

1996

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Zreiqat, H.; Standard, O. C.; Gengenbach, T.; Steele, J. G.; and Howlett, C. R. (1996) "The Role of Surface Characteristics in the Initial Adhesion of Human Bone-Derived Cells on Ceramics," *Cells and Materials*: Vol. 6 : No. 1 , Article 5.

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THE ROLE OF SURFACE CHARACTERISTICS IN THE INITIAL ADHESION OF HUMAN BONE-DERIVED CELLS ON CERAMICS

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(Received for publication May 6, 1996 and in revised form August 28, 1996)

Abstract

The tissue/biomaterial interactions of three biomaterials of potential use in bone implants were studied *in vitro*. The mechanism of cell adherence to various ceramic substrata has been investigated by measurement of short term (90 minutes) cell attachment and spreading rate. We have determined the effect of two serum glycoproteins, fibronectin and vitronectin, on the adhesion of human bone derived cells (HBD-cells) cultured on three different types of ceramics {alumina (Al₂O₃), hydroxyapatite (HAP) and yttria-doped tetragonal zirconia polycrystal (Y-TZP)}. The attachment of HBD-cells to alumina and hydroxyapatite was approximately 60% of that to Y-TZP. Furthermore, the requirement for serum adhesive glycoproteins vitronectin and fibronectin, for HBD-cell attachment to Al₂O₃, HAP and Y-TZP reveals a dependence upon serum vitronectin for the initial attachment of HBD-cells. There was no difference in the mechanism of initial adhesion between bioactive hydroxyapatite, as compared to the "bioinert" ceramics, alumina and Y-TZP, so the mechanism of the initial attachment of HBD-cells appears not to be dependent on the ceramic composition. The effect of surface roughness of alumina in the order of 8.3-70.7 nm on the adhesion of HBD-cells was also investigated. The Al₂O₃ disks with a root mean square surface roughness (roughness assessment: Ra) value of 8.3 nm had significantly fewer cells attached than those with an Ra of 70.7 nm.

Key Words: Cellular adhesion, bone-derived cells, fibronectin, vitronectin, alumina, hydroxyapatite, zirconia, topography.

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Introduction

Successful fixation of orthopaedic and dental prosthetic devices in clinical practice is dependent on formation of a strong and durable bond between implant and surrounding tissues, which involves direct and tight apposition of bone tissue to the surface of the implant. The response of host tissue to an implanted device is dependent upon a number of factors including the actual site of implantation, the mechanical loading upon it and the amount of micromotion at the interface [49]. Furthermore, certain characteristics of the implant material, particularly the surface chemistry and topography, also affect the extent of tissue bonding to the implant [39]. On this basis, ceramic implants have been classified as either "bioactive" or "bioinert", depending upon the host response elicited when implanted in skeletal tissues [52]. Alumina is described as a "bioinert" ceramic, as it does not bond directly to bone [22]. Similarly, the recently introduced ceramic implant material, partially-stabilized zirconia, can also be classified as bioinert [23, 26, 42]. In contrast to these, hydroxyapatite is a bioactive ceramic as it forms a direct chemical bond with bone [4, 17, 18, 50].

The "surface roughness" of a ceramic surface has been reported to affect the response of tissue to bone implants [39, 49]. Surface roughness may be defined as a random variation in the topography consisting of uniformly distributed asperities of more or less similar height and depth, but significantly smaller than the bulk geometry of the solid. However, although surface roughness is thought to be important, a correlation between bone cellular/tissue adhesion and surface roughness in the sub-micron size range has yet to be reported. Furthermore, it would be useful to correlate and thereby establish the relative impact of surface chemistry and surface roughness upon adhesion of bone cells to ceramic surfaces.

A comparison of the mechanism of bone cell adhesion to "bioinert" and "bioactive" ceramics should provide information useful in designing new biomaterials

for use in orthopaedics. The mechanism of initial adhesion of cells to biomaterials is most readily determined in a cell culture model under controlled conditions. Previously, we have studied the interaction of human bone-derived (HBD) cells with alumina and demonstrated that the initial attachment by cells to this ceramic was dependent upon adsorption of a serum glycoprotein, vitronectin [24]. The attachment and spreading of HBD-cells on alumina *in vitro* occurs effectively within the first 90 minutes after seeding. Spreading and growth of anchorage-dependent cells is known to be dependent upon the initial attachment reaction, and so, it is likely that the nature of the initial adhesion reaction is important for subsequent cellular processes of migration, proliferation and deposition of an extracellular matrix (ECM) [21, 45]. Therefore, understanding the biochemical mechanism of the initial adhesion of cultured bone derived cells to bioceramic surfaces may give some insight into the relationship between the surface characteristics of a prosthetic material and the early steps in forming an interface between an implant and bone.

In the present study, we have determined whether the initial attachment of HBD-cells to bioactive ceramic hydroxyapatite and a bioinert partially-stabilized zirconia (with equivalent surface roughness) is similar to alumina in being mediated by adsorbed vitronectin. We have also compared the relative importance of submicron surface roughness and the requirement for adsorbed vitronectin to the surface of polycrystalline alumina in the initial attachment and spreading of HBD-cells.

Materials and Methods

Source and preparation of ceramic substrata

Highly polished polycrystalline alumina (Al_2O_3), hydroxyapatite (HAP) and yttria-doped tetragonal zirconia polycrystal (Y-TZP) disks were used in this study. The polycrystalline alumina disks (Coors Ceramic Company, Golden, CO, USA) were polished on one face using routine metallographic techniques. The surface roughness was varied by polishing the disks to a different extent and four groups of disks of varying surface roughness were prepared. The polycrystalline alumina disks used in the depleted sera experiment were from the same batch as those used in the topography study. HAP disks were made from chemically prepared HAP powder [27]. Y-TZP disks were made from commercially-available high purity powder containing 2.82 mol% Y_2O_3 (Tosoh Corporation, Minato-ku, Tokyo, Japan). Ceramic powders were die-pressed into disks at 10 MPa and then cold-isostatically pressed at 200 MPa. The Y-TZP disks were sintered at 1500°C for 2 hours whereas HAP disks were sintered at 1200°C for 2 hours. Ceramic disks were polished on only one face by routine metallo-

graphic techniques involving a final polish on 0.06 μm colloidal silica suspension. Surface-modified polyethyleneterephthalate (PET) tissue culture coverslips (Nunc Medos, Sydney, NSW, Australia) were used as supplied as control substrata and were shown to have a roughness assessment (R_a) of 20 nm. The ceramic and PET disks were between 13 and 15 mm in diameter and 1 mm in thickness.

Characterisation of substrata

The bulk density and apparent porosity of the alumina disks was determined using a hydrostatic weighing method [2]. The phase composition was analysed by X-ray diffraction (XRD; model D5000, Siemens Pty. Ltd., Karlsruhe, Germany) using copper $K\alpha$ radiation. One disk was thermally-etched and the resultant microstructure examined using scanning electron microscopy (JXA-840, JEOL Ltd., Tokyo, Japan). The average grain-size was determined from micrographs using a line intercept method [32].

The root mean square (RMS) surface roughness of each group of disks was determined using a stylus-type surface profile analysis system (Dektak II, Sloan Technology Corp., Santa Barbara, CA, USA). The RMS surface roughness of the polished ceramic disks was also measured by scanning tunnelling microscopy (designed and built at CSIRO Division of Materials Science and Technology, Clayton, Vic., Australia). Disks were thoroughly cleaned and then sputter-coated with platinum. A minimum of 7 STM images, each 1.5 mm x 1.5 mm, were then digitally captured for each disk and from these STM images, the RMS surface roughness was calculated.

Contact angle measurements were made on all ceramics. Disks were cleaned using a non-ionic detergent, rinsed in flowing high purity distilled/deionised water and then dried using a jet of high-pressure, filtered nitrogen. Each measurement involved placing a droplet of liquid by syringe on to a clean ceramic surface and measuring the left and right contact angles within 30 seconds using a microscope equipped with a goniometer eyepiece (Erma, Tokyo, Japan). Four liquids spanning a range of surface tensions were used, including distilled-deionised water, glycerol, methylene iodide and quinoline. Typically, four droplets of each liquid were measured on each of three disks of each ceramic. The critical surface tension of each ceramic was determined from Zisman plots of the contact angle data [55].

Preparation of materials for cell attachment assays

The ceramic disks were ultrasonically washed, rinsed with copious amounts of reverse osmosis water and sterilized by autoclaving at 121°C. The three ceramic substrata were assayed separately, but PET disks were included in each experiment as a control material.

For each culture medium treatment, disks of each ceramic material were placed in triplicate wells of a 12 well polystyrene culture plate and PET disks were placed in a further 3 wells.

Culture of human bone-derived cells

HBD-cells were isolated from pieces of bone obtained during surgical operations from patients under 16 years of age. The selected patients did not suffer from any conditions known to affect the healing or metabolism of bone. As-received bone was trimmed of fat and soft connective tissue and then cut into small chips approximately 1 mm³ in size. The bone chips were rinsed several times in serum-free medium to remove red blood cells. Approximately 8 to 10 bone chips were placed in each 25 cm² tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing α -Minimum Essential Medium (α -MEM) (Gibco Laboratories, Life Technologies Inc., Grand Island, NY, USA), 5% (volume/volume, v/v) foetal calf serum (FBS), 2% (v/v) human AB serum and 2 mM L-glutamine (each from CSL, Ltd., Parkville, Vic, Australia), 30 μ g/ml penicillin, 100 μ g/ml streptomycin (Sigma Chemicals Co., St. Louis, MO, USA). This cultured medium was buffered with 1 M HEPES buffer (CSL) and bicarbonate; therefore, CO₂ was not used [24, 25]. The culture flasks were incubated at 37°C without CO₂ for approximately 14 days, during which time primary cell cultures of HBD-cells were established by the outgrowth of the HBD-cells from the bone fragments. HBD-cells were harvested using 0.1% (v/v) trypsin (Sigma Diagnostics, St. Louis, MO, USA) and 0.2 mM ethylenediaminetetraacetic acid (EDTA; Boehringer, Mannheim, Germany) in phosphate buffered saline (PBS). Cultures were maintained in an incubator without CO₂ at 37°C. Culture medium was changed three times a week.

Sibling cultures to those used in the attachment assays were stained for alkaline phosphatase activity (Sigma Diagnostics) to indicate osteogenic potential of cells. One-third of the cells stained positive with alkaline phosphatase. Immunohistochemical staining, using monoclonal antibodies (kindly provided by Drs. J. Werkmeister and J. Ramshaw, CSIRO Division of Biomolecular Engineering, Parkville Laboratories, Vic., Australia) which are antigen-specific for either collagen types I or III, was used to determine which collagen types (I or III) were being expressed by the cells. Collagen types I and III comprise approximately 95% and 5%, respectively, of the collagen in bone tissue and only type 1 collagen is expressed by osteoblastic cells.

Actively growing HBD-cells from passage numbers 3 to 6 were used in the assays. The HBD-cells were transferred to methionine-free α -MEM medium contain-

ing 10% (v/v) FBS, 2 mM L-glutamine, 1 M HEPES buffer (both from CSL), and 5-10 μ l/ml Trans-sulfur L-methionine, L-cysteine radioactive label (ICN Biomedicals Australia Pty. Ltd., Seven Hills, NSW, Australia) and incubated at 37°C without CO₂ for approximately 18 hours. The cells were then changed back to the normal serum supplemented medium and incubated for a further 2 hours. The HBD-cells were harvested using trypsin/EDTA solution, collected by centrifugation and suspended in serum free medium. The cells were collected in a single vial, counted using a hemacytometer and the cell concentration calculated.

Cell attachment and spreading assay

The cell suspension of metabolically-labelled HBD-cells from the third or fourth culture passage was seeded on to the substrata, in culture media containing α -MEM and 10% (v/v) sera. Three serum treatments were used for the cell attachment and spreading assay: untreated FBS; FBS depleted of fibronectin, obtained by passage of the serum through a gelatin-Sepharose affinity column [19]; and FBS depleted of vitronectin obtained by passage of the serum through an affinity column containing an anti-bovine vitronectin monoclonal antibody immobilized on Sepharose [51]. A fourth serum treatment, FBS depleted of both fibronectin and vitronectin by sequential passage over both affinity columns, was also used in certain experiments. The content of fibronectin or vitronectin in the depleted sera was typically less than 0.5% of that of the untreated serum, as determined by enzyme-linked immuno sorbent assays (ELISA).

HBD-cells labelled with ³⁵S-methionine were seeded on to the disks by adding 1 ml of a cell suspension (5 x 10⁴ cells/ml) to each well of the plate and incubated at 37°C in 5% CO₂ for 90 minutes. Neutral Red dye (at a concentration of approximately 12 μ g/ml) was added to the culture medium of the cell suspensions in order to assist in identifying cells which had adhered to the ceramics. The effect of this stain upon cellular viability, attachment and spreading was checked in pilot experiments using HBD-cells from the same source. No adverse effect of dye treatment was detected for these parameters, indicating that this vital stain did not influence the behaviour of these cells. After 90 minutes culture, images of cells settled on the disks were captured using a video system. This system consisted of a high resolution video camera and monitor linked to computer, attached to a reflectance optical microscope (Examet, Union Co., Tokyo, Japan) equipped with Nomarski interference optics. A minimum of 6 fields at 50X magnification (each equivalent to a surface area of 2.6 mm²) was captured from each disk. Immediately after capturing images, the culture medium was removed and disks gently rinsed two or three times with PBS to re-

Table 1. Physical characteristics of the ceramic substrata (values are mean \pm standard deviation).

Physical Property	Substrata		
	Y-TZP (tetragonal ZrO ₂)	Alumina (α -Al ₂ O ₃)	hydroxyapatite
Bulk density (g \cdot cm ⁻³)	6.05 \pm 0.01	3.85 \pm 0.05	3.12 \pm 0.01
% True density ^a	99.9	96.3	99.1
Apparent porosity (%)	0.01 \pm 0.02	0.14 \pm 0.08	0.13 \pm 0.07
Grain size (μ m)	0.39 \pm 0.02	1.42 \pm 0.18	1.21 \pm 0.09
Surface roughness (profilometer) (nm)	6.4 \pm 2.0	7.5 \pm 5.6	21.2 \pm 5.8
Surface roughness (STM) (nm)	2.6 \pm 0.6	6.1 \pm 1.5	5.7 \pm 2.4
XRD phase analysis	100%	100%	100%
Critical Surface Tension ^b (10 ⁻³ Nm ⁻¹)	42.5 \pm 2.4	42.2 \pm 2.1	43.4 \pm 2.9

^aTrue densities were calculated from lattice parameters determined from XRD phase analysis: Y-TZP = 6.053 g \cdot cm⁻³, alumina = 3.998 g \cdot cm⁻³, hydroxyapatite = 3.152 (g \cdot cm⁻³).

^bCritical surface tensions were calculated from contact angle measurements.

move non-attached cells. Disks were transferred to new culture plates and attached cells harvested by trypsinization. The ³⁵S level, which is proportional to the number of attached cells, was measured by liquid scintillation counting (model A300C, Packard Instruments, Downers Grove, IL, USA). Cells effectively spread during the first 90 minutes of culture [24]. The number of spread cells and the total number of cells on the ceramic disks were counted from the captured images after completion of the incubation. Spread cells were identified by the characteristic spreading and "ruffling" of the cytoplasmic membrane, whereas non-spread cells were taken to be those cells which remained well-rounded. This cell attachment and spreading assay was performed at least three times for each ceramic substrate and the HBD-cells used in each experiment were derived from a different patient. Additional disks were included in some experiments to study the morphology of attached HBD-cells. Cells on these disks were fixed in ethanol, allowed to dry slowly in air, sputter-coated with gold and examined using a scanning electron microscope.

A factor was applied to cell counts to correct for the differences in disk diameters. The effects of serum treatment and PET/ceramic on cell attachment were determined by analysis of variance (ANOVA), and significant differences between groups were determined using the Student-Newman-Keuls (SNK) multiple range test (SPSS Version 6.1, SPSS Inc., Chicago, IL, USA). The effects of serum treatment and substratum on cell spreading were determined using ANOVA and the SNK multiple range test after normalizing the spreading ratios

using a logistic transform function.

The experiment testing for an effect of surface roughness was done three times. HBD-cells cultured from different patients were used for each experiment. The effects of surface roughness on cell attachment were determined by analysis of variance (ANOVA). Significant differences between groups of substrata having different Ra were determined using the Student-Newman-Keuls (SNK) multiple range test.

Results

Effect of surface roughness and serum vitronectin upon cellular attachment to alumina

The physical properties of the alumina disks are listed in Table 1. The density and porosity data indicate that these disks were sintered to near-theoretical density and contained only a small amount of residual porosity. The Ra values determined by profilometry of four groups of were 8.3, 23.6, 51.7 and 70.7 nm. On these four topographically characterized alumina surfaces, the effect of the surface roughness upon *in vitro* cellular attachment was determined. HBD-cells were metabolically labelled and then seeded in culture medium containing FBS on to the disks, and the attachment and spreading of these cells was assessed after 90 minutes (Fig. 1). It has previously been observed that there is variability in the activities of early passage cultured HBD-cells as obtained from different patients, including differences in the metabolic uptake of radioactive label, as well as different propensities for cellular attachment (Howlett,

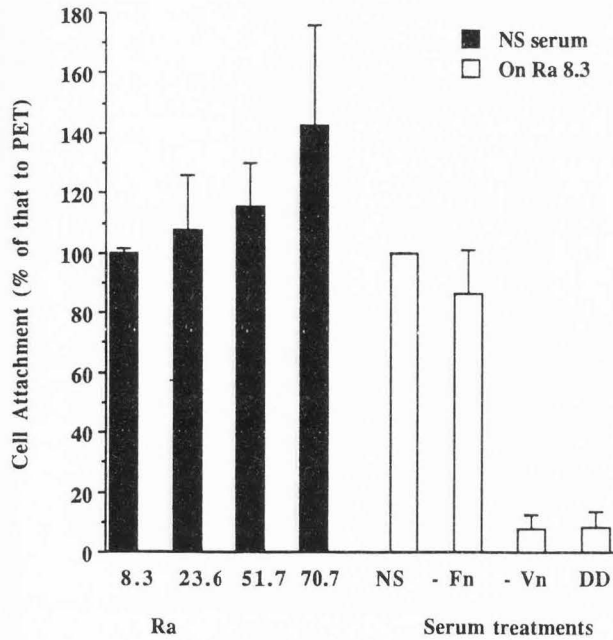


Figure 1. Effect of surface roughness and serum treatment on the attachment of HBD-cells on alumina substrata 90 minutes after seeding cells in normal serum (NS), fibronectin-depleted serum (-Fn), vitronectin-depleted serum (-Vn), and serum depleted of both fibronectin and vitronectin (DD). Attachment is shown relative to alumina disks with 8.3 nm surface roughness seeded in normal serum. Error bars correspond to \pm one standard deviation of the mean of three disks.

unpublished). To overcome any such potential artifacts, the numbers of cells attached to ceramics surfaces were compared directly to each other (or in the experiments described later in this section, directly compared to the control surface, PET). In each of the three experiments, the alumina disks with an Ra value of 8.3 nm had significantly fewer cells attached than those with an Ra of 70.7 nm. Furthermore, in two experiments, the disks with an Ra of 23.6 nm had significantly fewer attached cells than disks with an Ra value of 70.7 nm ($P < 0.01$). However, there was no significant difference in the number of cells attached to the disks of Ra 8.3, 23.6 and 51.7 nm.

A comparison of the requirement for serum adhesive glycoproteins, vitronectin and fibronectin, for HBD-cell attachment to alumina, reveals a dependence upon serum vitronectin for the initial attachment of HBD-cells (Fig. 1). This was tested by depletion of vitronectin or fibronectin, or both of these glycoproteins, from the FBS prior to addition to the *in vitro* culture assays. Indeed, depletion of vitronectin from the FBS used in the culture medium markedly reduced the number of HBD-cells attached to alumina, consistent with our previous findings

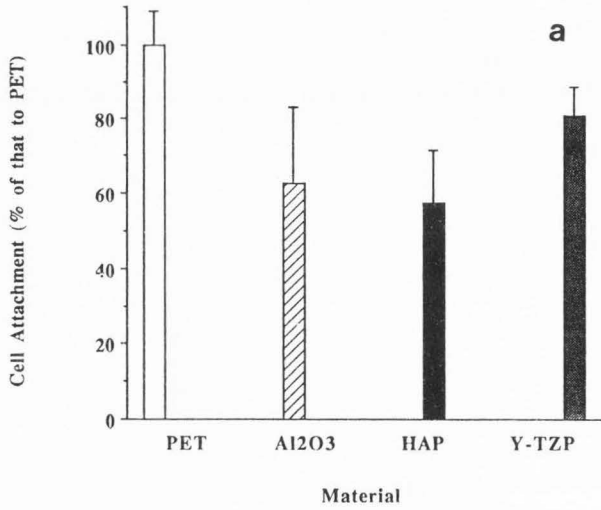
[24]. In contrast, depletion of fibronectin from the FBS had relatively little effect upon HBD-cell attachment. Figure 1 shows that the effect of the rougher surfaces on alumina was to enhance HBD-cell attachment to this surface, in comparison to the smoother surfaces. However, the attachment of cells to alumina is strongly dependent upon the vitronectin component of the FBS in the culture medium, and removal of this factor almost completely abolished attachment, despite the surface texture.

Comparison of the different ceramics for cellular attachment and spreading

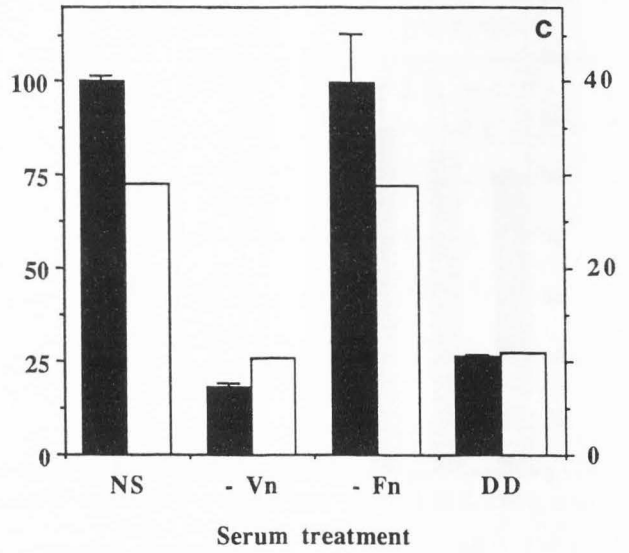
The physical properties of the alumina, hydroxyapatite and Y-TZP ceramic disks are listed in Table 1. The density and porosity results indicate that these ceramic disks were all sintered to near-theoretical density. X-ray diffraction analysis of their surfaces showed that the ceramics were all single phase. In particular, hydroxyapatite disks did not contain any other calcium phosphate phases, such as whitlockite or α - $\text{Ca}_3(\text{PO}_4)_2$. Critical surface tension for each ceramic was calculated from extrapolation of the linear regression lines of best fit to $\cos\theta = 1$ [55]. The three ceramics did not differ significantly in their critical surface tensions ($p > 0.1$). Examination of the highly polished surfaces by scanning electron microscopy revealed that they were all essentially featureless, although hydroxyapatite and alumina contained a small number of very fine intergranular pores, consistent with the very low values of surface roughness and apparent porosity. The surface roughness of the Y-TZP and hydroxyapatite (Ra between 6.4 and 21.2) measured below the range of Ra values for alumina, where an effect upon cell attachment occurred.

The relative ability of hydroxyapatite, Y-TZP and alumina ceramics to support the initial attachment of HBD-cells after seeding in culture medium containing intact FBS, is shown in Figure 2a. This figure depicts results for up to three experiments, where cells used in each experiment came from different patients. The attachment of HBD-cells to alumina and hydroxyapatite were approximately 80% of that to Y-TZP and PET.

The requirement for serum vitronectin and fibronectin in the processes of attachment and spreading of HBD-cells to Y-TZP and hydroxyapatite surfaces was tested. The attachment and spreading of HBD-cells to Y-TZP were virtually abolished by removal of vitronectin (Fig. 2b); in medium with serum depleted of fibronectin, cell attachment and spreading occurred almost at equivalent levels to those in medium containing intact FBS. Similar results were obtained for hydroxyapatite (Fig. 2c) when either vitronectin alone, or vitronectin and fibronectin were depleted; that is, the attachment and spreading of HBD-cells was reduced by approximately 75%. These results demonstrate that for hydrox-



Cell Attachment (■) (% of that in NS) Cell Spreading (□) (% of cells)



Cell Attachment (■) (% of that in NS) Cell Spreading (□) (% of cells)

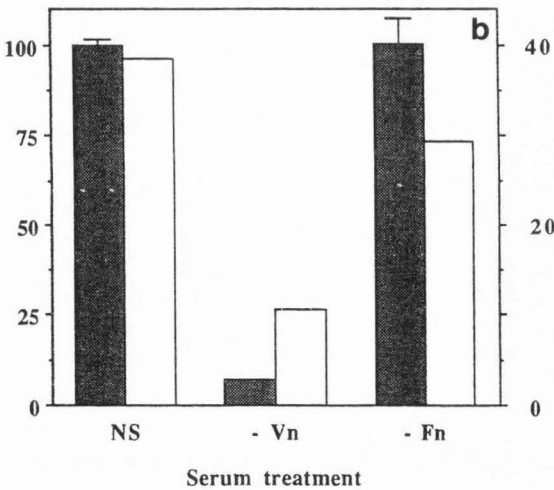


Figure 2. Histograms showing: (a) the relative attachment of HBD-cells on PET and ceramic substrata in normal serum. Attachment is shown relative to PET seeded in normal serum. Error bars correspond to one standard deviation of the mean of three disks; (b) the attachment and spreading of HBD-cells on Y-TZP 90 minutes after seeding in normal serum (NS), fibronectin-depleted serum (-Fn), and vitronectin-depleted serum (-Vn); and (c) the attachment and spreading of HBD-cells on hydroxyapatite 90 minutes after seeding in normal serum (NS), fibronectin-depleted serum (-Fn), vitronectin-depleted serum (-Vn), and serum depleted of both fibronectin and vitronectin (DD).

yapatite and Y-TZP, vitronectin from the FBS is critical in early adhesion of HBD-cells, as is the case for alumina and PET [24]. For the hydroxyapatite surface, these depletion experiments were conducted using cell preparations from 7 patients. Although, in all experiments the initial attachment of the HBD-cells was consistently dependent on vitronectin, with two of the seven cell preparations, cells that did attach to the hydroxyapatite substratum in the vitronectin-depleted medium were able to spread on this surface in this medium.

Figure 3 compares the effect of different serum treatments for the groups of surfaces upon HBD-cell attachment. Cellular attachment to the substrata was significantly different between vitronectin-depleted serum treatment and medium containing intact FBS ($P < 0.001$). HBD-cell attachment on to alumina and hydroxyapatite in serum depleted of both fibronectin and vitro-

nectin was not significantly different from that occurring in vitronectin-depleted serum, but was significantly less than that found when the medium contained either intact FBS or fibronectin-depleted serum. There were no significant differences in cell attachment to the PET and ceramic substrata ($P > 0.1$) within each culture medium composition, indicating that the biomaterial chemistry was not an overriding factor in early cellular adhesion. The number of cells attached to surfaces in medium depleted of vitronectin was typically less than 20% of that containing intact FBS. Furthermore, usually only 5% of those attached cells were spread in vitronectin-depleted serum, compared with approximately 40% spreading in normal serum.

The morphology of HBD-cells attached to the substrata 90 minutes after seeding was studied by SEM. The cells were similar in appearance when cultured in medium containing normal serum and fibronectin-depleted serum, being well spread and having cytoplasmic

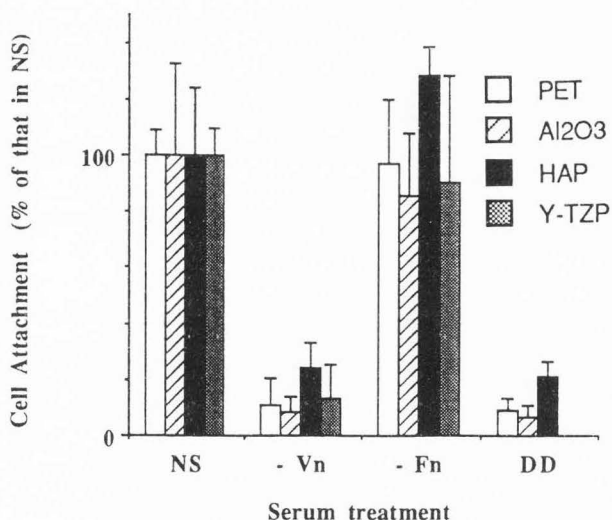


Figure 3. Histogram showing relative attachment averaged over experiments for each ceramic 90 minutes after cellular seeding in normal serum (NS), fibronectin-depleted serum (-Fn), vitronectin-depleted serum (-Vn), and serum depleted of both fibronectin and vitronectin (DD). Attachment to each material is shown relative to that in normal serum. Error bars correspond to one standard deviation of the mean.

membranes extending radially. Figure 4a displays a typical well spread cell on Y-TZP. Cell spreading was severely inhibited in vitronectin-depleted serum and in serum depleted of both fibronectin and vitronectin, regardless of the substrate on to which the cells were seeded (Fig. 4b). Indeed, those cells that were attached tended to remain rounded and showed few signs, if any, of spreading.

Discussion

The ceramics examined in this study have been described as being either "bioinert" (alumina and Y-TZP) [21, 22] or "bioactive" (hydroxyapatite) [16], as it is known from other studies that they elicit different tissue responses when implanted in hard tissue. However, there is little published work comparing cell attachment to these two classes of ceramics, and prior studies of the *in vitro* colonization of ceramic surfaces by bone cells have generally been qualitative ones. Hydroxyapatite has been shown to influence the behaviour of osteoblasts *in vitro* in terms of cell morphology [3, 5, 31], cell growth [1, 37], expression of alkaline phosphatase [1] and of bone-related proteins [20, 38]. In particular, it has been demonstrated that hydroxyapatite supports the formation of multiple cellular layers by cultured osteoblasts [10], and that osteoblasts can secrete and mineral-

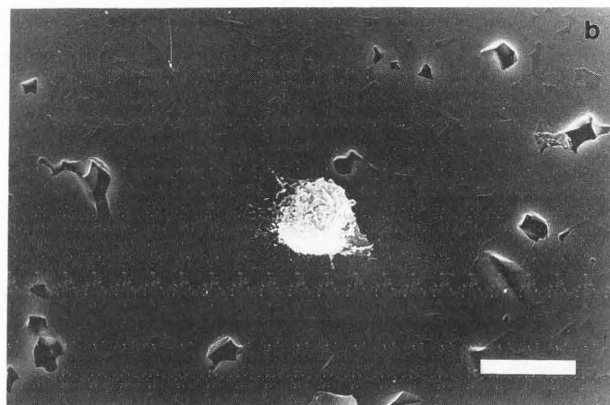
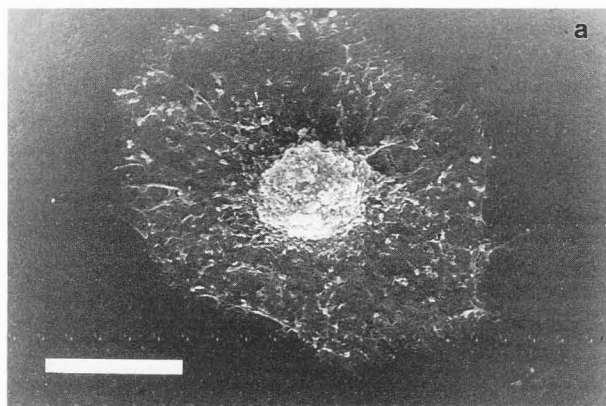


Figure 4. (a) HBD-cell spread on Y-TZP 90 minutes after seeding in normal serum, and (b) HBD-cell unspread on alumina (8.3 nm Ra) 90 minutes after seeding in vitronectin-depleted serum. Bar = 10 μ m.

ise an extracellular matrix which is chemically-bonded directly to the hydroxyapatite surface [40, 41]. Although less extensively studied, the adhesion of osteoblasts to alumina *in vitro* involves the formation of focal contacts, and the reorganization of the cytoskeleton [36], with corresponding changes in cell morphology [31]. The suitability of zirconia-based ceramics for the attachment and growth of HBD-cells has not been studied previously, although these materials have been evaluated for general biocompatibility. In cytotoxicity assays, it has been shown that mouse fibroblasts [33] and Chinese hamster fibroblasts [30] remain morphologically normal, and exhibit normal growth rates when cultured on dense Y-TZP substrata. Similarly, the growth rates of mouse fibroblasts on unspecified types of zirconia have been shown to be comparable with those of materials previously accepted as being biocompatible, including hy-

droxyapatite [47, 48].

The nature of the adsorbed protein layer is important in the interaction between cells and the surface and may influence the subsequent biological response to a material [14]. Two important serum proteins which mediate the adhesion of anchorage-dependent cells to substrata *in vitro* are fibronectin and vitronectin. Cell attachment to these glycoproteins is attributed to an arginine-glycine-aspartic acid (Arg-Gly-Asp or RGD) amino acid sequence, which is recognised by integrin receptors on many types of cultured cells, including HBD-cells [28, 34, 35, 44, 46, 54]. We have previously reported that the initial attachment and spreading of osteoblasts on alumina is strongly dependent upon vitronectin [24]. In this study, we have sought to determine whether the initial attachment of HBD-cells to the bioactive ceramic hydroxyapatite and bioinert zirconia (with equivalent surface roughness) are similar to alumina in being mediated by adsorbed vitronectin. We have also compared the relative importance of two factors, *viz.*, the impact of submicron surface roughness of the surface of polycrystalline alumina, and the requirement for adsorbed vitronectin, in the initial attachment and spreading of HBD-cells. The attachment of HBD-cells was studied over the first 90 minutes after seeding in culture, as this first contact by the cells with the surfaces is amenable to mechanistic analysis. Following this initial attachment reaction, the cells would commence to secrete extracellular matrix components, and possibly calcified afibrillar accretions, analogous to cement lines, would form on the substrata [15]. Moreover, it is conceivable that "bioinert" and "bioactive" materials would instigate different functional activities or different temporal patterns.

The alumina, Y-TZP, and hydroxyapatite ceramic disks used in this study were highly polished and had similar values of apparent porosity and surface roughness. In view of results in the present study concerning the effect of surface roughness upon cell attachment, it was considered that the small measured differences between the alumina, Y-TZP, and hydroxyapatite ceramic disks for surface roughness were unlikely to confound the interpretation of the effect of depletion of vitronectin or fibronectin. Moreover, it was found that the initial attachment and spreading of cultured HBD-cells to each class of ceramics was dependent upon vitronectin, and did not differ between bioinert (alumina and Y-TZP) versus bioactive (hydroxyapatite) ceramics. Similar attachment and spreading results were obtained when the medium was depleted of both fibronectin and vitronectin. Such results are consistent with those of our previous study, in which initial attachment and spreading of HBD-cells on alumina was strongly dependent upon serum vitronectin [24].

The observed effects of depletion of vitronectin or fibronectin upon HBD-cell attachment and spreading are consistent with their adsorption to surfaces in competition with other serum proteins [29]. Compared with other proteins found in serum, and most notably vitronectin, fibronectin has a relatively weak affinity for adsorption to surfaces *in vitro* [29, 43]. In culture medium containing less than about 2-3% (v/v) FBS, fibronectin is able to successfully compete with other serum proteins for protein binding sites on a substratum, and so may be involved in the attachment and spreading of various types of cells [29, 43]. However, at serum concentrations greater than about 2-3% (v/v), fibronectin is unable to adsorb on to surfaces in adequate quantities to promote cellular adhesion, due to competition from other proteins present in greater concentrations and/or having greater affinities for the substratum [29, 43]. Indeed, that fibronectin plays only a minor, if any, role in initial adhesion of HBD-cells is clearly demonstrated by the lack of effect of the medium depleted of fibronectin.

Vitronectin, in contrast to fibronectin, has a strong affinity for substrata, and is adsorbed at relatively high serum concentrations [43]. The presence of other serum proteins has a much less pronounced competitive effect upon adsorption of vitronectin than upon fibronectin adsorption. Consequently, it is vitronectin that is predominantly involved in the attachment and spreading of various cells to biomaterial surfaces in medium containing FBS at levels greater than 2-3% (v/v) [43]. The results of the present study of HBD-cell adhesion to "bioinert" and "bioactive" ceramics are therefore consistent with previous studies [43, 44, 51], in that initial cellular adhesion (attachment and spreading) of HBD-cells in medium containing 10% (v/v) FBS was dependent upon adsorption of vitronectin from the FBS.

Therefore, there appeared to be no difference between the mechanism of initial *in vitro* adhesion for the different ceramic substrata, a finding previously recorded for these cells adhering to polymers (PET), tissue culture polystyrene (TCPS) and metals [44]. Similarly, in a short-term *in vitro* assay, it was shown that the expression of alkaline phosphatase, fibronectin and type I collagen, as well as growth rates of osteoblast-like cells, did not vary greatly when cultured on sputter-coated thin films of alumina, hydroxyapatite and zirconia [53]. As the composition of the protein layer that becomes adsorbed on to a surface is believed to be strongly influenced by surface energy, which in turn determines the initial attachment and spreading of anchorage-dependent cells [24], it was of interest to compare the surface energy values for the different ceramic substrata. The critical surface tension values were similar for the ceramics used in this study (Table 1). Overall, it seems that the composition of the adsorbed proteins for the three ce-

amics are similar, leading to a similar mechanism of initial adhesion of HBD-cells.

The results of the present study suggest that the attachment of HBD-cells to a substratum is also influenced by variations in surface topography at the level of approximately 10-70 nm. It is known that the *in vitro* orientation and migration of isolated cells on substrata [6, 7, 8, 9, 11, 12] are influenced by micromachined grooves with dimensions of approximately 0.5-2 μm , an effect known as contact or topographic guidance (reviewed in [13]). We have studied the effect of surfaces topography in a very tight range (< 100 nm), the effect of topography shown in the present study is seen at the level of 10 - 70 nm, which is an order of magnitude smaller than any previous report of an effect of topography or contact guidance effects. Topographical features in this size range occur in most ceramic materials, and include micropores, microcracks, dislocations and grain boundary discontinuities. As such surface features may affect the adhesion of anchorage-dependent cells to ceramic substrata, it is important that surface structures not only be carefully controlled during fabrication, but also be fully characterised prior to assessing the comparative biocompatibility of any biomaterial. Furthermore, it is possible that the adhesion of other types of anchorage-dependent cells may similarly be influenced by such nanometre-sized surface topography.

Conclusions

It was found that initial *in vitro* adhesion (attachment and spreading) of HBD-cells to ceramic substrata was strongly dependent on the presence of vitronectin in serum used in culture medium. There was no difference in the mechanism of initial attachment between the bioactive hydroxyapatite as compared to the "bioinert" ceramics, alumina and Y-TZP, and so the mechanism of the initial attachment of HBD-cells appears not to be dependent on the ceramic composition. Submicron surface roughness had a significant effect on cellular attachment of HBD-cells to the Al_2O_3 disks, with those having a Ra value of 8.3 nm having significantly fewer cells attached compared with those disks with a Ra of 70.7 nm.

Acknowledgments

The authors gratefully acknowledge Mrs. Barbara James for her assistance in maintaining the tissue culture and Dr. Hans Griesser for use of the scanning tunnelling microscope at the Clayton CSIRO site.

References

[1] Alliot-Licht B, Gregoire M, Orly I, Menanteau

J (1991) Cellular activity of osteoblasts in the presence of hydroxyapatite: An *in vitro* experiment. *Biomaterials* **12**, 752-756.

[2] Australian Standards Association, Sydney (1989) Australian Standard 1774.5, Refractories and Refractory Materials - Physical Test Methods. Method 5: The Determination of Density, Porosity and Water Absorption.

[3] Bagambisa FB, Joos U (1990) Preliminary studies on the phenomenological behaviour of osteoblasts cultured on hydroxyapatite ceramics. *Biomaterials* **11**, 50-56.

[4] Blitterswijk CA von, Hesselting SC, Grote SC, Koerten HK, de Groot K (1990) The biocompatibility of hydroxyapatite ceramic: A study of retrieved human middle ear implants. *J Biomed Mater Res* **24**, 433-453.

[5] Bovell Y, Davies JE, Tarrant S (1986) Incident light and SEM observation of bone cell colonized bio-ceramics. *Histochem J* **18**, 583-586.

[6] Brunette DM (1986) Spreading and orientation of epithelial cells on grooved substrata. *Exp Cell Res* **167**, 203-217.

[7] Brunette DM (1986) Fibroblasts on micro-machined substrata orient hierarchically to grooves of different dimensions. *Exp Cell Res* **164**, 11-26.

[8] Chehroudi B, Gould TRL, Brunette DM (1989) Effects of a grooved titanium-coated implant surface on epithelial cell behaviour *in vitro* and *in vivo*. *J Biomed Mater Res* **23**, 1067-1085.

[9] Chehroudi B, Gould TRL, Brunette DM (1990) Titanium-coated micromachined grooves of different dimensions affect epithelial and connective tissue cells differently *in vivo*. *J Biomed Mater Res* **24**, 1203-1219.

[10] Cheung HS, Haak MH (1989) Growth of osteoblasts on porous calcium phosphate ceramic: An *in vitro* model for biocompatibility. *Biomaterials* **10**, 63-67.

[11] Clark P, Connolly P, Curtis ASG, Dow JAT, Wilkinson CDW (1987) Topographical control of cell behaviour: I. Simple step cues. *Development* **99**, 439-448.

[12] Clark P, Connolly P, Curtis ASG, Dow JAT, Wilkinson CDW (1990) Topographical control of cell behaviour: II. Multiple grooved substrata. *Development* **108**, 635-644.

[13] Curtis ASG, Clark P (1990) The effects of topographic and mechanical properties of materials on cell behavior. *CRC Crit Rev Biocomp* **5**, 343-362.

[14] Curtis ASG, Forester JV (1984) The competitive effects of serum proteins on cell adhesion. *J Cell Sci* **71**, 17-35.

[15] Davies JE, Ottensmeyer P, Shen X, Hashimoto M, Peel SAF (1991) Early extracellular matrix synthesis by bone cells. In: *The Bone-Biomaterial Interface*. Davies JE (ed.). University of Toronto Press, Toronto. pp. 214-228.

- [16] de Groot K (1981) Degradable ceramics. In: *Biocompatibility of Clinical Implant Materials*, Vol. 1. Williams DW (ed.). CRC press, Boca Raton, Florida. pp. 199-222.
- [17] de Lange GL, de Putter C, de Wijis FLJA (1990) Histological and ultrastructural appearance of the hydroxyapatite-bone interface. *J Biomed Mater Res* **24**, 829-845.
- [18] Denissen HW, de Groot K, Ch. Makkes P, van den Hooff A, Klopper PJ (1980) Tissue response to dense apatite implants in rats. *J Biomed Mater Res* **14**, 713-721.
- [19] Engvall E, Ruoslahti E (1977) Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int J Cancer* **20**, 1-5.
- [20] Grègoire M, Orly I, Menanteau J (1990) The influence of calcium phosphate biomaterials on human bone cell activities. An *in vitro* approach. *J Biomed Mater Res* **24**, 165-177.
- [21] Grinnell F (1978) Cellular adhesiveness and extracellular substrata. *Int Rev Cytol* **53**, 65-144.
- [22] Griss P, Heimke G (1981) Biocompatibility of high density alumina and its application in orthopaedic surgery. In: *Biocompatibility of Clinical Implant Materials*, Vol. 1. Williams DW (ed.). CRC press, Boca Raton, Florida. pp. 155-198.
- [23] Hayashi K, Matsuguchi N, Uenoyama K, Sugioka Y (1992) Re-evaluation of the biocompatibility of bioinert ceramics *in vivo*. *Biomaterials* **13**, 195-200.
- [24] Howlett CR, Evans MDM, Wildish KL, Kelly JC, Fisher LR, Francis BW, Best DJ (1993) The effect of ion implantation on cellular adhesion. *Clin Mater* **14**, 57-64.
- [25] Howlett CR, Evans MDM, Walsh WR, Johnson G, Steele JG (1994) Mechanism of initial attachment of cells derived from human bone to commonly used prosthetic materials during cell culture. *Biomaterials* **15**, 213-222.
- [26] Ichikawa Y, Akagawa Y, Nakai H, Tsuru H (1992) Tissue compatibility and stability of a new zirconia ceramic *in vivo*. *J Prosthet Dent* **68**, 322-26.
- [27] Jarcho M, Bolen CH, Thomas MB, Bobick J, Kay JF, Doremus RH (1976) Hydroxyapatite synthesis and characterization in dense polycrystalline form. *J Mater Sci* **11**, 2027-2035.
- [28] Jenne D, Stanley KK (1985) Molecular cloning of S-protein, a link between complement, coagulation, and cell-substrate adhesion. *EMBO J* **4**, 3153-3157.
- [29] Knox P. (1984) Kinetics of cell spreading in the fibronectin-depleted serum. *J Cell Sci* **71**, 51-59.
- [30] Kotoura Y, Oka M, Yamamuro T, Hyakuna K, Nakayama Y (1988) A method for toxicological evaluation of biomaterials based on colony formation of V79 cells. In: *Transactions of The Third World Biomaterials Congress*, Kyoto, Japan. Business Center for Academic Societies Japan, Tokyo, Japan. p. 177.
- [31] Malik MA, Puleo DA, Bizios R, Doremus RH (1992) Osteoblasts on hydroxyapatite, alumina and bone surfaces *in vitro*: Morphology during the first 2 h of attachment. *Biomaterials* **13**, 123-128.
- [32] Mendelson MI (1960) Average grain size in polycrystalline ceramics. *J Am Ceram Soc* **52**, 443-446.
- [33] Ohashi T, Inoue S, Kajikawa K, Ibaragi K, Tada M, Oguchi M, Kanematsu N, Kamegai A, Muramatsu Y, Kondo K (1988) Studies on mechanical strength, tissue affinity and cytotoxicity of UTZ-30 zirconia. In: *Transactions of The Third World Biomaterials Congress*, Kyoto, Japan. Business Center for Academic Societies Japan, Tokyo, Japan. p. 405.
- [34] Pierschbacher MD, Ruoslahti E (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33.
- [35] Pierschbacher MD, Hayman EG, Ruoslahti E (1983) Synthetic peptide with cell attachment activity of fibronectin. *Proc Natl Acad Sci USA* **80**, 1224-1227.
- [36] Puleo DA, Bizios R (1992) Formation of focal contacts by osteoblasts cultured on orthopedic biomaterials. *J Biomed Mater Res* **26**, 291-301.
- [37] Puleo DA, Holleran LA, Doremus RH, Bizios R (1991) Osteoblast responses to orthopedic implant materials *in vitro*. *J Biomed Mater Res* **25**, 711-723.
- [38] Puleo DA, Preston KE, Shaffer JB, Bizios R (1993) Examination of osteoblast-orthopaedic biomaterial interactions using molecular techniques. *Biomaterials* **14**, 111-114.
- [39] Ricci JL, Spivak JM, Blumenthal NC, Alexander H (1991) Modulation of bone ingrowth by surface chemistry and roughness. In: *The Bone-Biomaterial Interface*. Davies JE (ed.). University of Toronto Press, Toronto. pp. 334-349.
- [40] Sautier JM, Nefussi JR, Forest N (1991) Ultrastructural study of bone formation on synthetic hydroxyapatite in osteoblast cultures. *Cells Mater* **1**, 209-217.
- [41] Sautier JM, Nefussi JR, Forest N (1992) Surface-reactive biomaterials in osteoblast cultures: An ultrastructural study. *Biomaterials* **13**, 400-402.
- [42] Soumiya M (1984) Study of zirconia ceramics. In: *Orthopaedic Ceramic Implants*, Vol. 4. Oonishi H, Ooi Y (eds.). Japanese Society of Orthopaedic Ceramic Implants, Osaka-Minami National Hospital, Kawachinagano-city, Osaka 586, Japan. pp. 45-49.
- [43] Steele JG, Johnson G, Underwood PA (1992) Role of serum vitronectin and fibronectin in adhesion of fibroblasts following seeding onto tissue culture polystyrene. *J Biomed Mater Res* **26**, 861-864.
- [44] Steele JG, McFarland C, Dalton BA, Johnson

G, Evans MDM, Howlett CR, Underwood PA (1993) Attachment of human bone cells to tissue culture polystyrene and to unmodified polystyrene; the effect of surface chemistry upon initial cell attachment. *J Biomat Sci Polym Ed* **5**, 245-257.

[45] Stoker M, O'Neill C, Berryman S, Waxman V (1987) Anchorage and growth regulation in normal and virus-transformed cells. *Int J Cancer* **3**, 683-693.

[46] Suzuki S, Oldberg Å, Hayman EG, Pierschbacher MD, Ruoslahti E (1985) Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J* **4**, 2519-2524.

[47] Suzuki T, Toriyama M, Kawamoto Y, Yokogawa Y, Kawamura S (1991) The adhesiveness and growth of anchorage-dependent animal cells on bio-compatible ceramic culture carriers. *J Ferment Bioeng* **72**, 450-456.

[48] Tateishi T, Uchida T, Aoki H, Ikada Y, Nakamura M, Williams DF, Clark B, Stookey G, Christel P, Pizzoferrato A (1992) Round-robin test for standardization of biocompatibility test procedure by cell culture method. In: *Advances in Biomaterials, Volume 10: Biomaterial-Tissue Interfaces*. Doherty PJ, Williams RL, Williams DF, Lee AJC (eds.). Elsevier Science Publishers, Amsterdam, Netherlands. pp. 89-97.

[49] Thomas KA, Cook SD (1985) An evaluation of variables influencing implant fixation by direct bone apposition. *J Biomed Mater Res* **19**, 875-901.

[50] Tracy BM, Doremus RH (1986) High resolution electron microscopy evidence for the direct bonding of bone to hydroxylapatite. In: *NATO ASI Series, Series E: Applied Sciences, No. 116: Materials Sciences and Implant Orthopedic Surgery*. Kossowsky R, Kossovsky N (eds.). Martinus Nijhoff Publishers, Dordrecht, Netherlands. pp. 189-199.

[51] Underwood PA, Bennett FA (1989) A comparison of the biological activities of the cell-adhesive proteins vitronectin and fibronectin. *J Cell Sci* **93**, 641-649.

[52] Williams DF (ed.) (1987) *Progress in Biomedical Engineering, Vol. 4: Definitions in Biomaterials*. Elsevier, Amsterdam p. 24.

[53] Windeler AS, Bonewald L, Khare AG, Boyan B, Mundy GR (1991) The influence of sputtered bone substitutes on cell growth and phenotypic expression. In: *The Bone-Biomaterial Interface*. Davies JE (ed.). University of Toronto Press, Toronto. pp. 205-213.

[54] Zhao Y, Sane DC (1993) The cell attachment and spreading activity of vitronectin is dependent on the arg-gly-asp sequence. *Biochem Biophys Res Comm* **192**, 575-582.

[55] Zisman WA (1964) Relation of the equilibrium contact angle to liquid and solid constitution. *Adv Chem*

43, 1-51.

Discussion with Reviewers

J.-M. Sautier: These experiments were carried out after 90 minutes of interaction with the materials. Are 90 minutes enough for cell spreading? Have time studies been carried out over a longer interaction time?

Authors: In these experiments and previously [25], cells were able to attach and spread at the end of the 90 minute incubation period. We have previously published on the adhesion of human bone derived cells to modified surfaces at 24 hours of interaction [59].

H. Ohashi: The authors incubated HBD-cells on the disks for 90 minutes according to their previous paper. Can this incubation time be applied for both bioinert and bioactive materials?

Authors: Yes, in our assay at 90 minutes, the attachment of cells had reached similar levels regardless of whether the substratum was classified as bioinert or bioactive.

J.-M. Sautier: Have you any idea of the differentiation state of osteogenic cells which attached after only 90 minutes?

Authors: We did not measure this. From the published work, the cells tend to go into proliferative stage [63].

J.-M. Sautier: Have the authors noted the influence of calcium ion concentrations?

Authors: Calcium ion concentration has been maintained at a constant level throughout our experiment.

J.-M. Sautier: Several authors have reported the presence of osteopontin and bone sialoprotein at various bone/implant interfaces. What is your opinion concerning a possible role of these bone specific adhesive molecules in the attachment of bone cells to implantable materials?

Authors: This is a subject of ongoing research. *In vitro*, the dominant protein for attachment and cell spreading is vitronectin. *In vivo*, the vitronectin may also be involved [60].

H. Ohashi: Generally, cell-biomaterial interaction is brought about by the following steps: (1) by physical force (i.e., van der Waals force, hydrophobic interaction, electric charge); (2) by non-specific chemical bonds (i.e., covalent, polar and hydrogen bonding); and (3) by specific ligand-receptor interaction. Furthermore, the nature of the adsorbed protein layer is important in the reaction between cells and the surface.

Authors: Our studies have pointed strongly to the pre-

dominant role of vitronectin in the cellular adhesive process. The molecular mechanisms by which vitronectin becomes rapidly and preferentially attached to the surface of these materials are not presently known and would be difficult to determine, since it is a competitive process. However, the interactions could well include the physical forces and non-specific chemical interactions you have mentioned. For further discussion of these mechanisms of cellular adhesion to surfaces, please refer to the recent book by Horbett and Brash [58].

M.F. Sigot-Luizard: The scientific quality of the work is excellent, with close and well-conducted experimental protocols concerning material characterization as well as cell response analysis. Nevertheless, this study does not substantiate new elements concerning this relationship between vitronectin adsorption and cell attachment except perhaps its application to two ceramics different from alumina.

Besides, the conclusions related to cell attachment and surface roughness are not as clear as the authors claimed. Indeed, some papers dealing with this subject reported a better cell adhesion on smooth surfaces than on rough ones. It may probably depend on the cell and/or material used, but it would have been interesting to recall this point in **Discussion**.

Authors: We have studied the effect of surface topography on cellular adhesion in a very tight range ($R_a < 100$ nm). A similar trend has been recently found by Chehroudi *et al.* [57] who noted that rougher surfaces enhance mineralisation of osteoblasts in the range of 30-120 μ m. Moreover, studies focusing on cell attachment [56, 62] did reveal that better cell attachment occurred on the rougher surfaces.

In an elegant paper by Martin *et al.* [61], using human osteosarcoma cells (MG63), more cells were found on smoother surfaces (10 nm) when compared to rougher surfaces (30 nm). However, their study was terminated at 24 and 48 hours and, therefore, the number of cells that attach to the metallic surface is determinant in part by the rate of proliferation on the different topographies, whereas our study examines only the attachment rate to different topographical states of alumina.

Furthermore, the differences in these observations may be due to the different surfaces used, species, or source of cells human osteosarcoma cells (MG63) versus human osteoblast-like cells.

M.F. Sigot-Luizard: Do the authors have an explanation of the capability of the cells to attach and then to spread when cultured in vitronectin-depleted medium?

Authors: This is a very good question; we have yet to understand this mechanism. We speculate that those few cells which did attach and spread were capable of mounting an ill-understood endogenous mechanism of cellular attachment.

Additional References

[56] Bowers K, Keller J, Randolph B, Wick D, Michaels C (1992) Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. *Int J Oral Maxillofac Implants* **7**, 302-310. 1992.

[57] Chehroudi B, Qu J-L, Brunette DM (1996). Effects of implant surface topography on osteogenesis. In: *Transactions of Fifth World Biomaterials Congress, Vol. II*. Univ. Toronto Press, Canada. p. 119 (abstract).

[58] Horbett TA, Brash JL (eds.) (1995) *Proteins at Interfaces II. Fundamentals and Applications*. ACS Symposium Series 602. American Chemical Society, Washington DC.

[59] Howlett CR, Zreiqat H, O'Dell R, Noorman J, Evans P, Dalton BA, McFarland C, Steele JG (1994) The effect of magnesium ion implantation into alumina upon the adhesion of human bone derived cells. *J Mater Sci: Mater Med* **5**, 715-722.

[60] Kothari S, Douglas CWI, James PF, Hatton PV (1996) The binding of adhesive proteins to material surfaces. In: *Transactions of Fifth World Biomaterials Congress, Vol. II*. Univ. Toronto Press, Canada. p. 67 (abstract).

[61] Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J, Dean Jr DD, Cochran DL, Boyan BD (1995) Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). *J Biomed Mat Res* **29**, 389-401.

[62] Michaels CM, Keller JC, Stanford CM, Solursh M, Mackenzie IC (1989) *In vitro* connective tissue cell attachment to cpTi. *J Dent Res* **68**, 276 (abstract).

[63] Owen T, Aronow M, Shalhoub V, Brone L, Wilming L, Tessinari M, Kennedy M, Pockwinse S, Lian J, Stein G (1990) Progressive development of the rat osteoblast phenotype *in vitro*: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* **143**, 420-430.