Human osteoblast adhesion on titanium alloy, stainless steel, glass and plastic substrates with same surface topography

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Osteoblast adhesion on materials will depend on the surface aspects of materials which may be described according to their surface chemistry, surface topography or surface energy. To separate the effects of roughness and composition of materials on osteoblast response, we chose to compare substrates with various surface composition but with the same smooth surface. Ti6Al4V alloy, stainless steel, glass and standard tissue culture polystyrene were tested. Adhesion was evaluated using specific antibodies against adhesion proteins and by a quantitative cell detachment assay. After 1, 7 and 14 days, cells expressed extracellularly fibronectin fibers, and intracellularly type I collagen and osteopontin. Vinculin-labeled focal contacts were visible on all materials but were more frequent on glass and stainless steel surfaces. β_1-integrin subunit-labeled patches were visible on all surfaces at each delay. The quantitative cell detachment assay showed few differences between materials. Adhesion was higher on metallic substrates although cell proliferation was higher on glass and stainless steel compared to tissue culture polystyrene and Ti6Al4V alloy. Substrates with various surface composition but with the same surface topography did not induce significant differences of adhesion although cell proliferation was variable.

1. Introduction

Cells in contact with a material surface will firstly attach, adhere and spread. The quality of this adhesion will influence their morphology and their future capacity for proliferation and differentiation. Osteoblast adhesion on materials will depend on the surface aspect of materials, which may be described according to their surface topography [1–3] or chemistry [4–6].

Some authors attempted to correlate the influence of surface topography and surface composition on cell adhesion, proliferation and differentiation [7, 8]. They demonstrated their interaction on osteoblast differentiation [8]. To separate the effects of roughness and composition of materials on cell response, we studied in a previous experiment, the effect of roughness of a titanium alloy on human osteoblast adhesion [9].

In this experiment, we chose to compare substrates with various surface composition but with the same surface topography. We focused on the comparison of adhesion of human osteoblasts cultured on smooth titanium alloy (Ti6Al4V), stainless steel, glass and standard tissue culture polystyrene (Thermanox®) substrates. Adhesion was evaluated by a qualitative immunostaining of adhesion protein expressed by cells and by a quantitative cell detachment assay.

2. Materials and methods

2.1. Materials

Mirror-polished discs of a Ti6Al4V-ELI alloy (medical quality) and of stainless steel discs (14 and 15 mm in diameter, respectively, and 2 mm in height) were rinsed twice in absolute alcohol and once in demineralized water before heat sterilization. Round glass and Thermanox® coverslips were sterilized by γ-irradiation.

2.2. Cell culture

Human bone cells were obtained from explants of trabecular bone from the iliac crest of young patients. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Eurobio, Les Ulis, France) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in 75 cm^2 flasks until confluent. After trypsinization (second passage), cells were inoculated on materials in 24 well plates.

2.3. Cell morphology

Cell layers were fixed in 2% paraformaldehyde (w/v) in monosodic dipotassic 0.2 M buffer, rinsed, dehydrated in graded alcohol, critical-point dried with CO_2 (Emscope

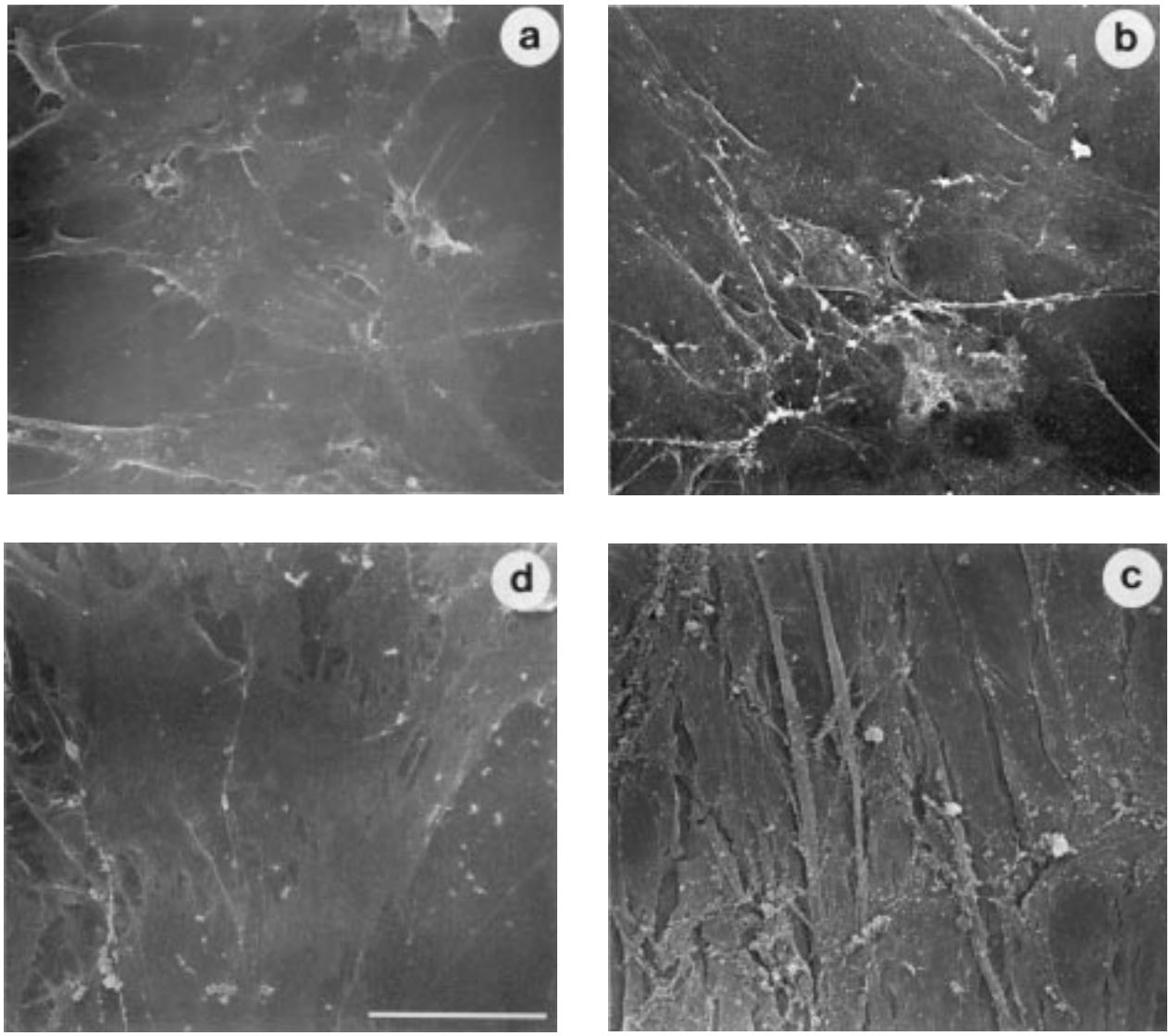

Figure 1 Scanning electron micrographs of cell layers after 7 days on (a) glass, (b) tissue culture polystyrene, (c) Ti6A14V alloy and (d) stainless steel (bar = 60 μm).

CPD 750, Elexience, Paris, France), sputter-coated (Emscope SC 500, Elexience, Paris, France) and examined using a Hitachi S520 scanning electron microscope (SEM) at an accelerating voltage of 25 kV (Elexience, Paris, France).

2.4. Immunohistochemistry

Cells were inoculated on the substrates at 1.5×10^4 cells/well. Ten samples of each material were inoculated for each incubation period (24 h, 7 days and 14 days). Following the incubation period, cells were fixed and processed for immunostaining for adhesion proteins as previously described [9]. Various antibodies against adhesion proteins were used: rabbit antihuman fibronectin immunoglobulin G (IgG) fraction (1 : 100; Institut Pasteur, Lyon, France), rabbit antihuman type I collagen IgG fraction (1 : 100; Institut Pasteur, Lyon, France), rabbit polyclonal antihuman osteopontin antibody (1 : 200; a generous gift from P. Marie, Hôpital Lariboisière, Paris, France), mouse monoclonal antihuman vinculin (1 : 200; Sigma, L'Isle d'Abeau, France), mouse monoclonal antihuman β_1 integrin subunit (1 : 200; Affiniti, Nottingham, UK), anti-α_2 and anti-α_3 integrin sub-units (1 : 50; Novocastra, Newcastle upon Tyne, UK). F-actin microfilaments were revealed directly by fluoroscein isothiocyanate (FITC)-conjugated phalloidin (25 μg ml^{-1}, Sigma, L'Isle d'Abeau, France).

Cells were fluorescently labeled by incubation with fluorescein-conjugated antibody: FITC–antimouse IgG antibody (Caltag, CA, USA) or FITC–antirabbit IgG antibody (Sigma, L'Isle d'Abeau, France). The labeled cells were examined using a Zeiss Axioskop microscope (Zeiss, Le Pecq, France) equipped for epifluorescence.

2.5. Quantitative adhesion test

Twenty samples of each surface were inoculated with 2×10^4 cells/sample. Four samples were analyzed after each incubation period: 24 h, 7 days, 14 days and 21 days. The cells were enzymatically detached from the samples by a diluted trypsin–ethylenediamine tetra-acetic acid (EDTA) (0.025% v/v) treatment as previously described [9]. The curve of percentage of released cells

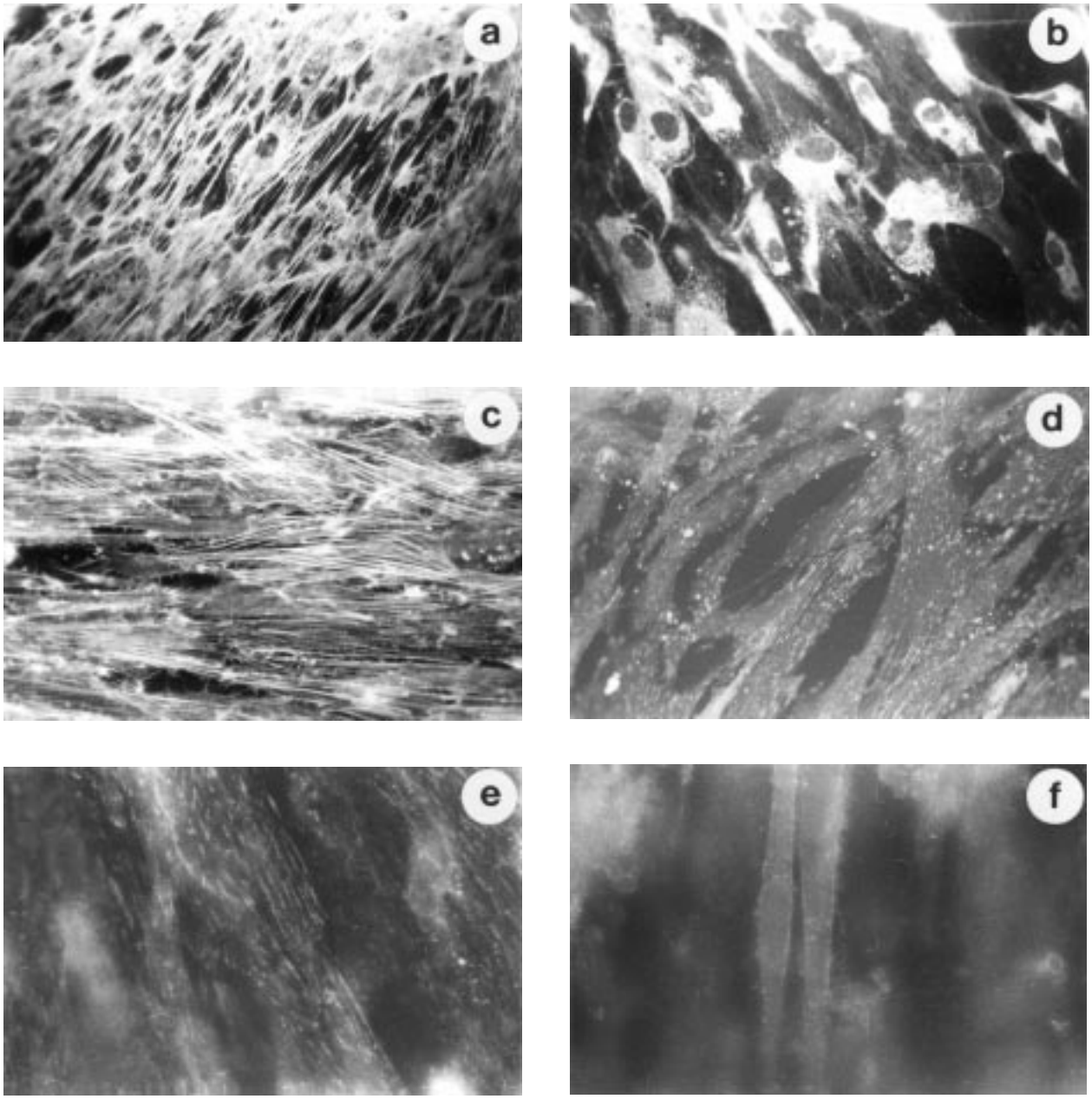

Figure 2 Expression by human osteoblasts after 7 days of (a) fibronectin (original magnification $\times 40$), (b) type I collagen ($\times 40$), (c) F-actin ($\times 40$), (d) vinculin ($\times 40$), (e) β_1-integrin subunit ($\times 100$), (f) $\alpha 3$ integrin subunit ($\times 100$). Cells were cultured on Ti6A14V alloy (a,b), stainless steel (c,f) or glass (d,e).

versus trypsination 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 the cell adhesion on the biomaterial. The experiments were reproduced three times.

The cell detachment index obtained on each surface was divided by the cell detachment index on the control surface (i.e. Thermanox® surface) to calculate the detachment index percentage (DIP).

2.6. Proliferation test

From the total detached cell count/cm^2 obtained at each delay during adhesion test, a proliferation rate was calculated by dividing the total detached cell number by the total inoculated cell number. The proliferation rate curves on the different materials were drawn.

3. Results

3.1. Cell morphology

The appearance of the cells was comparable on glass and stainless steel (Fig. 1a, d). Cells were very spread out, with few dorsal ruffles. On tissue culture polystyrene and Ti6Al4V alloy, cells were less spread out, with more filamentous extensions (Fig. 1b, c). On Ti6Al4V alloy, extracellular fibers were visible on the cell layer (Fig. 1c).

3.2. Immunohistochemistry

After 24 h, an intracellular labeling was obtained with antifibronectin, collagen and osteopontin antibodies. Fibronectin labeling of extracellular matrix was elevated after 7 days, although type I collagen labeling was still intracellular from 7 days to 14 days (Fig. 2a, b). Histochemical staining of F-actin revealed cells containing many thick stress fibers in a parallel arrangement

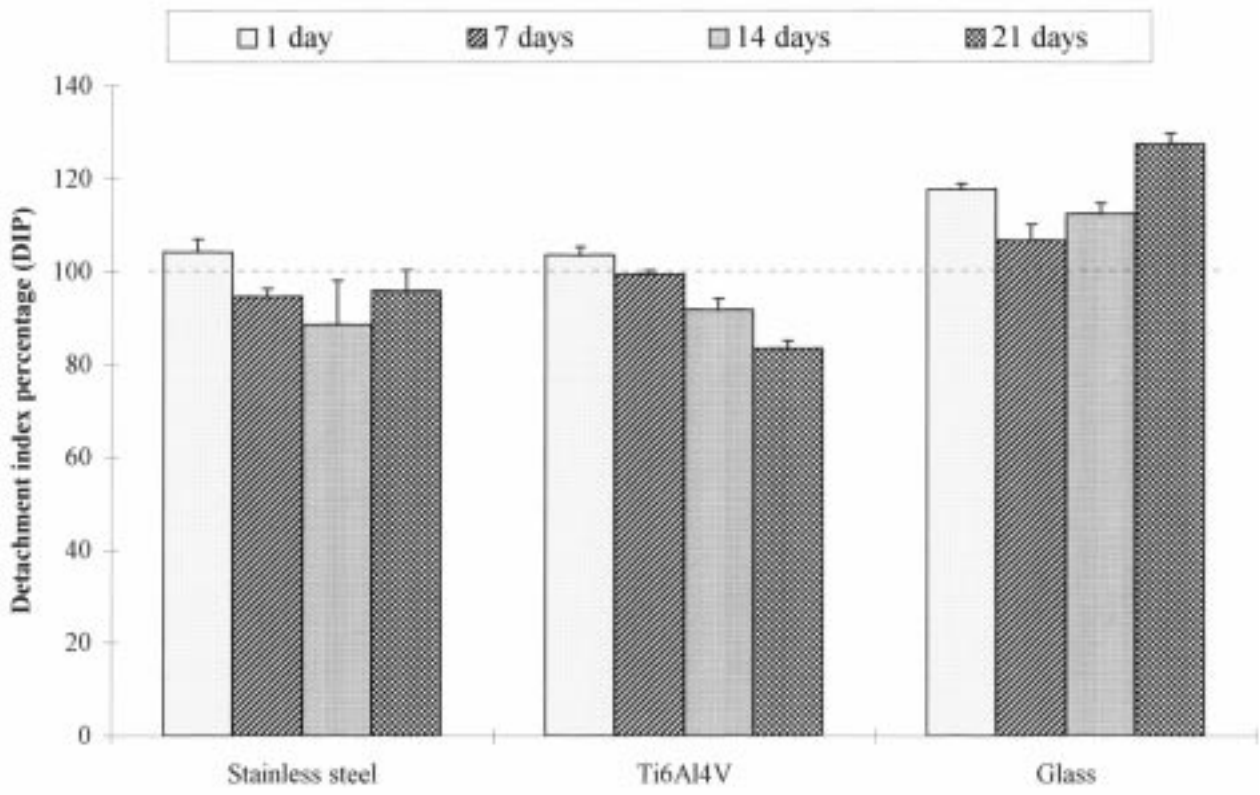

Figure 3 Detachment index percentage (DIP) on stainless steel, Ti6A14V alloy and glass after 1, 7, 14 and 21 days.

(Fig. 2c). At each delay, vinculin-labeled focal contacts did form short and dense patches, evenly distributed on the membrane surface, which were in contact with the substratum (Fig. 2d). Vinculin-labeled focal contacts appeared more frequent on glass and stainless steel surfaces compared to Ti6Al4V surfaces. However, after 14 days, no more patches were observed on glass. The β_1 integrin subunit was expressed by all cells at each delay on all substrates and appeared as thin focal contact-like patches as well as thin filaments (Fig. 2e). The α_2 integrin subunit was not expressed, although the α_3 integrin subunit showed a pericellular membrane labeling after 7 and 14 days on all substrates (Fig. 2f).

3.3. Quantitative adhesion tests

An intersurface comparison showed a stronger adhesion (lower DIP) on Ti6Al4V and stainless steel compared to glass after 1, 7, and 14 days (Fig. 3).

The DIP was always over 100% for glass, indicating that the adhesion on glass was lower than on plastic. The DIP on Ti6Al4V decreased with time: the adhesion on Ti6Al4V was lower than on control after 1 day and higher than on control after 14 and 21 days. The DIP on stainless steel was lower than on control after 1 day and slightly higher than on control after 7 days. Contrary to results on Ti6Al4V, no increase of adhesion was observed with time on stainless steel.

3.4. Proliferation test

The proliferation rate was higher on glass and stainless steel samples than on Ti6Al4V and Thermanox® (Fig. 4).

4. Discussion

A few studies were interested in quantitative evaluation of cell adhesion on various materials [5, 10]. Frequently, cell adhesion was evaluated by cell attachment some hours after inoculation [4, 5, 10]. We chose to study cell adhesion after delays longer than 24 h because, as previously noted, the initial cell attachment evaluation would be of limited value as an end point for a screening assay of potential material surfaces [11]. Moreover, we previously observed that cell attachment after 1 day did not discriminate between Ti6Al4V surfaces with variable roughness, although later adhesion evaluation discriminated well the variable roughnesses. In our experiment, we observed a stronger adhesion on metallic substrates compared to glass from 1 to 21 days after inoculation. The adhesion on Ti6Al4V and stainless steel was comparable at each delay, except after 21 days when adhesion on Ti6Al4V was increased related to stainless steel. These observations are consistent with previously published results [5, 10]. The adhesion measurement after these longer delays (7, 14 and 21 days) did evaluate the strength of the cell/matrix/interface; this strength was mainly related to extracellular matrix organization and we attempted to appreciate it using specific antibodies.

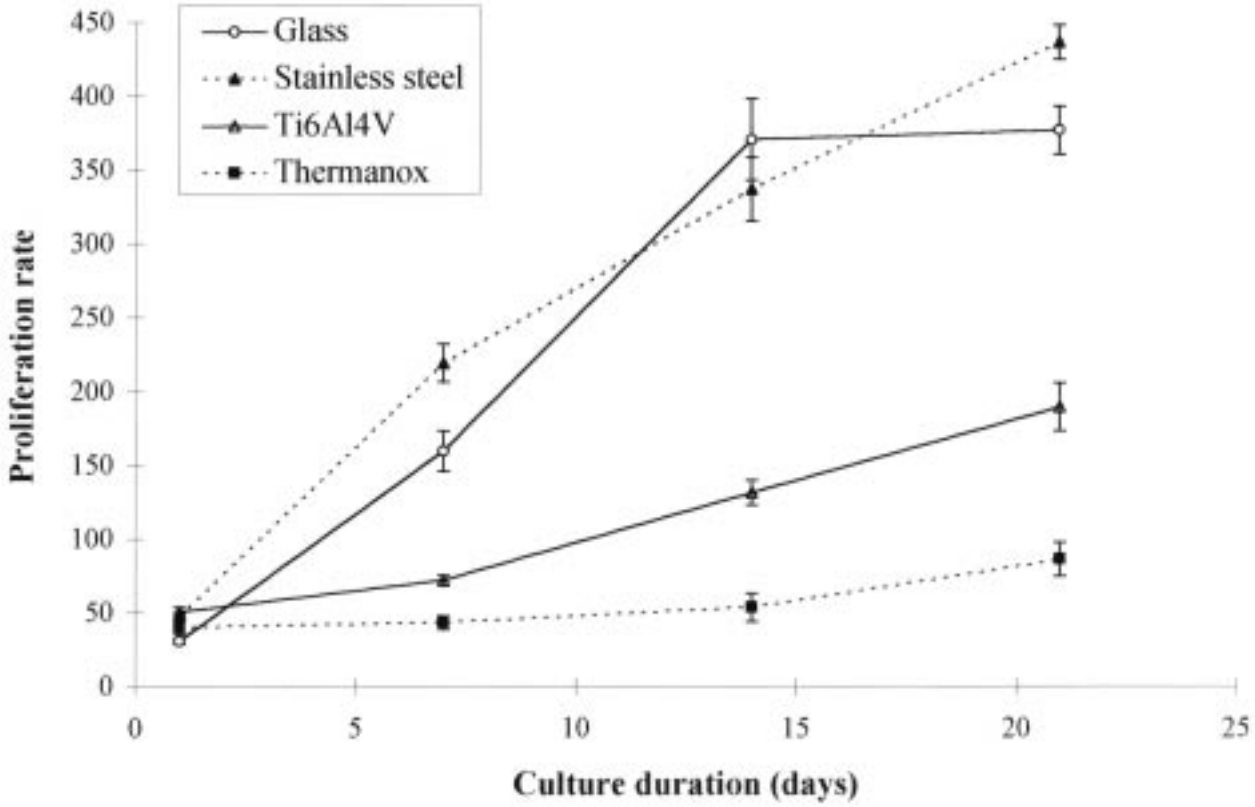

Figure 4 Proliferation rate of human osteoblasts on glass, stainless steel, Ti6A14V alloy and tissue culture polystyrene.

We observed, as previously described, the extracellular expression of fibronectin and the intracellular expression of type I collagen [9]. No differences concerning fibronectin or collagen expression were visible between the various materials tested. However, the immunofluorescence technique we used did not allow us to conclude on an eventual quantitative difference of protein expression relating to substrates.

Concerning the expression of cytoskeleton proteins, Sinha *et al*. showed after short delays (under 24 h) a higher focal contact area on CoCrMo compared to titanium substrates and a higher rate of actin reorganization on titanium compared to CoCrMo and plastic surfaces [5]. These very early phenomena were not visible after some days. In our experiment, we did not observe any significant modification of the actin fibers and vinculin patch morphology at any delay. Vinculin-labeled patches appeared only more frequent on stainless steel than on Ti6Al4V at each delay.

We observed a higher proliferation rate on glass and stainless steel than on Ti6Al4V and Thermanox®. Surprisingly, this higher proliferation rate was related to a more spread out aspect and a closer contact of cells with glass and stainless steel. Previous studies failed to demonstrate any difference of proliferation on these same materials [2, 6, 8].

5. Conclusion

In our experiment, the chemical composition of substrates with comparable surface topographies did not significantly influence adhesion protein expression by primary human osteoblastic cells. Although cell proliferation was comparable between glass and stainless steel, adhesion was higher on the latter. Adhesion of human osteoblasts was comparable on smooth TA6V and stainless steel surfaces and higher than on glass and tissue culture polystyrene.

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Received 13 May
and accepted 2 June 1999