and generate meaningful conclusions, the established primary HGF cell line was characterized. The obtained data revealed that the cells were expressing mesenchymal stem cell and fibroblast-specific markers, exhibited polygonal, spindle-shaped cell morphology, and possessed high viability on all specimen surfaces. Also, more cells were found around laser ablated holey structures and even inside the holes. Dunn and Brown reported that fibroblasts could form actin fiber terminations both in the grooves and on the ridges.[34] It may be speculated that collagen produced by fibroblasts has better mechanical interlocking on rougher surfaces as well as better resistance for lateral shear force due to distinctive rough surface grooves that act as anchoring sites. Still, this hypothesis should be confirmed by additional physicochemical studies.

Next, the adhesion strength of HGFs on differently treated Ti discs was evaluated. It has been proven that the surface characteristics influence cellular attachment and spreading.[35] A recent study showed that distinctive surface roughnesses created by laser can improve fibroblast cell attachment.[36] But our previous studies did not reveal any significant differences in early HGF adhesion (30, 60, 120 min postseeding, data not presented) on laser-modified Ti surfaces. This study showed that the cell attachment to all laser-treated surfaces 24 h postseeding was statistically and significantly better as compared with controls. These results disagree with the data of Cochran et al., who found that initial adhesion of periodontal fibroblasts was greater on smooth titanium; however, after 5 days of incubation, there was no difference in cell number between all surfaces. Meanwhile, Meyle reported that sand-blasted titanium surface enhanced fibroblast as well as osteoblast proliferation and adhesion, whereas epithelial cells showed a decreased quality of latter features on the same surfaces.[37,38] Current study did not detect any significant difference in cell adhesion strength among groups with different laser treatment scenarios. In summary, a controversy still exists among various researchers on surface roughness topic in regard to cell adhesion quality. Moreover, we examined cell proliferation as a marker of biocompatibility of laser-treated Ti surfaces. The data obtained revealed that smoother surfaces stimulated cell growth better as compared to rougher surfaces only after 72 h. Latter findings are in agreement with previous studies.[39] In another *in vitro* study, higher number of focal contacts and a better organization of the cytoskeleton including stronger network of actin fibres of fibroblasts was observed with smooth titanium surfaces and surfaces with fine irregularities as compared to rougher surfaces.[40] Lauer et al. found that human gingival epithelial cells attached, spread, and proliferated on glossy polished Ti surfaces with the greatest extension than on sand-blasted or plasma-sprayed titanium surfaces.[41] These findings partially contradict Mustafa et al. observations, which showed that more human gingival fibroblasts initially attach to polished Ti abutments, but display a higher rate of proliferation on rougher Ti surfaces blasted with TiO_2 particles.[42]

The evaluation of expression of focal adhesion kinase, which plays an important role in cell adhesion revealed significant upregulation of FAK in HGFs grown on modified Ti surfaces. It is known that activation of FAK is essential for many cellular functions. Oates et al. reported that fibroblasts are sensitive to changes in surface roughness and hydrophilicity, with adhesive interactions mediated through FAK, an important modulator of fibroblast response.[43] The study of Miura and Takebe revealed that fibroblasts have the capacity to adhere to the material, which markedly improves expression of focal adhesion molecules and enhances the fibroblast phenotype.[44] Such corelation has been established in our study. On the other hand, as was shown by Oates et al., loss of FAK did not significantly affect fibroblast attachment to any surface, but attenuated spreading.[43] Michael et al. reported that FAK autophosphorylation is required for the enhancement of adhesion strength and integrin-binding responses.[45] Our results showed upregulation of pFAK level in HGF grown on sand-blasted Ti surface. These findings were not consistent with cell adhesion data.

Besides surface topography alteration, laser treatment may have effects on surface chemical and physical properties, including surface wettability, oxidation state of titanium, and so forth. These characteristics, along with surface topography, may influence biocompatibility of materials.[46,47] Further studies are needed to elucidate the mechanisms of different surface treatment strategies on HGF behavior.

In conclusion, it may be suggested that the laser-modified titanium surfaces exhibited nontoxic effects and further promoted HGF adhesion. Thus, titanium surface treatment can significantly affect human fibroblast behavior. Further studies are needed to research and improve soft tissue attachment around dental implants.

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