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Karine Anselme, Maxence Bigerelle, B. Noël, A. Iost, P. Hardouin. Effect of grooved titanium substratum on human osteoblastic cell growth. *Journal of Biomedical Materials Research*, 2002, 60 (4), pp.529-540. 10.1002/jbm.10101 . hal-02566304

HAL Id: hal-02566304

<https://hal.science/hal-02566304>

Submitted on 17 Apr 2024

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Effect of grooved titanium substratum on human osteoblastic cell growth

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Abstract: Various surface treatments have been developed to increase the clinical performance of titanium-based implants. Many *in vitro* tests have been carried out on substrates with varied surface topography for a complete understanding of osteoblasts. In previous research, we made the observation that surface roughness must be taken into account, not only in terms of amplitude but also in terms of organization. In this study, we tested the adhesion and proliferation of human primary osteoblasts on grooved titanium surfaces with various amplitudes and organizations of topography. The roughness was described at a scale above (macro-roughness) or below (micro-roughness) the cell size.

We observed better orientation and proliferation of human osteoblasts on surfaces with a micro-roughness characterized by a lower Order (parameter describing the organization of topography) and by a higher Ra and Rz (parameters describing the amplitude of topography). It appears that cultured human osteoblasts prefer surfaces with relatively high micro-roughness amplitude and with a low level of repeatability.

Key words: titanium; human osteoblasts; surface topography; grooved surface; cell adhesion

INTRODUCTION

For efficacy of orthopedic or dental implants, it is essential to establish a mechanically solid interface with complete fusion between a material's surface and the bone tissue with no fibrous tissue interface. Various surface treatments have been developed to increase the clinical performance of titanium-based implants.¹⁻³ As Vercaigne et al. noted, a distinction has to be made between macro- and micro-surface features. The first is related mainly to gross biomechanical stress and strain transfer between implant and bone although the second can affect cell-implant interaction more directly.⁴ Thus a complete understanding of osteoblast adhesion on materials with varied surface topography is essential to improve the bone/biomaterial interface.

In previous studies, we made the observation that

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Contract grant sponsor: European Funds for Regional Development (FEDER); contract grant number: Obj.2-99.2-01b-n°78

surface roughness must be taken into account, not only in terms of amplitude but also in terms of organization. We demonstrated lower adhesion and proliferation on less organized surfaces, such as sand-blasted Ti6Al4V surfaces, compared to polished ones.⁵ In order to verify these observations, we tested osteoblast adhesion and proliferation on organized grooved surfaces obtained by machine tooling of Ti6Al4V bars.

Previous reports have shown that osteoblasts are influenced by grooved surfaces.⁶⁻⁹ Chesmel et al. demonstrated that on smooth surfaces bone cells are oriented randomly but they are lined up parallel to the grooves in an end-to-end fashion in 5- μm -deep grooves. In contrast, they "ignored" the surface topography on a 0.5- μm grooved surface.⁶ Microgrooved polylactic acid surfaces have been shown to induce a higher mineralized extra-cellular matrix deposit by rat bone marrow cells on grooves with a depth of 1 μm and a width of 1 and 2 μm .⁹

In this study, we compare three different machine-tooled surfaces with different amplitudes and organizations of topography. A qualitative kinetic analysis using F-actin labeling reveals the orientation of cells in grooves at between 4 h and 6 days. A quantitative evaluation of cell adhesion and proliferation is corre-

lated with parameters describing surface roughness at a scale above (macro-roughness) or below (micro-roughness) the cell size.

MATERIALS AND METHODS

Surface preparation

Titanium alloy Ti6Al4V bars were machine-tooled under different conditions to obtain samples with three different roughness amplitudes (Ra) (Table I). Some of these samples were polished (I, MP).

Titanium alloy Ti6Al4V bars (14 mm in diameter) were machine-tooled in our laboratory (Machine Tooling Laboratory, ENSAM, Lille, France) using a classic Cazeneuve HB725 lathe to obtain samples measuring 2 mm in thickness. The conditions for machine-tooling were calculated to obtain an organized and regular grooved surface with various roughness amplitudes (A, B, H).

To obtain such a surface, we chose a turning tool in WCo (ref H13A) with a radius of 1.2 mm. We selected a rotation speed of 2500 rpm. As the rotation speed is constant, the linear cutting speed will decrease from the extremities to the center of the bar. If the linear speed is under 20 m/min., the material will be pulled out rather than cut and roughness will become more chaotic. Calculations show that this speed will lead to a damaged circular zone of 2.54 mm in diameter in the center of the samples. To obtain two different values of depth and width of grooves, we retained the radius speed of 0.15 mm/rotation (sample A) and 0.2 mm/rotation (sample B) that lead to groove widths of 150 μm and 200 μm , respectively, for a theoretic Ra of 0.6 μm and 1.2 μm , respectively. To obtain a chaotic machined surface, the radius speed should be equal to 1 mm/rotation and should give, without pulling out of the material, a theoretical Ra of 25 μm and a groove width of 1000 μm (the formula used is $Ra = f^2/0.039$, where f is the radius speed). However, as friction during machining process occurred, the damage of the surface decreased dramatically the theoretical roughness amplitude, which in the end measured $Ra = 3.35 \mu\text{m}$.

Using an automatic PEDEMAX 2 polishing machine, the samples were polished using grade 80 silicon carbide paper (I) or were mirror-polished, successively using grade 80, grade 120, grade 500, grade 1200, and grade 4000 silicon carbide paper and 3- μm and 1- μm diamond paste (MP).

Thermanox® round coverslips were used as the control for adhesion tests (Fisher, Elancourt, France).

TABLE I
Prepared Surfaces

Ra (μm)	Machining	Polishing
0.81	A	
1.21	B	
3.35	H	
0.31		I
~0		MP

Cell culture

Human osteoblasts were obtained from trabecular bone taken from the iliac crest of young patients. Cells were initially cultured in Dulbecco's modified essential medium (DMEM, Eurobio, France) containing 10% fetal bovine serum, 100 units/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin until confluence. The cells then were preserved in liquid nitrogen in complete DMEM + 10% dimethylsulfoxide (DMSO; Sigma, L'Isle d'Abeau, France) for several months. The cells then were thawed and cultured in 75- cm^2 flasks. At confluence, the cells were harvested using trypsin-EDTA and inoculated onto samples in 24-well plates. The medium was changed twice a week.

Scanning electron microscopy

Cell layers cultured on samples for 4 h, 1, 2, 3, or 6 days were fixed at 4°C in 2% paraformaldehyde (w/v) in monosodic dipotassium 0.2M buffer, rinsed in monosodic dipotassium 0.175M buffer, dehydrated in graded alcohol, critical-point dried with CO₂ (Emscope CPD 750, Elexience, Paris, France), sputter-coated (Emscope SC 500, Elexience, Paris, France), and examined using an Hitachi S520 scanning electron microscope at an accelerating voltage of 25 kV (Elexience, Paris, France).

Cell labeling

The cells were inoculated on the substrates at 4×10^4 cells/well in DMEM containing 10% FBS and 1% penicillin-streptomycin. Five incubation periods were chosen: 4 h and 1, 2, 3, and 6 days. Following the incubation period, the substrates were rinsed with PBS (phosphate-buffered saline without calcium and magnesium chloride). The cells were fixed for 20 min in 2% paraformaldehyde in monosodic dipotassium 0.2M buffer at room temperature, followed by three rinses with PBS. The cells then were permeabilized with 0.2% (v/v) Triton-X100 (Sigma, L'Isle d'Abeau, France) for 15 min at room temperature and rinsed three times with PBS. Nonspecific binding sites were blocked by incubating the samples in a 1% (w/v) bovine serum albumin in PBS for 20 min at room temperature. The cells were stained directly by FITC-conjugated phalloidin (25 $\mu\text{g}/\text{mL}$; Sigma, L'Isle d'Abeau, France) to reveal F-Actin microfilaments. The substrates were mounted on a microscope slide under glass cover slips using glycerin/PBS (50/50) containing 1.4-diazobicyclo-(2,2,2)-octane (DABCO, 25 mg./mL; Sigma, L'Isle d'Abeau, France) for photobleaching reduction.

The labeled cells were examined using a Zeiss Axioskop microscope (Zeiss, Le Pecq, France) equipped for epifluorescence.

Quantitative adhesion test

Samples of each surface were inoculated with 2×10^4 cells/sample. Four samples were analyzed after each incu-

bation period: 24 h, 7 days, 14 days, and 21 days. The cells were detached enzymatically from the samples by a diluted trypsin–EDTA (0.025% v/v) treatment, as previously described.^{5,10} The curve of percentage of released cells versus trypsinization time was established. The area included between the curve and the X-axis was evaluated. The areas obtained were considered as a detachment index, inversely proportional to cell adhesion on the biomaterial.

The cell detachment index obtained on each surface was divided by the cell detachment index on the control surface, that is, the Thermanox® surface, to calculate the detachment index percentage (DIP). Each of these experiments was reproduced twice, and the results were expressed as the DIP average of the two experiments.

The proliferation curves of human osteoblasts were established from the total detached cell count obtained during the cell adhesion test after each incubation period.

Roughness measurement

Roughness was measured using a tactile profilometer (Perthen M4PI, Mahr Measure, Göttingen, RFA) on the surfaces of the samples. The vertical and lateral resolutions were 12 nm and 0.4 μm, respectively. Five measurements were made on each of the six samples per roughness. For the machine-tooled surfaces, the scanning length was equal to 4 mm to avoid the damaged zone in the center of the sample. The scan was made perpendicular to the grooves. For other samples, the scanning length was equal to 12 mm and taken randomly on the surface. Profiles were digitized into 8000 points and analyzed on a computer, using personal software. A hundred roughness parameters were computed. Initially, no filtering was introduced into the data processing.

To characterize the roughness, ten different parameters were used. They can be classified in two categories: the amplitude parameters that represent a vertical measure of the roughness (depth of the grooves) and the frequency parameters that represent a horizontal measure of the roughness (width of the grooves).

Amplitude parameters

- Ra: Average roughness. This value did represent the mean height of the roughness (μm).
- Rt: The range of the roughness (maximum height–minimum height) (μm).
- Rz₁–Rz₅: The profile was divided into five identical parts. For each part, the local Rt (range amplitude Rz_i) was calculated. The more homogeneous the roughness, the closer the Rz values (μm).
- Rz: The mean between Rz₁ and Rz₅ (μm).

Frequency parameters

- SM: Mean spacing between the profile irregularities (μm).
- Peak: Number of peaks per inch of profile at the zero threshold.

- Autocorrelation parameter: First, we defined a normalized autocorrelation function and found the integer (*i*) such as

$$R(i) = \frac{1}{Rq^2 (N - i)} \sum_{j=1}^{N-i} y_j y_{j+i}$$

where y_i were equidistant discontinuous points in N points and Rq was the well-known standard deviation of the amplitude.

- LAC: Autocorrelation length L , such as $L = x_{i+1} = 1/e$.
- PAS: If the autocorrelation function was periodic, we calculated the period (PAS) by the discrete Fourier transform. PAS did represent the width of the periodic grooves (μm).
- Order: First, we defined a correlation integral J such as

$$J = \int_{x=0}^{x=L} R(x) dx$$

which represented a type of fundamental as regards a function symbolizing a certain order power. Second, we defined the K series I_k of integrals

$$I_k = \int_{x=kL}^{x=(k+1)L} |R(x)| dx$$

which represented a type of successive harmonics of the order power of profiles. Finally, the order parameter was defined as

$$Order = 100 \sum_{i=1}^K I_k / (K J)$$

and lay between 0 (white noise profiles) and 100 (perfect periodic profiles without noise).

A statistical analysis of the roughness effects on cell proliferation and cell adhesion was carried out using SAS® software (SAS Institute, Cary, NC).

RESULTS

Topographical aspects of surfaces before culture

Macro-roughness characterization

The measurements were made avoiding the central damaged zone. No filtering was done. Roughness profiles and autocorrelation functions are shown in Figure 1. Profiles A and B obtained the same morphologies. They were quite regular and periodic. By Fourier analyses, the periodicity between grooves was 151 μm for A samples and 205 μm for the B samples. We observed that these values were near the theoretic values explained by the machine-tooling process (150 μm and 200 μm, respectively). The maximal depth of the grooves was well characterized by the Rz parameters and due to the good homogeneity of the Rz₁ values. The Rz was greater on B samples than on A samples

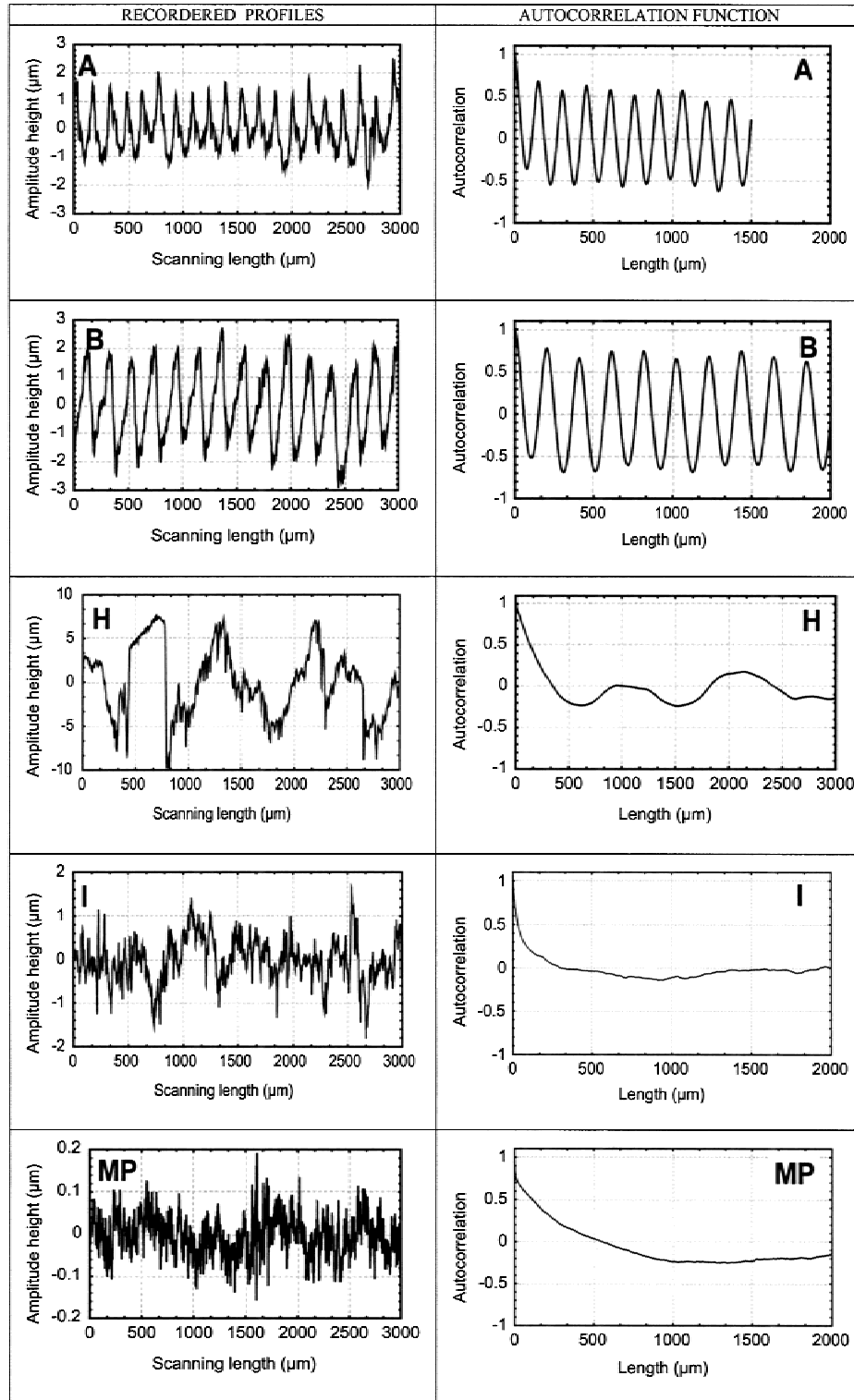


Figure 1. Macro-roughness profiles (left column) and autocorrelation function (right column) for each surface.

($5.30 \mu\text{m}$ versus $3.97 \mu\text{m}$). Both A and B samples displayed a very periodic surface, with the Order being around 50% (Table II).

The roughness of the machine-tooled H samples presents a more chaotic aspect (Fig. 1). The Order on the H surfaces was lower than on the A and B surfaces

(27%). However, relative to the autocorrelation function, we observed a period of $PAS = 1000 \mu\text{m}$ but with a low correlation ($r \sim 0.2$), indicating that very large grooves did exist. The roughness amplitude on H also was higher than on A and B surfaces ($Rz = 14.56 \mu\text{m}$) (Table II). The profile on H contained two compo-

TABLE II
Macro-Roughness Parameters of the Different Surfaces

	Ra	Rt	Rz ₁	Rz ₂	Rz ₃	Rz ₄	Rz ₅	Rz	LAC	Order	Peak	Pas	SM
A	0.8	5.23	4.06	3.90	3.99	3.76	4.14	3.97	33	47	194	152	68
	<i>0.07</i>	<i>0.37</i>	<i>0.04</i>	<i>0.3</i>	<i>0.4</i>	<i>0.3</i>	<i>0.4</i>	<i>0.32</i>	2	2.5	16		6
B	1.21	6.53	5.27	5.18	5.23	5.33	5.48	5.30	41	59	146	205	165
	<i>0.09</i>	<i>0.41</i>	<i>0.51</i>	<i>0.5</i>	<i>0.4</i>	<i>0.5</i>	<i>0.5</i>	<i>0.2</i>	3	4	10		12
H	3.35	22.2	15.7	14.22	14.04	13.40	17.41	14.96	211	27	138	1000	183
	<i>0.8</i>	<i>1.1</i>	<i>0.6</i>	<i>0.6</i>	<i>0.6</i>	<i>0.6</i>	<i>1.5</i>	<i>0.4</i>	20	1.7	10.5		11.5
I	0.31	3	2	2	1.90	1.90	2.17	1.99	63	22	451	—	58.3
	<i>0.09</i>	<i>0.1</i>	<i>0.1</i>	<i>0.1</i>	<i>0.1</i>	<i>0.1</i>	<i>0.1</i>	<i>0.06</i>	8	1.2	20		2.7
MP	0.07	0.73	0.44	0.44	0.46	0.46	0.45	0.45	192	36	1025	—	26
	<i>0.005</i>	<i>0.02</i>	<i>0.02</i>	<i>0.01</i>	<i>0.03</i>	<i>0.02</i>	<i>0.02</i>	<i>0.01</i>	30	2	103		2

Numbers in italics represent the standard error of the mean.

nents: a periodic one and a stochastic one (Fig. 1). Consequently, it became very difficult to characterize the roughness of H surfaces at only one scale.

For the polished surfaces, the Order was low (around 22%), and the autocorrelation function did not indicate the periodic component, suggesting that the grooves were not regularly spaced (Fig. 1). Roughness amplitude was low ($Rz = 1.99 \mu\text{m}$) and was very homogeneous because of the low dispersion of the Rz values (Table II). As scanning direction was chosen at random on the surface, the low dispersion of the parameter demonstrated that the surface was highly isotropic, meaning that no roughness direction could be found on the polished surface.

Micro-roughness characterization

Roughness parameters were computed on a given length that was the scanning length. They related to roughness between $0.5 \mu\text{m}$ (the sampling rate) and 4 mm or 12 mm (the sampling length). However, it was difficult to characterize the roughness at all the scales. For example, samples of H presented grooves with an elevated height ($Rz = 15 \mu\text{m}$) and with a large width ($PAS = 1000 \mu\text{m}$). This width was much larger than the cell size. However, in these large grooves, a micro-roughness did exist. The previous computation of the parameters did not take account this micro-roughness. For this reason, we attempted to analyze the micro-roughness that was at the scale of the cells ($<100 \mu\text{m}$). For this purpose we filtered the roughness profiles through a high-pass filter.

We used the Fourier Transform and retained only those frequencies greater than the inverse of the size of one cell ($1/50 \mu\text{m}$). By the inverse Fourier transform, a new profile was created that represented the roughness seen “under the cell” (Fig. 2). From these filtered profiles, we then calculated new “micro-roughness” parameters (Table III) using a mathematic formulation identical to those used in Table II, which we called “macro-roughness” parameters.

For the regular machine-tooled surfaces A and B, using the Sheffe statistical test at the confidence level of 0.05%, it was shown that the amplitude of the micro-roughness characterized by the Rz and the Ra did not differ significantly and lay around $Rz \sim 1.19 \mu\text{m}$ versus $1.79 \mu\text{m}$ and $Ra \sim 0.15 \mu\text{m}$ (Table III). Moreover, they were also identical to the Rz and Ra of the P80 polishing surface (respectively 1.62 and $0.18 \mu\text{m}$). On the other hand, the irregular machine-tooled H surfaces had a micro-roughness amplitude greater than that of the A and B surfaces ($Rz = 4.13 \mu\text{m}$, $Ra = 0.37 \mu\text{m}$). As was observed on experimental profiles, the micro-grooves were deeper for the H samples (Fig. 2).

The number of micro-peaks was equal for all machine-tooled surfaces and lay around 1100 peaks per inch. The mean average spacing between micro-grooves (SM) for all the tooled samples did not differ statistically and was around $21 \mu\text{m}$. This was confirmed by autocorrelation length values that also were similar and were around $6 \mu\text{m}$ (Table III).

However, as could be observed by the autocorrelation function, the microgrooves were rather periodic on A and B samples and unperiodic on H samples (Fig. 2). This was confirmed by the Order parameter that was 23% for surface B, 15% for surface A, and less than 10% on surface H (Table III). This parameter being highly discriminating, we concluded that the periodicity was $B > A > H$.

Scanning electron microscopy of cells after culture on the samples

The orientation and shape of cultured cells were determined by scanning electron microscopy at 4 h, 24 h, 3 days, and 6 days on machined and polished surfaces. On grooved surfaces, the cells were spread at 4 h on B surfaces and had a polygonal morphology [Fig. 3(b)], but on H surfaces the cells had a rather more fusiform morphology [Fig. 3(a)]. After 24 h, they appeared to be oriented along the grooves [Fig. 3(d,e)],

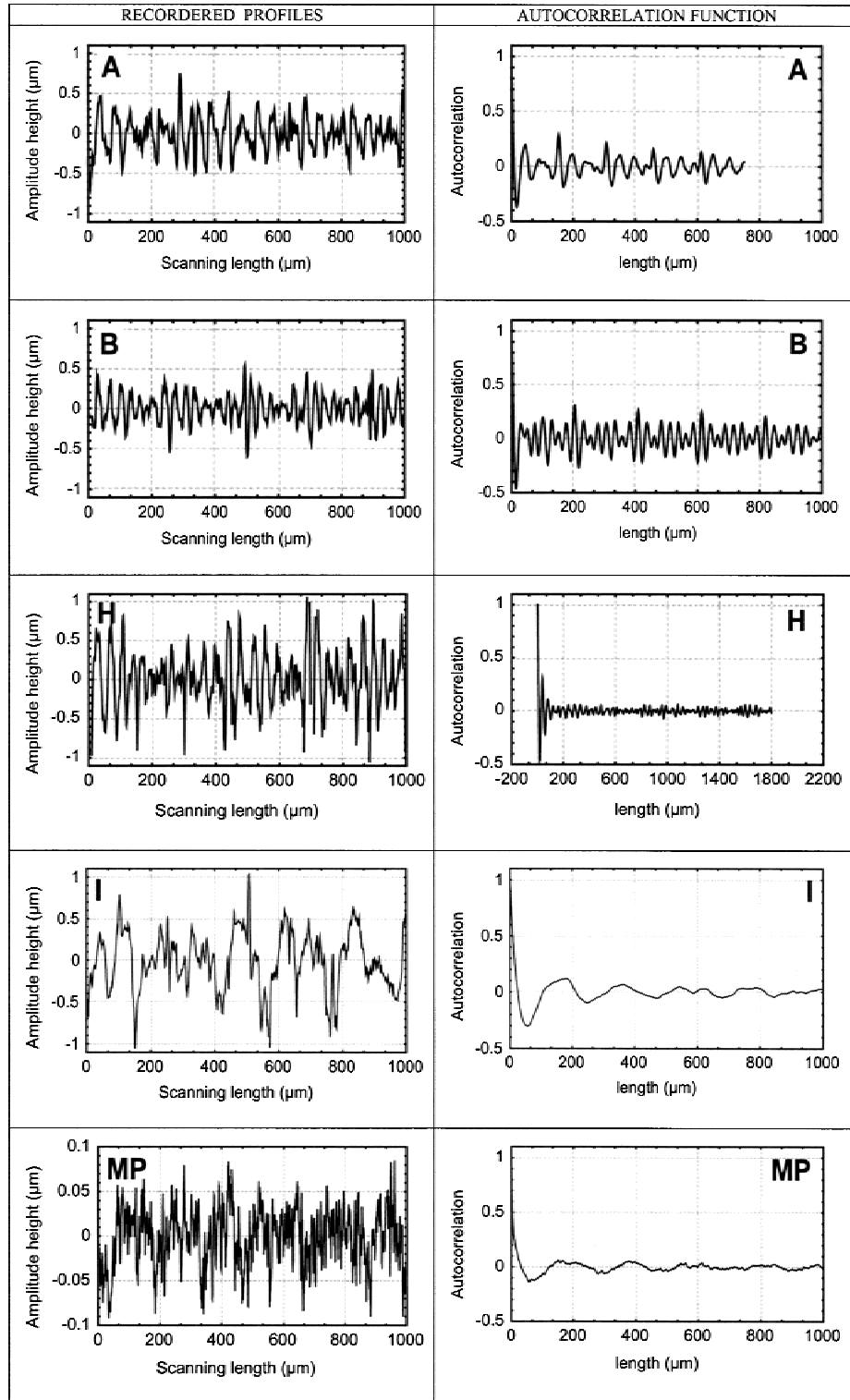


Figure 2. Micro-roughness profiles (left column) and autocorrelation function (right column) for each surface.

and sometimes at higher magnification the cells were seen to be aligned in deeper grooves with little lateral spreading [see inset in Fig. 3(d)]. On polished surfaces, osteoblasts were widely spread and randomly oriented at 24 h [Fig. 3(c)]. At later time points, as their

density increased, they became more elongated but continued to show random orientation. At later times, cells on grooved surfaces remained oriented along the grooves but re-covered the surfaces with a confluent layer [Fig. 3(f)].

TABLE III
Micro-Roughness Parameters of the Different Surfaces

	Ra	Rt	Rz ₁	Rz ₂	Rz ₃	Rz ₄	Rz ₅	Rz	LAC	Order	Peak	Pas	SM
A	0.15 <i>0.003</i>	1.87 <i>0.18</i>	1.39 <i>0.12</i>	1.01 <i>0.08</i>	0.99 <i>0.06</i>	1.12 <i>0.08</i>	1.45 <i>0.13</i>	1.19 <i>0.07</i>	6.02 <i>0.20</i>	23.4 <i>2.1</i>	1207 <i>35</i>	45	20.9 <i>0.5</i>
B	0.15 <i>0.007</i>	4.49 <i>0.61</i>	2.89 <i>0.34</i>	1.05 <i>0.06</i>	1.06 <i>0.05</i>	1.11 <i>0.05</i>	2.85 <i>0.37</i>	1.79 <i>0.14</i>	6.05 <i>0.10</i>	15.2 <i>1.2</i>	1084 <i>12</i>	30	23.2 <i>0.2</i>
H	0.37 <i>0.001</i>	6.30 <i>0.72</i>	4.71 <i>0.39</i>	3.69 <i>0.20</i>	3.96 <i>0.21</i>	3.42 <i>0.22</i>	4.89 <i>0.58</i>	4.13 <i>0.20</i>	5.95 <i>0.001</i>	9.60 <i>0.3</i>	1145 <i>15</i>	37	21.9 <i>0.3</i>
I	0.18 <i>0.001</i>	2.11 <i>0.12</i>	1.69 <i>0.12</i>	1.45 <i>0.09</i>	1.61 <i>0.13</i>	1.67 <i>0.15</i>	1.68 <i>0.12</i>	1.62 <i>0.10</i>	13.5 <i>0.49</i>	12.2 <i>0.6</i>	726 <i>24</i>	—	35.1 <i>1.2</i>
MP	0.03 <i>0.001</i>	0.66 <i>0.09</i>	0.34 <i>0.04</i>	0.28 <i>0.03</i>	0.34 <i>0.06</i>	0.43 <i>0.10</i>	0.49 <i>0.08</i>	0.37 <i>0.04</i>	6.61 <i>0.87</i>	6.80 <i>0.4</i>	2049 <i>41</i>	—	12.1 <i>0.2</i>

Numbers in italics represent the standard error of the mean.

Organization of cells on grooved surfaces

Kinetic observation of cell growth on grooved surfaces using F-actin labeling showed the orientation of cells along the grooves at 4 h on H surfaces but not on

A and B surfaces (Fig. 4). On H surfaces, cells appeared more elongated than on A and B surfaces (Fig. 5). Soon after 3 days, on A and B surfaces a confluent cell layer re-covered all surfaces except central zones, where confluence was attained after 6 days. At 6 days,

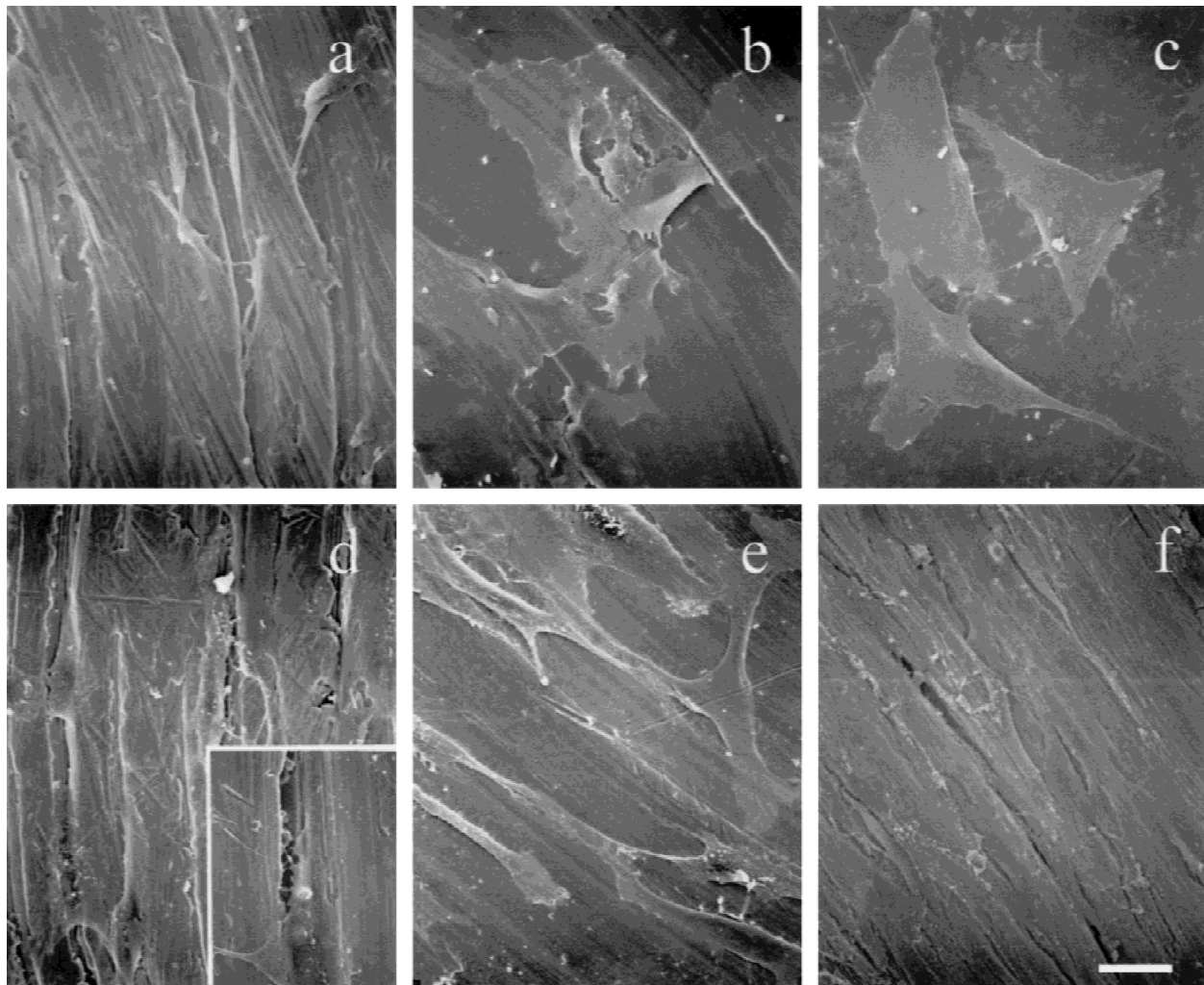


Figure 3. SEM micrographs of surfaces after culture: (a) surface H after 4 h of culture, bar = 32 μm ; (b) surface B after 4 h of culture, bar = 32 μm ; (c) surface I after 24 h of culture, bar = 32 μm ; (d) surface H after 24 h of culture, bar = 32 μm ; (e) inset: surface H after 3 days of culture, bar = 23 μm ; (f) surface B after 24 h of culture, bar = 32 μm ; and (g) surface H after 6 days of culture, bar = 32 μm .

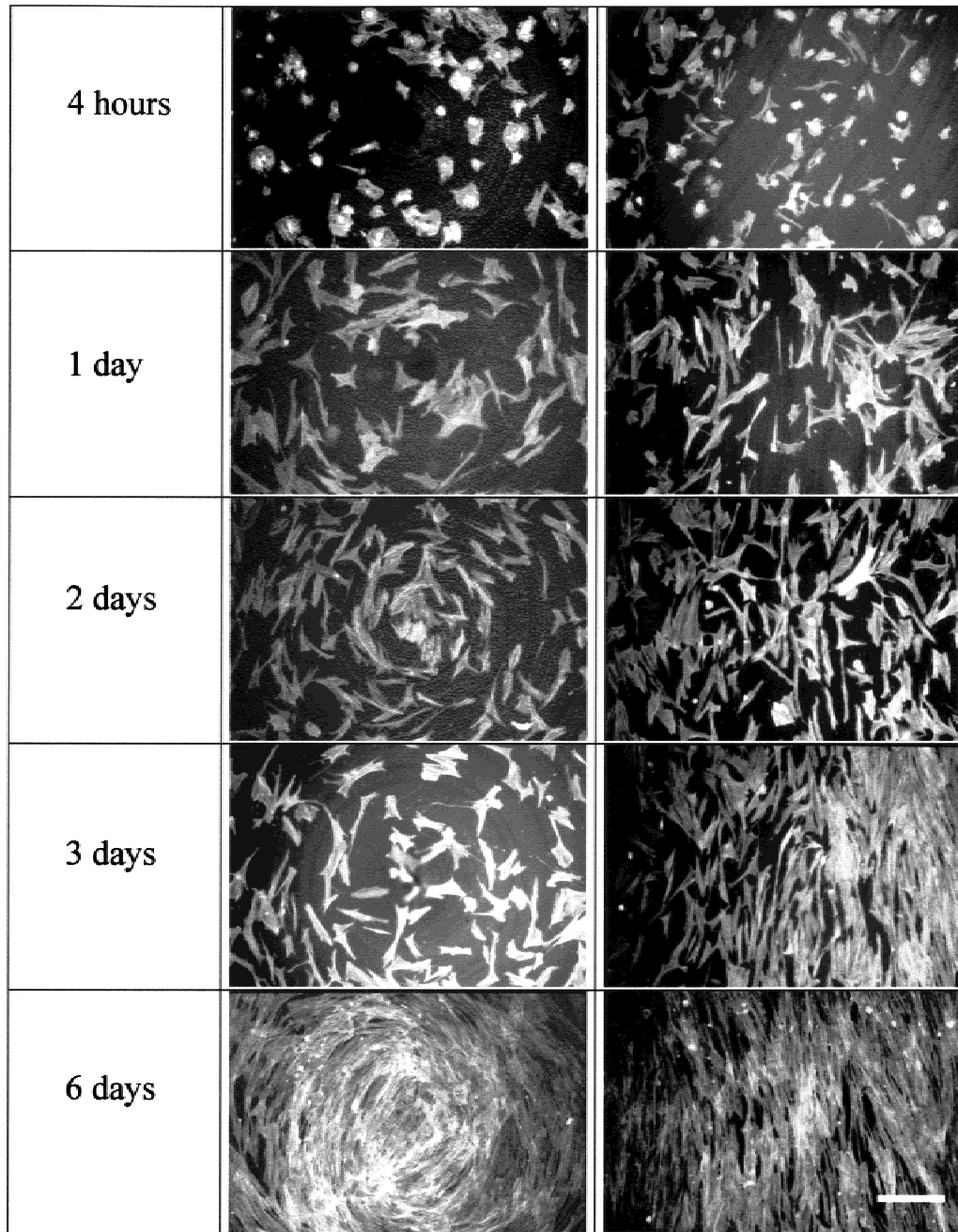


Figure 4. Kinetic observation of cell orientation on A surfaces after 4 h, 1, 2, 3, and 6 days. The cells were labeled by phalloidin-FITC to reveal the F-actin fibers. The center (left column) and the periphery of the surfaces (right column) were observed. Bar = 250 μm .

a cell layer with a circular orientation surrounded a central zone where cells were oriented in a parallel manner (Figs. 4 and 5).

Effects of roughness on cell adhesion and proliferation

As previously described,^{5,10} we observed an increase of cell adhesion (lower DIP) as a function of time (Fig. 6). Adhesion on titanium-based substrates

was equivalent to that of the control (DIP ~100%) after 1 and 7 days and became higher at 14 days. At later times, DIP appeared slightly lower on P80 polished surfaces (I) and coarse machined surfaces (H) (Fig. 6). However, the intersurface comparison did not demonstrate any significant difference of adhesion between titanium-based machined and polished surfaces at each time point.

Proliferation was the same on all surfaces except on H surfaces. Proliferation was higher on H surfaces compared to other surfaces, notably after 21 days of culture (Fig. 7).

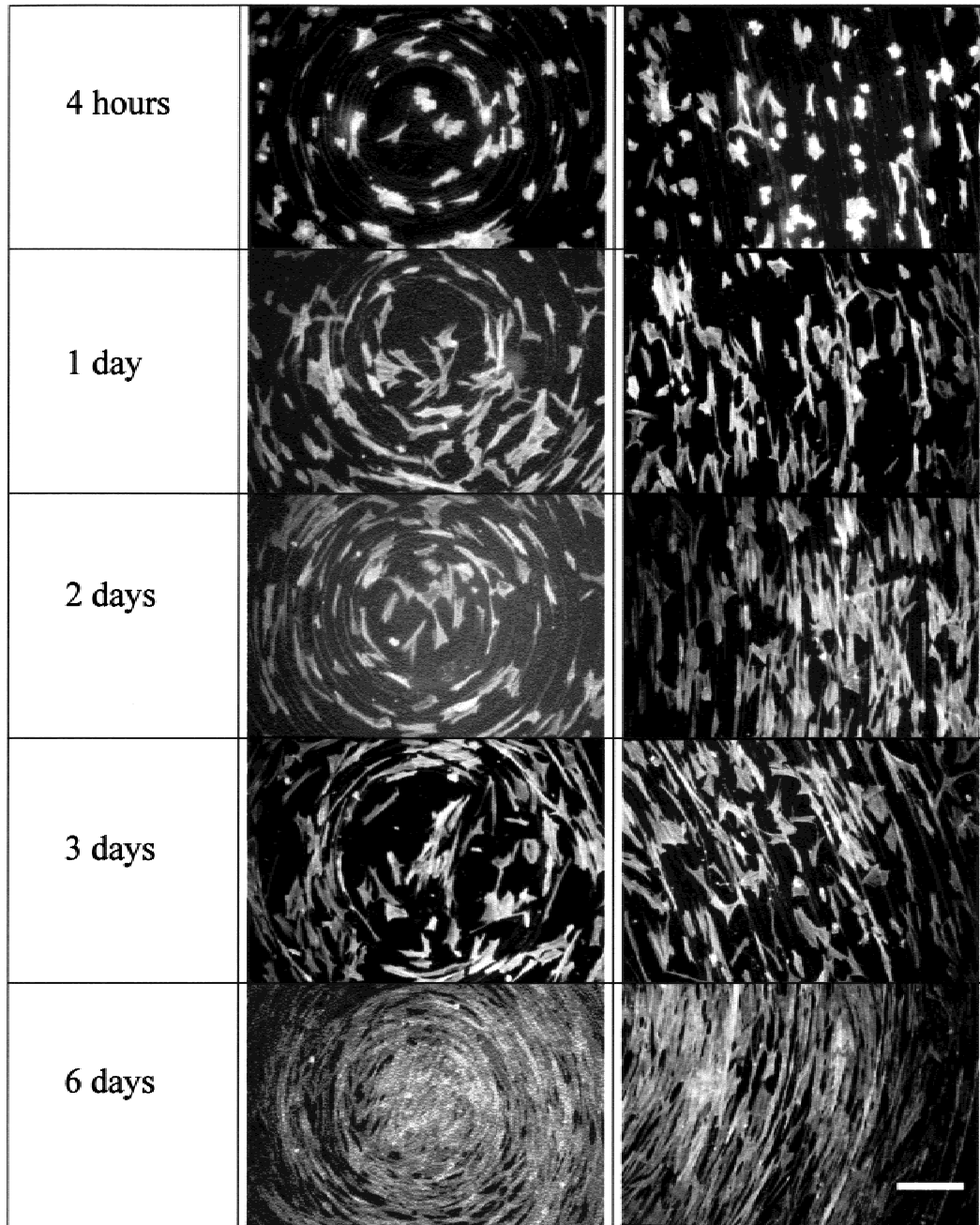


Figure 5. Kinetic observation of cell orientation on H surfaces after 4 h, 1, 2, 3, and 6 days. The cells were labeled by phalloidin-FITC to reveal the F-actin fibers. The center (left column) and the periphery of the surfaces (right column) were observed. Bar = 250 μm .

DISCUSSION

It is now well known that osteoblasts react differently relative to a substrate's roughness.¹¹ Notably, Boyan et al. demonstrated that the MG-63 human osteoblast-like osteosarcoma cell line respond to increasing titanium surface roughness with decreased proliferation and increased osteoblastic differentiation.^{12–15} Keller et al. have shown that the highest level of rat osteoblast cell attachment was obtained with rough, sandblasted Ti6Al4V surfaces compared to grooved ones although their Ra values were identical.¹⁶ These

results led them to the hypothesis that surface roughness amplitude per se may not be as important as other surface properties.¹⁷ Likewise, in a previous work we demonstrated that the amplitude of roughness is not the only influential parameter in human osteoblast response; roughness organization parameters also significantly correlate with proliferation and adhesion parameters.⁵

In this work we used a test for cell adhesion based on an enzymatic cell detachment.⁵ In our opinion, it was necessary to evaluate not only attachment at an early stage of culture but also to evaluate cell adhesion

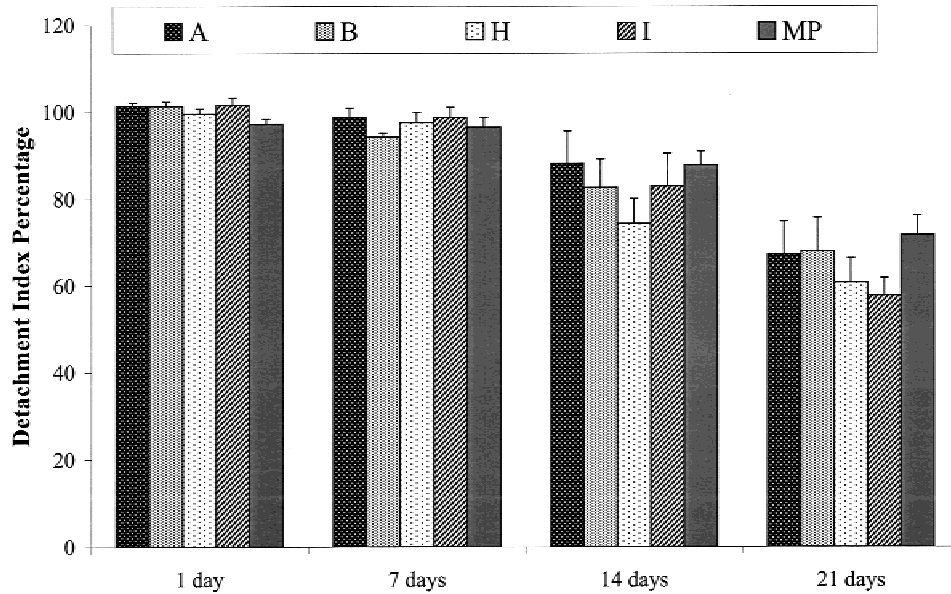


Figure 6. Detachment index histogram of the various tested surfaces as a function of time.

after several days. An *in vitro* evaluation of cell attachment 12 h after inoculation is not sufficient to anticipate the future integration of a material several weeks after implantation. *In vivo*, the biomaterial integration implies the establishment of a cell/matrix/material interface, and the future integration of the implant depends on its solidity. Then we chose to develop a progressive enzymatic cell detachment method to assess cell adhesion by measuring the cell/matrix bond strength at time points greater than 1 day.

This test is a measure of the sensitivity of the cells to trypsin and consequently is related to the amount of protein. Just after inoculation cells adhere directly onto the surface of the material (cell/material interface) and later onto a layer of extracellular matrix proteins (cell/ECM/material interface). Thus our mea-

sure corresponds early to the detachment of cells from materials and later from the protein layer. Finally, we evaluate the quality of the cell/material interface. A lower detachment after several days suggests that the interface is evolved and is closer to the *in vivo* cell/matrix/material interface.

Therefore, in the work described here, we chose to compare the effects on human osteoblast adhesion, orientation, and proliferation of grooved titanium surfaces (same organization) with various depths and widths of grooves (various amplitudes). We did not observe any visible morphologic differences between cells growing on A surfaces and those growing on B surfaces. Major differences did exist between these surfaces and H surfaces. On H surfaces, cells appeared more fusiform. SEM observations displayed pictures

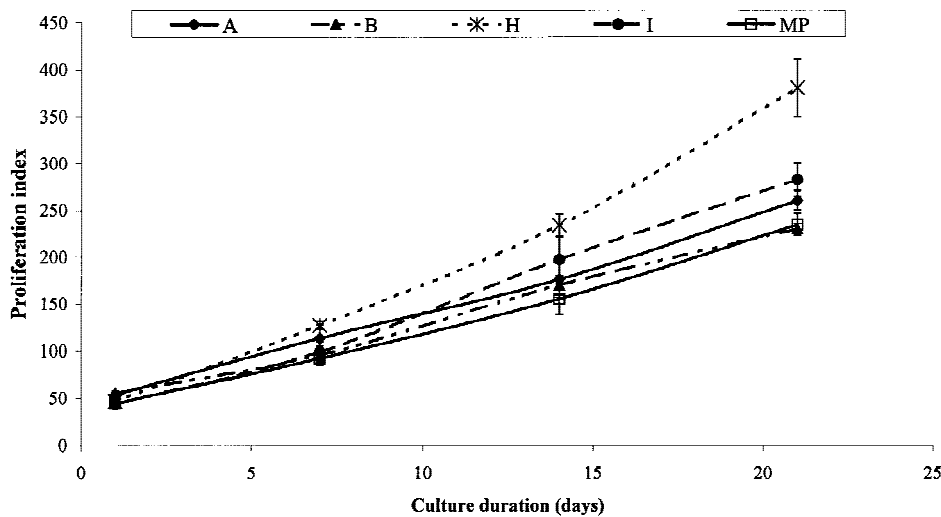


Figure 7. Proliferation index of human osteoblasts cultured on Ti6Al4V samples with varied surface roughnesses.

of cells looking as if they had “fallen into cracks” on the surface. This morphologic aspect is coherent with those described by Walboomers et al. They showed that rat fibroblasts cultured on microgrooved titanium substrates need grooves with a minimal width of 10 μm in order to contact the floor of the grooves.¹⁸

Moreover, quantitative evaluation of proliferation showed higher values for cells grown on H surfaces. We did not observe any significant differences in proliferation and adhesion between the A and B surfaces although these two surfaces were different in their macro-roughness parameters (Ra, Rz, Order . . .). When micro-roughness was considered, no more differences in roughness appeared between the A and B surfaces. On the other hand, the roughness parameters of H surfaces were very different at each scale compared to A and B surfaces. These observations indicate that cells react more to roughness at a scale of 10–100 μm than to roughness at the higher scale of 100–1000 μm . This was described previously by Curtis and Wilkinson⁸ and agrees with previous results describing various responses of cells to surfaces presenting grooves of 1 to 10 μm in width.^{6,7,9,18–22}

Larsson et al.²³ have demonstrated that even surface topography on the submicrometer is important. Fibroblasts, macrophages, and monocytes have been shown to react with alignment to a surface microstructure 1 μm in width and 1 μm in depth although keratinocytes and neutrophils did not.^{24,25} Concerning osteoblasts, Chesmel et al. have shown that cells align themselves in an end-to-end fashion parallel to the direction of the grooves within a 5- μm -deep groove although cells have been found to “ignore” the surface topography and span the width of the groove on a 0.5- μm -deep groove.⁶ Brunette et al. observed that the osteoblast-like cells assume an elongated shape on a 18- μm -deep grooved substratum and migrate in the direction of the grooves.⁷

The phenomenon of contact guidance has been largely studied by Jansen’s group using rat dermal fibroblasts cultured on microtextured silicone, polystyrene, titanium, or poly-L-lactic acid (PLA) substrates.^{18–22,26} They measured orientation between 1, 2, 5, and 10 μm in width and 0.5, 1, and 1.5 μm in depth and observed that the rate of orientation increases drastically when the grooves are made deeper. Although the surface with the deepest grooves has a total available surface greater than the others, the highest number of cells is not found in the deepest grooves because the cells bridge the grooves.²⁶ They demonstrated the same phenomenon for the behavior of rat bone-marrow cells on microgrooved PLA and polystyrene surfaces. Moreover, PLA surfaces with a groove depth of 1 μm and groove widths of 1 and 2 μm induced the most mineralized extracellular matrix.⁹

The correlation study we have done shows that the

higher the micro-roughness amplitude, the higher the proliferation. Another difference exists between A,B surfaces and H surfaces and concerns a frequency parameter, that is, the Order parameter. This parameter, which describes the repeatability of a roughness pattern, was much higher on A and B surfaces than on H surfaces, whatever the scale of analysis. Thus it appears that cells prefer surfaces with a relatively high micro-roughness amplitude (Ra = 0.37 μm and Rz = 4.13 μm) and with a low level of repeatability (Order ~10%).

The response to H surfaces may be related to the hypothesis of Curtis and Wilkinson, who proposed that cells react to discontinuities, with H surfaces presenting more discontinuities than do A and B surfaces.²⁷ They hypothesized, as did Jansen and co-workers, that discontinuities allow the condensation and the nucleation of actin.^{8,26}

These authors proposed that the dynamics of actin polymerization could be the explanation of contact guidance by way of a mechano-receptive response. Cells may tend to find a state in which internal and external forces will be favorably balanced for their differentiation. Cells cultured on micro-grooved substrata could be subjected to a certain pattern of forces in which the equilibrium of forces induces an aligned cellular shape.²⁶ At the front edges of cells the lamellipodia contain actin microspikes. When a spike faces a ridge, it is faced with an unfavorable force and will not give rise to actin polymerization. Consequently, actin filaments will form and elongate, orienting along the groove direction.²⁶ They theorize that the depth of the applied grooves is a determining factor in establishing the reaction of the cells toward microtexture. They observed that the orientation of cells on substrates does not depend (or depends only slightly) on the spacing of the surface grooves.²⁶

In our experiment, the presence of deeper grooves (or discontinuities) at the micro-roughness level is certainly the reason for the good osteoblastic adhesion and proliferation on the H surfaces since the spacing between grooves is the same as that on the A and B surfaces. The larger number of discontinuities on H surfaces could explain the faster alignment of human osteoblasts on these surfaces compared to A and B surfaces.

CONCLUSIONS

We compared the morphology, adhesion, and proliferation of human osteoblasts on three different machine-tooled surfaces with different amplitudes and organizations of roughness. We observed a better orientation, adhesion, and proliferation on H surfaces with a micro-roughness characterized by a lower Or-

der and a higher Ra and Rz. It appears that cultured human osteoblasts "prefer" surfaces of a relatively high micro-roughness amplitude and a low level of repeatability. The response to H surfaces may be related to the hypothesis of Curtis and Wilkinson, who propose that cells react to discontinuities and that the H surfaces present more discontinuities than do the A and B surfaces.²⁷

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