An investigation of the initial attachment and orientation of osteoblast-like cells on laser grooved Ti-6Al-4V surfaces

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ABSTRACT

This paper presents the results of an experimental study of the initial cell spreading and adhesion on longitudinally- and transversally-oriented micro-grooves produced by the laser irradiation of laser grooved Ti-6Al-4V surfaces. The initial spreading and orientations of human osteosarcoma (HOS) cells were observed and quantified after 15-min, 1-hour, 4-hour and 24-hour cell culture periods. Immuno-fluorescence staining of adhesion proteins (actin and vinculin) was then used to study the spreading and adhesion of HOS cells in 1 hour and 4 hour culture experiments. The initial cell adhesion was also quantified using enzymatic detachment tests. The results showed that cell spreading and adhesion were enhanced by longitudinally- and transversally-oriented micro-grooves. The effects, which increase with time, were not remarkable after 1 hour, but obvious after 4 hours. Contact guidance was found to promote cell adhesion due to the increase in interactions between the focal adhesions and the patterned extra-cellular matrix (ECM) proteins on the laser micro-grooved surfaces.

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1. Introduction

Ti and Ti-6Al-4V alloys are widely used in orthopedic and dental implants because of their excellent mechanical properties and exceptional biocompatibility [1,2]. Since current orthopedic implants have relatively short lifespans, which result from the loosening between the implants and surrounding bone [2,3], there is a need for improved adhesion between Ti implants and bone. This has stimulated prior work on the effects of surface modification techniques, such as grit blasting and porous coating [4–7].

More recently, however, laser-ablated micro-grooves have been used to modify the surface characteristics of biomaterials and influence cellular behavior [8–14]. Unlike blast-textured surfaces that give rise to random cell orientations [5,7], laser micro-grooved Ti-6Al-4V surfaces have been shown to promote contact guidance (cell alignment) [10,12], a phenomenon that involves the alignment and migration of cells along micro-grooves. This has been shown to reduce the extent of scar tissue formation and promote osseointegration [8,9].

Although enhanced cell attachment and obvious cell alignment have been observed on laser-ablated micro-grooved surface after a few days culture [8–14], the actual mechanism of contact guidance and cell adhesion enhancement on laser grooves remains unclear. There is, therefore, a need to develop a fundamental understanding of the mechanisms of cell spreading and adhesion that are associated with contact guidance. These will be explored in this paper using laser micro-grooved geometries with well controlled groove spacings and surface textures, since prior work has shown that both groove spacing [8–10,12] and groove surface texture [11,13,14] are important factors that affect the spreading and attachment of cells onto grooved Ti-6Al-4V surfaces.

Unlike prior studies [8–14] that focus on cell spreading after 1 to 7 days of cell culture, the current work examines the phenomena that occur during the first few hours (0.25 h, 1 h, 2 h, 4 h and 24 h) of cell culture on laser grooved titanium surfaces. The focus on the early stages of cell spreading is important because they are thought to be a major determinant of the long-term bone-biomaterial interfacial response that is associated with the subsequent spreading and growth of osteoblasts on implant surfaces [7]. Hence, an improved understanding of the bone-biomaterial response may lead to faster and more extensive implant integration and improved long-term stability [13–16].

This paper presents the results of an experimental study of the initial stages of cell spreading and adhesion to laser micro-grooved Ti-6Al-4V surfaces with well-controlled groove spacings and surface textures. Cell spreading and orientation were studied using scanning electron microscopy (SEM) and analyzed using image analysis software (Imagej, NIH, Bethesda, MD). Enzymatic detachment tests were also used to quantify cell adhesion after 1-hour and 4-hour cell culture periods. Based on these results, the relationship between cell contact guidance and enhanced cell adhesion (on laser-irradiated...
surfaces was elucidated) for laser textured surfaces. The implications of the results are also discussed for biomedical applications of laser textured Ti-6Al-4V surfaces.

2. Materials and methods

2.1. Surface modification

The material used in this study was a mill annealed Ti-6Al-4V billet, fabricated and supplied by Wyman Gordon, Houston, Texas. The rectangular specimens, with approximate dimensions of 28 mm × 15 mm × 5 mm, were cut and ground using a series of sand papers (400 grit, 600 grit, 800 grit, 1200 grit). They were then mechanically polished using a 50 nm colloidal silica suspension (as the final polishing step). Polishing was done to a surface finish with an RMS (root mean squared) surface roughness of ~3.4 nm, which was measured using a Digital Instruments Nanoscope IIIa AFM (Veeco Instruments, Santa Barbara, CA).

Two groups of micro-grooved specimens were produced by irradiating the polished surfaces with 30 nanosecond laser pulses generated by a frequency tripled YHP40 Q-switched Nd:YVO₄ laser at 355 nm.

The first group consists of three kinds of grooved surfaces that have a very similar groove pattern referred to as pattern 0. They were produced using a single laser pass with the same laser processing parameters as those used in Ref. [12]. These laser parameters were used to produce groove spacings of 20, 40 and 60 µm (Fig. 1a–f). The second group of laser-irradiated surfaces was produced by multiple laser passes. These include three kinds of grooved surfaces that have the same groove spacing of 20 µm. However, in this case, different groove surface textures, referred to as groove patterns 1, 2, and 3, were produced (Fig. 1g–i). The laser processing parameters that were used to produce the second group of micro-grooves are summarized in Table 1.

All the grooves on these surfaces had depth of about 8–12 µm and width about 11–13 µm. These dimensions were selected based on the

![Fig. 1. SEM images of micro-textured surfaces: UV laser-irradiated micro-grooves top-views of (a) 20 µm, (b) 40 µm, (c) 60 µm groove spacing with groove pattern 0, and side-views of (d) 20 µm, (e) 40 µm, and (f) 60 µm groove spacing with groove pattern 0; UV laser-irradiated micro-grooves top-views of (g) groove pattern 1, (h) groove pattern 2, (i) groove pattern 3, which all have 20 µm groove spacing.](image)

<table>
<thead>
<tr>
<th>Processing parameters</th>
<th>Pattern 1</th>
<th>Pattern 2</th>
<th>Pattern 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal spot size (µm)</td>
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<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Pulse repetition rate (Hz)</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Pulse duration (ns)</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Scan speed (mm/s)</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Scan translation distance (µm)</td>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Scan fluence (J/cm²)</td>
<td>21.2</td>
<td>21.2</td>
<td>21.2</td>
</tr>
</tbody>
</table>
results from earlier studies [12] that suggested that the optimal groove dimensions for osteoblast ingrowth are on the order of 8 μm to 12 μm, which also promote contact guidance and osseointegration. Further details of the prior work on nanosecond laser-irradiation of Ti-6Al-4V are presented in Ref. [17].

2.2. Surface characterization

Pre- and post-processing examination of the textured surface regions were performed using SEM (Philips XL30 FEG-SEM), stylus profilometry (KLA-Tencor P15 Surface Profiler) and atomic force microscopy (Digital Instruments Nanoscope IIIa AFM). The geometries of grooves (widths and depths) were measured using profilometry, while the groove inside roughness values were measured using AFM.

The adsorption of extracellular matrix proteins to biomedical surfaces is very sensitive to the surface chemistry [1,2,18]. It is, therefore, important to reveal the possible effects of laser irradiation on surface chemical composition. In addition to using energy dispersive spectroscopy (EDS), which characterizes the underlying microchemistries in regions within 5 μm from the top surfaces [12], x-ray photoelectron spectroscopy (XPS) was used to examine the surface chemical compositions within 10 nm of the top surfaces.

XPS spectra were collected using a VG Scientific ESCALAB2 spectrometer in which mono-chromated Mg Kα radiation (hv = 1253.6 eV) was applied to the Ti-6Al-4V surfaces before and after laser irradiation. A pass energy of 100 eV was used for wide range scans (survey scans), while a 20 eV pass energy was used for high resolution measurements of Ti 2p, Al 2p, O 1s and C 1s regions.

For all measurements, the binding energy scale was normalized to the position of the C 1s peak at 285 eV. Curve fitting of the core-level XPS lines was carried out using CasaXPS software (Casa Software Ltd., UK) with a Gaussian–Lorentzian product function and a non-linear Shirley background subtraction. A Gaussian–Lorentzian mixing ratio was taken as 0.3 for all lines.

2.3. Cell culture and SEM observation

To investigate the effects of surface texture on initial cell spreading and adhesion, osteoblast-like cells human osteosarcoma (HOS; ATCC, Manassas, VA) were incubated with the micro-textured specimens for 15-min, 1-hour, 4-hour and 24-hour culture periods. It has been shown that these cells exhibit a developmental sequence that is similar to osteoblasts in bone tissue [18].

The HOS cells were cultured in 25 cm² flasks (Becton-Dickinson, Franklin Lakes, NJ), and maintained in an incubator at an incubation temperature of 37 °C regulated with 5% CO2, 95% air, and a saturated humidity. A Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B was used as the cell culture medium (Quality Biological, Gaithersburg, MD). At confluence, the cells were sub-cultured by splitting. Prior to cell seeding, 50 pieces of Ti-6Al-4V specimens were cleaned, passivated, and sterilized in order to create a clean, titanium-oxide surface for cell proliferation. Briefly, each sample was ultrasonically cleaned in a solution of double distilled water (dd H2O) and detergent for 20 min. This was followed by a rinse in dd H2O. The surfaces were subsequently sonicated in acetone for 20 min and rinsed with dd H2O. Each sample was then passivated in 30% nitric acid for 15 min and rinsed with dd H2O. All samples were then sterilized in 100% ethanol for 5 min and dried with nitrogen gas before being placed in multi-well culture plates (Becton-Dickinson, Franklin Lakes, NJ). After sterilization treatment, HOS cells were trypsinized, harvested, and seeded onto these specimens at a concentration of 5 x 10⁴ cells/cm², as determined by a hemocytometer count. The multi-well plates were then incubated in a water saturated atmosphere of 95% air – 5% CO₂ at 37 °C.

After culturing for 15 min, 1 h, 4 h and 24 h, the samples were rinsed with 0.1 M phosphate-buffered saline (PBS). They were then fixed in 3% glutaraldehyde solution for 1 h at room temperature. Subsequently, the samples were rinsed with 0.1 M PBS before dehydrating them at room-temperature in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 min at each concentration. Finally, samples were critical point dried, sputter-coated with a 20 nm thick gold layer, and examined using SEM (Philips XL30 FEG-SEM).

2.4. Immunofluorescence staining

After the 1-hour and 4-hour incubation periods, immunofluorescence staining (IF) was performed using protocols described in detail in [12]. These were used to stain for vinculin (focal adhesions) and the actin cytoskeletal structure. Briefly, the specimens were first fixed for 15 min in a 3.7% formaldehyde solution. Then they were permeabilized through exposure to a 0.5% Triton solution for 15 min. After that, they were then exposed to a primary antisera, containing the primary anti-vinculin antibody. The samples were incubated in the primary antisera for 30 min in a humid, 37 °C atmosphere. The samples were then rinsed and incubated in a secondary antisera, containing rhodamine phalloidin and a secondary anti-vinculin. The samples were incubated while exposed to the secondary antisera for 30 min at 37 °C. IF microscopy was subsequently carried out using a Nikon 50i research microscope, with an EPI-fluorescence attachment and images were taken using a Nikon Instruments DXM 1200F color digital camera (Nikon, Melville, NY). Confocal IF microscopy images were taken using a PerkinElmer spinning disk confocaleica SP5 spectral system (PerkinElmer, Waltham, Massachusetts).

2.5. Enzymatic detachment

A 0.25% (v/v) trypsin enzymatic detachment test was used to quantify the adhesion of HOS cells cultured on the micro-textured surfaces for durations of 1 and 4 h. To ensure that the samples were free of nonviable cells, the micro-textured specimens were rinsed 5 times in a phosphate buffered solution. Exposure to the trypsin solution lasted 2.5 min at room temperature. This was followed by a 1 ml rinse of PBS. The fluid was collected and saved, and the samples were again exposed to trypsin solution. This process was repeated four more times with trypsin exposure periods of 2.5, 5, and 10 min.

Each portion of the fluid was then examined for cellular content using a hemocytometer. At the end of the experiment, any remaining cells were removed from the specimens by long-term (over an hour) exposure to a 0.5% trypsin solution and physical agitation. A cell count was then performed again, and normalized with respect to the original total number of cells on each surface. The data obtained in this manner was used to establish a curve of the percentage of cells detached against trypsinization time.

2.6. Statistical analysis

For measurements of the extent of cell contact guidance (alignment), 3 samples per group were subjected to the ImageJ software analyses of cell areas, with at least 100 cells per sample analyzed. For all cell adhesion measurements using enzymatic detachment, 5 specimens per group were evaluated. Data were plotted as mean ± s.e.m. (standard error of mean), and comparison between the mean values of the experimental groups was made by the two-sample t-test, which assumes that the two sampled populations are normal and have equal variance. A confidence level of 95% (p<0.05) was considered significant.

3. Results

3.1. Surface texture

The SEM images in Fig. 1 show the micro-textured surfaces investigated in this study. Fig. 1(a)–(c) show the surface morphologies
of the three grooved surfaces in group 1. Fig. 1(d)-(f) are side-view micrographs obtained from these three surfaces. The laser-ablated micro-grooved surfaces produced using single laser pass were found to exhibit relatively uniform surface morphologies with splatter patterns of resolidified material within and around the micro-grooved regions [12]. In this study, the surface morphology of these grooves is referred to as groove pattern 0.

Ulerich [19] indicated that, instead of using a single laser pass with high laser fluence, multiple laser passes, with relative low laser fluence can be used to fabricate micro-grooves with 8–12 µm groove geometries (widths and depths), but with different surface micro textures. Fig. 1(g)–(i) show three grooved surfaces produced using multiple laser passes. They all have similar primary geometries (Table 2), but very different textures within the grooves (Fig. 1(g)–(i)). These are denoted, respectively, as laser groove patterns 1, 2 and 3.

Pattern 1 (Fig. 1(g)) is very similar to pattern 0 (Fig. 1(a)). It was obtained by varying the laser processing parameters until the morphologies of the single and multipass grooves were similar. By adding the third laser pass with low energy, striations were obtained normal to groove direction (Fig. 1(h) and (i)). The striations were about 8 µm in length and 2 µm in width, in the two cases presented in Fig. 1(h) and (i). The spacing of the striations is approximately equal to the translation distance of the third pass, which also affects the height of the striations.

In pattern 2 (Fig. 1(h)), the striations are about 10 µm apart and ~1–2 µm in height. However, in pattern 3 (Fig. 1(i)), the striations are about 8 µm apart and ~2–3 µm in height. The striations, which are referred to as secondary surface features, were produced to explore their potential effects on the initial cell behavior and adhesion.

The multi-scale surface features produced by laser irradiation are presented in Fig. 2. Besides the intended groove geometries of ~10 µm (Fig. 2(a)), pillars, bumps and striations (with sizes of ~1 µm) were produced in the grooves (Fig. 2(b)). The RMS surface roughness of the flat areas in the grooves was measured using AFM that was operated in the tapping mode. These were determined to be about 50–100 nm (Fig. 2(c)). The surface metrology characterization of all of the micro-textured surfaces is summarized in Table 2.

3.2. Surface chemistry

Typical XPS survey spectra obtained from the Ti-6Al-4V surface (before and after a laser treatment) are presented in Fig. 3. The main Table 2

<table>
<thead>
<tr>
<th>Surface type</th>
<th>Groove width (µm)</th>
<th>Groove height (µm)</th>
<th>RMS surface roughness in grooves (nm)</th>
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<tbody>
<tr>
<td>Polished (control)</td>
<td>–</td>
<td>–</td>
<td>3.4 ± 0.5</td>
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<tr>
<td>Groove pattern 0 with 20 µm spacing</td>
<td>11.4 ± 0.6</td>
<td>10.2 ± 0.5</td>
<td>380 ± 42</td>
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<tr>
<td>Groove pattern 0 with 40 µm spacing</td>
<td>12.2 ± 0.6</td>
<td>9.5 ± 0.5</td>
<td>399 ± 37</td>
</tr>
<tr>
<td>Groove pattern 0 with 60 µm spacing</td>
<td>12.7 ± 0.7</td>
<td>9.0 ± 0.5</td>
<td>256 ± 34</td>
</tr>
<tr>
<td>Groove pattern 1 with 20 µm spacing</td>
<td>10.9 ± 0.5</td>
<td>10.1 ± 0.5</td>
<td>394 ± 52</td>
</tr>
<tr>
<td>Groove pattern 2 with 20 µm spacing</td>
<td>11.2 ± 0.6</td>
<td>9.8 ± 0.5</td>
<td>345 ± 46</td>
</tr>
<tr>
<td>Groove pattern 3 with 20 µm spacing</td>
<td>11.6 ± 0.6</td>
<td>10.2 ± 0.5</td>
<td>356 ± 55</td>
</tr>
</tbody>
</table>

Fig. 2. Multi-scale surface features induced by laser irradiation: (a) groove geometries (~10 µm), (b) micro pillars, bumps and striations (~1 µm) in grooves, (c) relatively smooth surfaces in grooves with RMS of ~80 nm measured using AFM.
chemical elements that were observed include: C, Ti and O, with small amount of Al. Fig. 4 shows high resolution Ti 2p, Al 2p and O 1s photoelectron spectra obtained from Ti-6Al-4V samples before (black curve) and after (red curve) laser treatment. A Ti 2p3/2 peak, on as-received sample, was obtained at 458.9 eV (Fig. 4a) with 5.8 eV splitting between the Ti 2p1/2 and Ti 2p3/2 peaks. This indicates that the surface was primarily in the form of metallic TiO2. Narrow scan spectra of the Al 2p region were obtained for Ti-6Al-4V samples before and after laser treatment. These are presented in Fig. 4b. The Al 2p spectrum for the as-received Ti-6Al-4V sample was resolved into two components; the main peak was located at 74.6 eV and attributed to Al2O3, while an additional small peak was located at 72 eV. The latter is attributed to elemental Al (metal). After laser treatment, the same two peaks were obtained on the Al 2p spectrum. The presence of vanadium oxides was not revealed for reasons that are already discussed in literature [20].

High resolution scans of the O 1s region (Fig. 4c) revealed two peaks. The oxide peak at 530.6 eV corresponds predominantly to the form of TiO2. In addition, a high intensity peak was obtained at 531.8 eV, which corresponds to hydroxyl groups in the form of Ti-OH. No significant changes were obtained in the O 1s spectra after laser treatment.

Hence, based on the above results, the laser processing used in this study does not significantly change the surface chemical composition of Ti-6Al-4V. The surface composition remains as TiO2 before and after laser treatment.

3.3. Observations of initial cell spreading and alignment

SEM images of the initial stages of cell spreading are presented in Figs. 5 and 6. These show how HOS cells spread and align on laser-
irradiated surfaces during the first 4 h of cell culture. For the grooved surfaces from group 1, little spreading was observed on the 20 µm micro-grooved surfaces after 15 min of cell culture (Fig. 5(a)). The cells were almost spherical and the nuclei were easy to resolve at this stage. There was also no evidence of contact guidance at this stage.

Fig. 5. SEM images of HOS cells cultured on micro-textured surfaces with groove pattern 0: (a) 20 µm, (b) 60 µm groove spacing.

Fig. 6. SEM images of HOS cells cultured on micro-textured surfaces: (a) groove pattern 2, (b) groove pattern 3 after 1 h of cell culture, and (c) groove pattern 2, (d) groove pattern 3 after 4 h of cell culture.
After 1 h, the cells were observed to spread within the grooves and above the grooves (Fig. 5(a)). There was clear evidence of cell stretching along and across the grooves. Many of the cells were observed to cover two adjacent grooves. However, the dominant direction of all stretching was along the grooves.

The stretching and alignment was more evident after 4 h of cell culture (Fig. 5(a)). Compared to the many cells that covered the two grooves after 1 h, most of the cells were found to spread within a groove (in a groove or along the ridges of a groove). The cells appeared to shrink from the cross groove direction and stretched more along the groove direction in durations between 1 and 4 h. The cell orientation was clearly evident after 24 h of cell culture, which resulted in almost complete coverage of the surfaces. Similar results were observed on the surfaces with groove separations of 40 and 60 µm. However, the randomness of the cell orientations between the grooves increased with increased groove spacing.

For grooved surfaces in group 2, we focus on groove patterns 2 and 3, since the observed cell behavior on grooved pattern 1 was very similar to that on groove pattern 0 with 20 µm spacing. Different from what we saw on groove patterns 0 and 1, after 1 h, a number of cells were observed to stretch along a direction perpendicular to the groove orientation. This is in contrast to the longitudinal groove orientation observed in groove patterns 0 and 1. The transverse orientation is more noticeable on pattern 3 than on pattern 2. However, this effect changes with culture time. After 4 h of cell culture, obvious cell contact guidance was observed along the grooves for both patterns 2 and 3 (Fig. 6(c) and (d)).

In order to quantify the effects of contact guidance, the SEM images were examined with respect to cell orientation. Orientation was measured by the angular separation between the groove axis and the major cell axis, as described in Ref. [12]. This was done using the ImageJ software package. About 100 cells were measured on each sample. A smaller orientation angle means a better cell alignment (contact guidance).

Fig. 7 shows the average orientation angle plotted against culture time. This was done for each of the micro-textured surfaces. Note that an average orientation angle close to 45° and with big standard deviation suggests a random distribution of cell orientation. An average angle close to zero and with a small standard deviation indicates good alignment. The results show that the orientation angles dropped very quickly (from about ~41° to 15°) on laser-grooved surfaces during the initial 4 h. In the next 20 h, the angles only decreased about 3°.

For group 1, strong trends were observed, with cells cultured on smaller groove spacings exhibiting more alignment along the groove axis than the cells growing on larger groove spacings. This is attributed to the lack of cell guidance on the smooth surfaces between grooves (Fig. 5(b)). In comparison, the HOS cells cultured on the group 1 of laser-irradiated micro-grooves exhibited much more contact guidance in the 4-hour culture, with the distributions narrowing, as the groove spacing decreased.

In the case of group 2, the cells cultured on groove pattern 1 exhibited faster contact guidance than those on the groove pattern 3. This was especially true for 1 h of cell culture. Also, the difference between the orientation angles on patterns 1 and 3 was about 35% (8.9°). However, the difference of orientation angles decreased with culture time, decreasing from about 35% (8.9°) after 1 h to 22% (2.8°) after 4 h. The same trend was observed on groove pattern 2, although the magnitudes of the orientation changes were much less. Therefore, the transverse alignment of the cells (normal to groove orientation) on groove patterns 2 and 3 resulted in slower cell alignment along the groove direction. Furthermore, the differences between the alignments on patterns attributed to differences in the striation spacings and heights.

Fig. 8 shows representative images of the stained HOS cells after 1 h and 4 h of incubation (actin and vinculin expressed as red and green, respectively). The staining of actin revealed differences in cell orientation (Fig. 8(a) and (c)). Anti-vinculin staining (focal adhesions considered as bright, short, and dense patches) was used to determine the mode of HOS cell adhesion on the various surfaces (Fig. 8(b) and (d)). This was determined by assessing the focal adhesion distributions. On the smooth surfaces, focal adhesion points were distributed randomly around the cell [12]. However, on the laser-grooved surfaces, focal adhesion points aggregated at the bottoms of the grooves or ridges where the cells were anchored. On all laser-irradiated surfaces, focal adhesion points increased with culture time.

Confocal microscopy images are presented in Fig. 9. Vinculin was stained green again. These show three-dimensional (3D) structures of two representative cells on the grooves. The focal plane moved from the bottom of the groove to the top of the cell with an increment of 0.6 µm. An image was taken at each position. Fig. 9(a) shows a cell on groove pattern 1 elongated along the groove. Both the bottom and top halve of the cell stretched in the same direction (the groove direction). However, in the case of a cell on groove
pattern 3 (Fig. 9(b)), the bottom half of the cell stretched along the direction perpendicular to the groove (the striation direction), while the top half of the cell elongated along the groove. This shows clearly the initial contact guidance was guided by the transverse striations. Furthermore, once the transversely guided cells stretch across the grooves, they oriented themselves along the longitudinal grooves, and were guided subsequently by the longitudinal groove orientation.

Fig. 8. IF staining images of HOS cells cultured on: (a, b) laser grooved surfaces with groove pattern 1 after 1 h of cell culture, and (c, d) laser grooved surfaces with groove pattern 1 after 4 h of cell culture. (red: actin; green: vinculin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Confocal IF staining images (green: vinculin) of HOS cells cultured on: (a) laser grooved surfaces with groove pattern 1, (b) laser grooved surfaces with groove pattern 3 after 4 h of cell culture (the value on each image is the height of the focus plane from the bottom of the groove); SEM images of HOS cells cultured on: (c) laser grooved surfaces with groove pattern 1, (d) laser grooved surfaces with groove pattern 3 after 4 h of cell culture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 9(c) and (d) show corresponding SEM images (not exactly the same cells) on these two surfaces after 4 h of cell culture to give readers a clear view of how cells look like from the top on these two types of surfaces.

3.4. Initial cell adhesion measurement

The enzymatic detachment experiments revealed several trends with respect to cell adhesion and surface topology. Figs. 10 and 11
show the cell detachment rates, as a percentage of the total number of cells detached on all the laser-irradiated surfaces. At a given time, a higher percentage of detached cells indicate a higher cell detachment rate and correspondingly weaker cell adhesion. The cell detachment results obtained from the laser-irradiated surfaces showed that the HOS cell detachment rates decreased, as the culture time increased from 1 to 4 h. These suggest that cell adhesion increases with increasing cell culture duration. However, for the grooved surfaces in group 1, cell adhesion on laser-irradiated surfaces with smaller groove separations of ~20 µm increased at much faster rates than those on laser-irradiated surfaces with larger groove separations (40 and 60 µm). Following the initial 5 min of the detachment test, the cell detachment rates on micro-grooved surfaces with 20 µm separations dropped by about 24% (from 46 to 22%), as the cell culture duration increases from 1 to 4 h. Detachment rates on 40 µm micro-grooved surfaces decreased by 15% (from 53 to 38%), while those on the 60 µm micro-grooved surfaces decreased by about 10% (from 63 to 53%).

The above cell alignment results (Fig. 7) and cell adhesion measurements (Figs. 10, 11) show that there is a positive correlation between the initial cell alignment and the extent of cell attachment to the laser-irradiated surfaces (Fig. 12). The greater the cell alignment, the greater the overall level of adhesion indicated by the trypsin detachment results. This correlation is more obvious for the grooved surfaces in group 1 than in group 2. For the grooved surfaces in group 2, there were no significant statistical differences between the cell detachment rates on the patterns 1, 2 and 3. However, a reduction in cell alignment was observed on patterns 2 and 3 due to the striations within grooves.

4. Discussion

Cell alignment on biomedical surfaces could result from two kinds of stimuli, chemical and topographic [18]. In this study, the XPS results showed that there were no noticeable microchemical changes on the Ti-6Al-4V surfaces after laser processing. Hence the analyses of the cell alignment in this study on laser grooved Ti-6Al-4V surfaces focus on surface topography.

Material surface topography can be divided into roughness and texture [21]. Here “roughness,” corresponds to non-standardized discontinuities, while “texture” refers to a standardized and controlled pattern [22]. Several prior studies have examined the effects of surface texture on cell behavior [21–31]. The studies have provided insights into the behavior of different cell types, such as osteoblasts, fibroblasts, epithelia etc, on a wide variety of material substrates such as silicon, glass, Ti and polymers etc.

In many of these studies, the grooves were introduced onto the surfaces using photolithography, since photolithography allows for the accurate control of groove geometries on different substrates [21–25, 27]. Furthermore, the surfaces are often very smooth, and hence the results were not strongly affected by the presence of surface discontinuities that are typically found on the surfaces of laser micro-grooves. In the absence of such surface discontinuities, the rate of cell spreading and contact guidance is slower, since there are fewer surface features for the focal adhesions to grab onto. Also, in such cases, the results show that the groove depth is more important than the groove width [23, 24, 27]. Alignment is also diminished when the spacing is much larger than the breadth of the cell [27]. Continuous edges (sharp edges) are better than discontinuous edges [27].

However, the scenarios are somewhat different for laser grooves. Laser grooves are typical discontinuous grooves (Fig. 1). Hence, after 4 h, most of the cells (over 90%) are aligned (Fig. 7). Even on grooves with 60 µm spacing, obvious contact guidance was observed after 4 h (Fig. 7). To explain this difference, the inherent surface roughening induced by laser irradiation must be considered. It is also important to note here that the induced roughness is multi-scale in nature. Hence, the surface features vary from about 50 nm to 1 µm (Fig. 2). Depending on their sizes and spacings, such features may provide sites for focal adhesions to grab onto during cell locomotion and spreading.

Bailly [32] pointed out that when new focal adhesion points form between cell receptors and surface ligands, inner tension forces inside the cells are caused by the stretching of the cytoskeleton. These detect some weak adhesion points and enable the cells to move towards new stronger focal adhesion points. The above mechanism by Bailly [32] was confirmed by the observations of initial cell spreading on laser-grooved surfaces, especially cells shrinking from the cross groove direction and stretching more along the groove direction from 1 to 4 h of cell culture because it is much easier for cells to find better sites to form stronger focal adhesion points along the groove axis direction than any other directions. Thus, it is reasonable to expect a positive correlation between cell orientation and cell adhesion (Fig. 12). This was observed on micro-grooved surfaces in groups 1 and 2. In the case of the grooves with the 60 µm wide groove spacings (groove pattern 0 in Fig. 7), the randomness of the cell orientations on relative smooth surfaces between the grooves decreases the overall adhesion compared to that on surfaces with 20 µm separation (Fig. 7). Also, the combined multi-storey transverse and longitudinal cell orientations observed in groove pattern 3 do not have a significant effect on the overall cell detachment/adhesion (Figs. 10 and 11).

Further work is clearly needed to establish the optimal nanoscale surface features that are required for optimal adhesion to laser micro-grooved surfaces. Nevertheless, the current results are of importance, since they provide new insights into how longitudinal and transverse micro-scale surface features affect the earliest stages of cell immobilization and spreading on discontinuous laser micro-grooved geometries. The results also suggest that cell contact guidance on laser-irradiated Ti-6Al-4V surfaces is caused by nano- and micro-scale asperities that provide the sites for focal point adhesion to grab onto during cell locomotion and spreading.

The current results also show that cell contact guidance is promoted by the geometrical confinement provided by the micro-groove geometries. The resulting contact guidance (Figs. 5–7) and increased cell adhesion (Fig. 8) can have significant impact on the extent of scar tissue formation [9] and micromotion [8] during the earliest stages of wound healing associated with the insertion of orthopedic and dental implants. Animal studies are clearly needed to investigate adhesion, wound healing and osseointegration in vivo animal models containing titanium surfaces with transverse and longitudinal groove geometries.
5. Conclusion

This paper presents the results of an experimental study of the initial cell spreading and adhesion of HOS cells on laser-irradiated Ti-6Al-4V surfaces with well-controlled micro-groove spacings and orientations. The spreading and adhesion of HOS cells was investigated during the first 24 h of cell culture. Salient conclusions arising from this study are summarized below.

1. Relatively straight and uniform micro-grooves are produced on Ti-6Al-4V using a UV laser operated at wavelength of 355 nm. These grooves have micron-scale (~1 µm) and sub-micron-scale (~0.1 µm) surface features on the ridges between the grooves, and along the grooved surfaces that affect subsequent cell spreading and adhesion.

2. No significant changes in surface microchemistry occur for the range of laser processing parameters that was employed in this study. The microchemical analyses show that the surfaces consist of TiO₂ layers in all cases.

3. Longitudinal cell contact guidance (alignment along the groove directions) was observed on all of the six types of laser-irradiated micro-grooved surfaces that were examined in this study. Also the extent of longitudinal cell contact guidance increased with decreasing longitudinal groove spacing.

4. Some short-term (1–4 h) transverse cell contact guidance was also promoted by transverse patterns, although long-term contact guidance occurred in the longitudinal direction after ~4 h of cell culture.

5. The enzymatic detachment results showed that enhanced attachment occurred on the micro-grooved Ti-6Al-4V surfaces during the initial 4 h of cell culture. The overall adhesion (measured by a trypsin detachment test) almost doubles, as the cell culture period increases from 1 to 4 h. There exists a positive correlation between cell orientation and cell adhesion.

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