Combined RLM, ESEM and AFM Study of Osteoblasts on Titanium Alloy Surfaces

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INTRODUCTION

The examination of the morphology, adhesion and growth of cells cultured on opaque substrates presents a particular challenge to microscopists [1-3] that is not evident when using transparent culture plates. If we make use of other techniques such as SEM, TEM, cutting, grinding and polishing, etc., with the aim of eradicating this problem, the number of artifacts introduced by each of them leads to their exclusion. Thus, we can only use one of them to assess the cellular morphology [4, 5].

This work describes the use of a combined microscopical technique, which has given us the opportunity to calculate the number of cells, to identify morphological patterns, and even to analyse these patterns in totally opaque samples and always with the same samples/cells. We have observed cellular morphology by reflected light microscopy, environmental scanning electron microscopy and atomic force microscopy techniques allowed the study of the morphology of cells adhering to these opaque materials.

The results show the value of the application of these techniques for the correct evaluation of morphological changes and the possibility of quantifying them in relation to medium and long term cellular surface evolution.

KEYWORDS
osteoblast, morphology, titanium alloys, surface treatment, reflected light microscopy, environmental scanning electron microscopy, atomic force microscopy

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ABSTRACT

The relationship between the morphology of adherent osteoblasts and the surface treatment of their titanium alloy substrates was studied. An ingenious combination of reflected light microscopy, environmental scanning electron microscopy and atomic force microscopy techniques allowed the study of the morphology of cells cultured on opaque substrates presents a particular challenge to microscopists [1-3] that is not evident when using transparent culture plates. If we make use of other techniques such as SEM, TEM, cutting, grinding and polishing, etc., with the aim of eradicating this problem, the number of artifacts introduced by each of them leads to their exclusion. Thus, we can only use one of them to assess the cellular morphology [4, 5].

This work describes the use of a combined microscopical technique, which has given us the opportunity to calculate the number of cells, to identify morphological patterns, and even to analyse these patterns in totally opaque samples and always with the same samples/cells. We have observed cellular morphology by reflected light microscopy, environmental scanning electron microscopy (ESEM) [6] and atomic force microscopy (AFM) [7,9].

MATERIALS AND METHODS

Titanium alloy disc substrates

The Ti substrates were obtained from Technalloy S.A., a Spanish supplier of alloys to orthopaedic companies for use as dental, hip or knee implants (as bulk material). The discs were manufactured by Inasmet and were cut from a metal bar forming discs 15 mm (w) × 1 mm (h).

Disc surface treatments

Ion implantation was carried out on the polished side of each disc in a Danfysik high-current implanter 1090. Samples were ultrasonically degreased and cleaned prior to ion-implantation treatment, which was performed with a Chordis ion source at doses from 0.5 to 5 × 10¹⁷ ions/cm² and energies from 40 to 100 keV. Implantation was performed at low temperature (~170°C) and at a vacuum better than 4 × 10⁻⁵ Pa.

Before carrying out the cell assay, the titanium discs were sterilized with UV light. Two types of surface treatment by ionic implantation to the Ti6Al4V were assayed, and the results were compared to those obtained for the base material without treatment (Table 1).

Surface treatments were: 16 discs of Ti6Al4V, treatment: 5CO5O (type A); 16 discs of Ti6Al4V, treatment: 3C5O5O (type B); 16 discs of Ti6Al4V, treatment: 3C5O + backfilling CO₂ (type C). 16 discs of Ti6Al4V without treatment (type D).

Cell cultures

The ATCC, CRL-11372 line of hFOB 1.19 human bone cells was used. Samples to be assayed were plated with 1.5 × 10⁴ cells/tube in 6-well microtitre culture plates for 4 hours at 37°C and 5% CO₂. After that time, samples were removed, washed with phosphate buffered saline, fixed with methanol and stained with the monochromatic dye crystal violet. Once the stain was prepared, a qualitative assessment of the morphology of osteoblasts adhering to these substrates was carried out with reflected light microscopy. The results show the value of the application of these techniques for the correct evaluation of morphological changes and the possibility of quantifying them in relation to medium and long term cellular surface evolution.

Figure 1: Relationship between number of adherent osteoblasts and surface treatment.
ent to the titanium samples was performed using reflected light microscopy, ESEM and AFM techniques.

**Reflected light microscopy**

Cell images were taken on a Reichert-Jung MEF3 inverted microscope using reflected light optics and 50x magnification of a real area of 3.05 mm². The number of adherent cells was quantified by image analysis using the Buehler Omnimet system.

**Environmental scanning electron microscopy**

Electron microscopy and microanalysis was performed with a JEOL ISM-5910 LV scanning electron microscope, and energy-dispersive spectroscopy (EDS), using the following parameters: accelerating voltage 15kV, chamber pressure 30 Pa, and secondary electron imaging.

**Atomic force microscopy**

Atomic force microscopy was performed with a Digital Instruments (Veeco) Multimode atomic force microscope (AFM). Images were obtained by the tapping mode technique in air. A silicon probe with reference RTESP (rotated tapping mode etched silicon probe) was used, with a resonant frequency of 300 kHz.

**RESULTS**

Once the cells were stained, a quantitative assessment of the grade of cellular adhesion to the different samples was performed using the image analysis system to obtain the percentage area of each sample occupied by the adherent cells and the percentage area occupied by a single cell. The results are represented as treatment type versus mean cellular density (mean of number of adherent cells/mm²) of sample.

As shown in Figure 1, it was observed that type A treatment was the one that produced the least osteoblast adhesion (27.2% less than on type C material). However, a similar grade of adhesion was produced in type B and C treatments (only 0.6% less adhesion was observed). Furthermore, no significant differences were observed related to the adhesion between different surfaces (taking into account that the standard deviation of the results was very high).

**Light microscopy**

The images obtained by reflected light microscopy showed the morphology of cells adhering to the Ti6Al4V substrates.

In images taken of the three sample types (Fig. 2) it was observed that there were three different types of cell morphology depending on the material to which they adhered. As it can be seen in the images, a round morphology was adopted by cells adhering to type C material, while for type A, cells acquired a more spread-out and star-shaped morphology (Fig. 2b). In addition, cells adhering to type B material showed an enlarged shape (Fig. 2c).

**DISCUSSION AND CONCLUSIONS**

Fixation with alcoholic solution followed by staining preserved the morphology of the cells. As it can be seen, although osteoblasts cultured on polycarbonate plates usually maintain a fusiform aspect, when they are cultured on titanium, the form they adopt depends on the surface treatment received by the metal [6]. This study was carried out using an opaque material, where it is clearly more difficult to observe the adhered cells than with traditional transmittance techniques. As biomaterials used in orthopaedics mainly correspond to non-transparent materials, this study had to be done with microscopical techniques that study the light reflected by opaque materials. In order to use the above-mentioned technique, it is necessary to stain those cells adhering to the material surface, otherwise the morphology adopted by a cell population could not be observed.

If we compared these morphologies to histological sections that show us the bone cells in their natural form, we would observe that the dominant morphology in the type B treatment strongly resembles the form of periosteum osteoblasts, which are powerfully polarized usually forming long cellular chains generating bone matrix. By contrast, the main cell morphology on the type A surface was more spread out, as can be found in osteoblasts trapped inside the more opaque bone material, resembling in a way the spreading of activated platelets. Finally, type C results are very similar to that commonly observed in polymer plate cultures without surface treatment. As there is a known relationship between the form adopted by osteoblasts when proliferating over a surface and their sensibility to different growth and differentiation factors [10, 11], we consider that it could be of major interest to correlate the different morphologies found with the expression of specific products of osteoblasts such as alkaline phosphatase.
and with their long term behaviour (e.g. apoptosis markers).

We can conclude that the adhesion and morphology of cells on opaque materials can be studied using the techniques discussed in this article, in such a way that original cell morphology after the adhesion process to the material remains intact.

REFERENCES


Table 2:
Morphological measures of osteoblasts depending on the material to which they adhered. Digital Instruments Nanoscope Software version 5.12 data analysis and treatment system was used to determine cellular morphology. This system permits three-dimensional measures on the images captured by AFM. Measurable maximum distance between cell edges was considered the major axis. Transverse direction was considered the minor axis.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Major Axis (µm)</th>
<th>Minor Axis (µm)</th>
<th>Maximum Height (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type C</td>
<td>61.535</td>
<td>38.637</td>
<td>0.917</td>
</tr>
<tr>
<td>Type A</td>
<td>62.986</td>
<td>44.371</td>
<td>1.136</td>
</tr>
<tr>
<td>Type B</td>
<td>70.978</td>
<td>12.845</td>
<td>1.281</td>
</tr>
</tbody>
</table>

Figure 3:
Environmental scanning electron microscopy images of different morphologies of osteoblasts on Ti6Al4V material. (a) Type C. (b) Type A. (c) Type B.

Figure 4:
Tapping-mode atomic force microscopy 3D images of cellular morphologies on Ti6Al4V material. (a) Type C. (b) Type A. (c) Type B.

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