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The effect of magnesium ion implantation into alumina upon the adhesion of human bone derived cells

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Our group is investigating the potential of modifying the surface atomic layers of biomaterials by ion beam implantation in order to stimulate adhesion of bone cells to these treated biomaterials. In this study alumina that had been implanted with magnesium ions (Mg)–(Al_2O_3), was compared to unmodified alumina (Al_2O_3) for the adhesion of cells cultured from explanted human bone. The attachment and spreading of cultured human bone derived cells onto (Mg)–(Al_2O_3) was significantly enhanced as compared to Al_2O_3. The role of adsorption of serum adhesive glycoproteins fibronectin (Fn) and vitronectin (Vn) in the adhesion of human bone derived cells to (Mg)–(Al_2O_3) was determined. The requirement for Fn or Vn for the attachment and spreading of bone-derived cells onto the Al_2O_3 and (Mg)–(Al_2O_3) surfaces was directly tested by selective removal of Fn or Vn from the serum prior to addition to the culture medium. The cell adhesion to both the alumina and the (Mg)–(Al_2O_3) surfaces in the presence of FBS was dependent upon serum Vn.

1. Introduction

Total joint replacements give excellent short-term results and have gained an important place in the treatment of fractured necks of femurs and severe joint disease. However, the major long-term problem encountered is aseptic loosening of these cemented prostheses. During the last decade, some surgical units have turned to press-fit prostheses where reliance is placed upon the interference fit inducing permanent fixation of the device by ingrowing skeletal tissue. Therefore the bone–prosthesis interface remains the critical region for study in order to improve long-term fixation of a device within the skeleton particularly as considerable stresses are applied to the tissue–implant interface of those prosthetic joints [1]. In the search for methods to enhance the adhesion of bone tissue to implant surfaces, little attention has been directed towards the potential use of surface chemistry modification. Such technology could at a later date be combined with designed topographic contours to enhance and encourage tissue ingrowth.

Changes which facilitate cellular adhesion are likely to increase the tissue differentiation. The modification of surfaces by ion beams is a versatile method whereby ions of a chosen element are fired at high energy under high vacuum into a surface. These ions become buried in the near surface region of this material. There is extensive literature on ion beam modification of silicon wafers from their use in the semiconductor industry to fabricate the integrated circuits used in computer chips.

Ion beam implantation alters the surface atomic layers of the substratum and is capable of altering the chemical and electrical nature of a material [2]. Although ion beam bombardment with nitrogen has been used to improve corrosion and hardness in hip joints thereby decreasing the wear of Ti-6Al-4V alloys by a factor of 400 [3], little investigation has been aimed at the potential use of this technology in improving the biocompatibility of materials. Recently, however, three groups (Rostlund *et al.* [4]; Lee *et al.* [5] and Howlett *et al.* [6]) have shown that surfaces modified by ion implantation alter the response of cells to metallic, ceramic and polymeric surfaces. Furthermore, the migratory morphology, mode of adhesion and synthetic activity of osteoblasts have been influenced by substratum charge [7]. More importantly, however, these authors considered that the specific chemistry (functional groups) on the polymeric surface determined the degree of colonization by osteoblasts whereas charge influenced calcification [7].

In this study, we have evaluated ion beam implantation of surfaces with the divalent ion magnesium, as a method of enhancing the adhesion of bone-derived

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cells. Our specific aim was to determine whether the attachment of human bone derived cells to alumina (Al_2O_3), which is commonly used in orthopaedic devices, could be enhanced by implantation of the surface with magnesium ions, (Mg)–(Al_2O_3). For the experimental determination of bone cell adhesion, we used a culture assay to screen for enhanced cell attachment and spreading during the initial period after contact between the cells with the surfaces.

The serum (FBS) used in cell culture adhesion assay systems contains two glycoproteins, fibronectin (Fn) and vitronectin (Vn), and in their purified form both of these are capable of stimulating the attachment of human bone-derived cells (HBDC) to material surfaces [8, 9]. Previously we have shown that when HBDC are seeded in medium containing FBS, their initial attachment onto prosthetic metals, ceramics and tissue culture polystyrene (TCPS) is a result of adsorption onto the material surface of serum Vn [8, 9]. HBDC are, however, very responsive to Fn when it is adsorbed onto a surface [8]. In a second aspect of this study, we have tested for the role of serum Vn and Fn in the initial attachment and spreading of HBDC to the ion implanted alumina. The relative contribution of Fn and Vn to the initial attachment of HBDC to the Al_2O_3 and (Mg)–(Al_2O_3) surfaces was directly determined by selectively depleting these components from the FBS used in the culture medium.

2. Materials and methods

2.1. Preparation of implanted alumina

The alumina (Al_2O_3) used to fabricate the discs was 99.5% pure alumina. These polycrystalline discs of 15 mm diameter having a density of 3.89 gm cm^{-3} were purchased, with one surface highly polished, from Coors Ceramic Company, Colorado, USA. An assessment of roughness (RA) was measured by profilometry (Dektak II Surface Profile Measuring System, Sloan Technology, Corp., Santa Barbara, CA, USA) of 8.3 $\pm$ 10 nm.

2.1.1. Ion implantation of alumina

The ion beam modified discs were the same discs as described above, but implanted with Mg ions to a nominal dose of 1.1×10^{17} ions cm^{-2} using a metal vapour vacuum arc (MEVVA) ion source [10]. For magnesium, this type of source generates approximately equal amounts of the 1 + and 2 + charge states [11] which, in conjunction with the applied extraction voltage of 15 kV, yielded an average ion energy of 22.5 keV. Ion trajectory calculations using the TRIM computer code [12] yielded a projected range of 25 nm for this average energy. The ion implantation was done in two manners, either diffusely or as a grid pattern with implanted zones of 4 mm^2.

The polyethyleneterephthalate (PET) used as a positive control was surface modified PET discs (sterile Thermanox tissue culture covers, Nunc, Inc., Naperville, USA) used as supplied.

2.1.2. Characterization of surfaces by Rutherford backscattering (RBS) analysis

RBS analysis of selected samples was performed with a 2 MeV^4He^+ incident beam, which was normal to the target, and a scattering angle of 169°. A typical RBS spectrum of a Mg implanted specimen is shown in Fig. 1. For comparison purposes, the spectrum of an as-received disc is also displayed. The signal due to the implanted Mg is clearly evident in Fig. 1, superimposed on that from the aluminium of the substrate. The implant dose determined from this spectrum by means of the RUMP program [13] was calculated to be 1.1×10^{17} ions cm^{-2}.

2.2. Cell adhesion assay

2.2.1. Cells

The HBDC were osteoblast-like cells released from cultured pieces of bone and passaged in culture using 0.1% (v/v) trypsin, 0.2 mM EDTA. The cells were cultured in medium α-MEM (Minimum Essential Medium, Nunc, Inc., Naperville, USA) with 5% (v/v) human AB serum (CSL Ltd., Parkville, Victoria, Australia) supplemented with 2 mM *L*-glutamine (Nunc. Inc., Naperville, USA), 30 μg ml^{-1} penicillin 100 μg ml^{-1} streptomycin (Sigma Chemical Co., St. Louis, USA) [6, 9].

2.2.2. Visual attachment assay using microscopy

For the assay of attachment and spreading of the HBDC, a cell suspension was prepared using trypsin/EDTA solution [9] and the cells were seeded and cultured on the substrata at about 0.5×10^5 cells/well of the cluster plate. During the attachment assay, a FBS concentration of 10% (v/v) was used. The 15 mm sample discs were placed in the bottom of 12-well

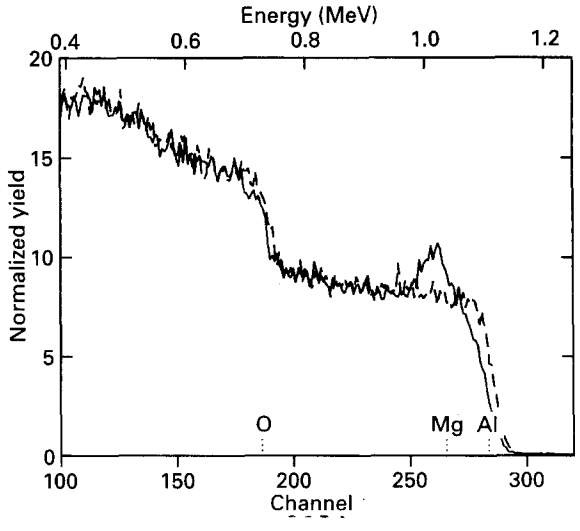

Figure 1 A composite of Rutherford backscatter spectra of a native high density (99.5% pure) polycrystalline alumina disc as received from the manufacturer (– – –), and a typical spectrum from one such disc after implantation of magnesium ions to a dose of 1.1 $\times 10^{17}$ ions/cm^2 (———).

tissue culture cluster wells. After 24 h culture, the culture medium was removed and the adherent cells were fixed using 2.5% glutaraldehyde in phosphate-buffered saline (PBS). For the measurement of cellular adhesion, the total number of adhering cells was determined on ceramic surfaces by relfectance microscopy using Nomarski interference optics fitted to an Examet microscope (Union Co., Tokyo, Japan) with a 5× objective lens, to give a final magnification at viewing of 50×. The microscope was fitted with a video camera and the images (each equivalent to a surface area of 2.6 mm^2) were projected on a screen and the number of cells were counted manually from each image, until the entire surface of each disc was scanned and counted. Apart from surfaces where Vn was not supplied all discs had in excess of 1200 cells per disc.

2.2.3. Attachment assay using metabolically labelled cells

HBDC were metabolically labelled for 15–22 h in a medium consisting of methionine-free α-MEM medium supplemented with *L*-[^{35}S] methionine (ICN Flow Laboratories, Seven Hills, Australia), at a final concentration of 56 μCi ml^{-1}. Following a further 2 h culture in medium which did not contain radioactive methionine, cultures were harvested using trypsin/EDTA solution and labelled cells recovered by centrifugation. A suspension of metabolically labelled cells was prepared and seeded onto the test material discs. After culture for 90 min or 24 h as described in the text, the medium was removed and the discs were flushed twice with phosphate-buffered saline (PBS) to remove non-adherent cells. The discs were placed in a clean 12-well culture plate and the adherent cells were harvested from each test surface using 0.1% trypsin/EDTA in PBS and collected into scintillation vials. Scintillant (9.0 ml of PCS-II scintillant) was added and the amount of cellular [^{35}S] was measured in a beta-counter (Packard Instruments, Illinois, USA). The relative number of cells attached onto the materials was calculated from the radioactive counts and for each culture medium treatment, these counts were expressed as a proportion of the number of cells attached to the PET surface with the culture medium containing intact FBS.

2.2.4. Treated sera

Different sera were used in these assays [14]. These sera were either entire FBS or FBS stripped of Vn (− Vn), made by passage over a column containing an immobilized monoclonal antibody against bovine Vn [15] or depleted of Fn(− Fn), by passage over gelatin-Sepharose. [16].

2.3. Determination of the adsorption of Vn from FBS onto Al_2O_3 and (Mg)–(Al_2O_3) surfaces

2.3.1. ^{125}I-Vn

^{125}I-Vn was prepared by the chloramine T method [17], and the proportion of this radioactivity which was adsorbable to Dynatech PVC trays was determined at the time of the adsorption assay [18].

2.3.2. Detection of relative amounts of ^{125}I-Vn in situ

The amounts of Vn which adsorbed onto the surfaces from culture medium containing FBS were measured using ^{125}I-Vn. FBS depleted of both Fn and Vn (double depleted FBS) was used to make FBS containing 50 μg Vn/ml by the addition of Vn, and this solution was used to make up different concentrations (0–30% v/v) of FBS in culture medium. ^{125}I-Vn was added to these solutions and triplicate wells were incubated with 80 μl (containing 9×10^5 cpm) for 90 min at 37 °C. The wells were washed three times with PBS, then the relative amounts of ^{125}I on each sample was determined using a phosphoimage detection system by exposing a phosphor screen to the samples before scanning the screen in the phosphor imager (Molecular Dynamics Pty. Ltd., Melbourne, Australia).

2.3.3. Detection of ^{125}I radioactivity

Following detection of ^{125}I *in situ*, the ^{125}I was removed from each sample by sequential treatement with 1% (w/v) trypsin in PBS at 37 °C for at least 2 h followed by treatment for 1 h at 60 °C with 0.5% (w/v) NaOH containing 0.1% (w/v) sodium dodecylsulphate [14]. The total amount of adsorbable ^{125}I was calculated from the total amount of radioactivity added per well and the proportion of this radioactivity which was determined to be adsorbable to Dynatech PVC trays [18]. This total amount of adsorbable label, the amount of tracer which was recovered from the wells, the known Vn concentration and the surface area of the well in contact with solution (0.566 cm^2) were used to calculate the amounts of Vn adsorbed on the surfaces, which were expressed as ng/cm^2.

2.4. Statistical analysis

The cellular attachment results on uniform discs objectively measured by radioactive counts were analysed using one-way analysis of variance. The *a priori* contrast between Al_2O_3 and (Mg)–(Al_2O_3) was tested by *t*-test using the pooled within-group variance [19].

In the experiments with patterned discs the difference between the number of cells adhered to the Al_2O_3 untreated discs (Mg)–(Al_2O_3) was assessed using the unpaired *t*-test. Differences between the implanted and unimplanted zones of the patterned (Mg)–(Al_2O_3) discs were assessed using the paired *t*-test. In experiments concerned with the adsorption of Vn the differences between Al_2O_3 and (Mg)–(Al_2O_3) discs were assessed using the unpaired *t*-test.

3. Results

3.1. Effect of implantation with magnesium upon cell adhesion to alumina

3.1.1. Alumina implanted in a patterned manner

The attachment of HBDC to Al_2O_3 was determined with discs which had been implanted with magnesium

ions in a patterned format. Cellular adhesion at 24 h to untreated Al_2O_3 and to (Mg)–(Al_2O_3) regions was determined by direct examination of each disc in its entirety using a reflectance microscope fitted with Nomarski interference optics. The images were captured by a video camera and viewed on a TV monitor. The screen area was equivalent to 2.6 mm^2. There was a significant increase in cells adhering to (Mg)–(Al_2O_3) zones ($p < 0.01$) over the unimplanted zones of Al_2O_3, whereas there was no significant difference between the number of cells adhering to native discs and the unimplanted zones in the patterned prepared discs. Each disc was scanned and all cells adhered to either the implanted or unimplanted zones were counted.

Each disc had over 1200 cells attached to it, except those in serum culture medium depleted of Vn. Fig. 2 is a representative image of the interface of implanted and unimplanted zones and the cells attached to these adjacent zones.

As the implantation of Mg into Al_2O_3 created a more reflective region, cellular morphology was more easily visualized using reflectance microscopy. However, it was still apparent that more cells adhered to the implanted zone than the unimplanted zone and that these cells were more voluminous, had spread further and had longer and more prominent cytoplasmic processes. None appeared to be disc-shaped and were either fusiform or stellate shape. In contrast cells on the unimplanted zones were smaller having either a disc-like or fusiform shape (Fig. 2). This morphological difference was also apparent when the discs were uniformly implanted (c.f. Fig. 3a and 3b).

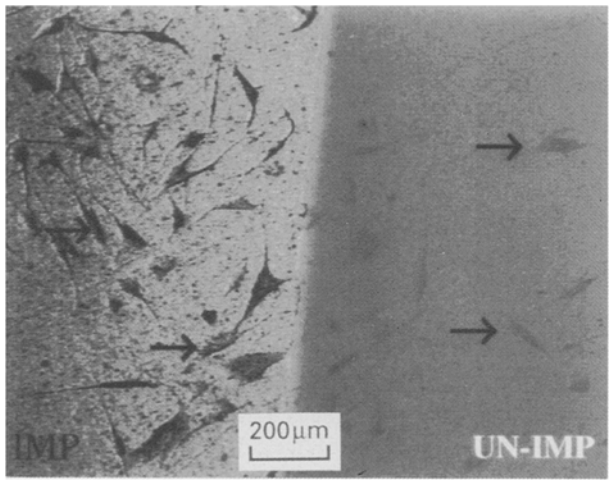

Figure 2 A photomicrograph of the boundary of implanted (IMP) and unimplanted (UN-IMP) zones after patterned implantation of Mg into high-density Al_2O_3 discs. The implantation of magnesium ions into the Al_2O_3 makes the surface highly reflective and as a result the cells are more visible on the implanted surface than on the unimplanted Al_2O_3 polycrystalline surface. Differing cellular morphology of the HBDC (arrows) adherent to each zone after 24 h of culture is apparent. Those on the implanted zone were spindle or stellate shaped and larger in comparison to those on the unimplanted zone, which were either disc or fusiform shape. The cells were fixed using 2.5% glutaraldehyde in PBS and viewed by reflectance microscopy.

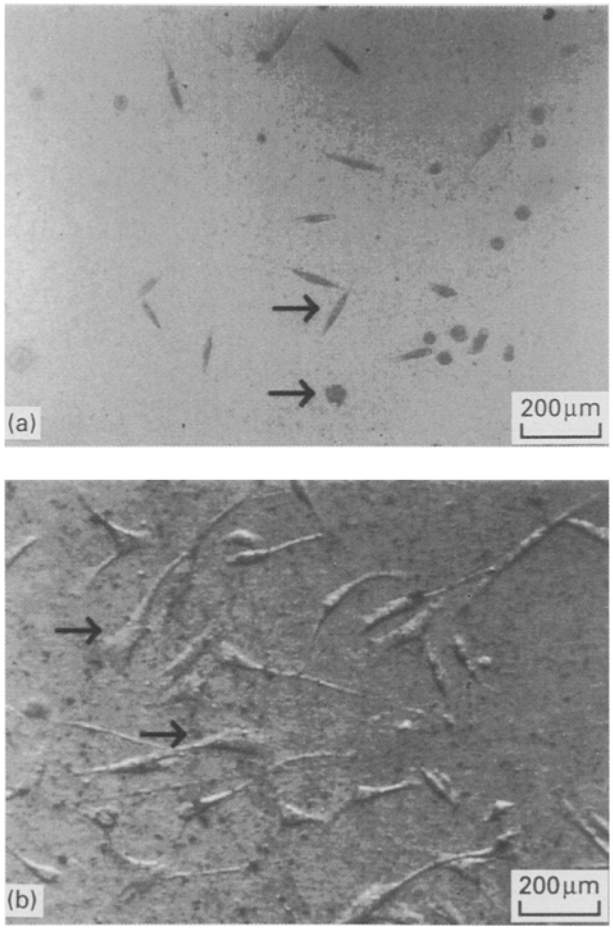

Figure 3 These photographs depict HBDC cultured for 24 h in media containing 10% (v/v) FBS. (a) The HBDC on Al_2O_3 controls, the cells being spread in either a fusiform or disc like manner. On the (Mg)–(Al_2O_3) surfaces (b) all cells (arrow) appeared to be about 100% larger than those in (a) and were of either fusiform or stellate shape.

3.1.2. Diffusely implanted alumina

The enhanced HBDC adhesion seen with the patterned implants was also observed with (Mg)–(Al_2O_3) samples where the implantation of magnesium was diffusely applied over the entire surface. HBDC were metabolically labelled and then seeded in culture medium containing FBS and cultured for 90 min. The relative attachment of HBDC onto (Mg)–(Al_2O_3) as compared to Al_2O_3 and PET surfaces is shown in Fig. 4. Attachment of HBDC to Al_2O_3 was 60% of that to PET, consistent with results obtained in our previous study [9]; cellular attachment onto (Mg)–(Al_2O_3) was higher than that for Al_2O_3 and equal to that for PET (Fig. 4).

3.1.3. Diffusely implanted alumina at 24 h

The enhancement of cellular adhesion was even more apparent in experiments conducted using uniformly implanted (Mg)–(Al_2O_3) substrata (Fig. 3a and b). Fig. 3a and 3b is representative of the cellular morphology of living cells cultured for 24 h on the two different substrata. In this case, the morphology of the cells was not modified by shrinkage which occurs during fixation of cells with glutaraldehyde. Fig. 3 clearly shows that the HBDC on (Mg)–(Al_2O_3)

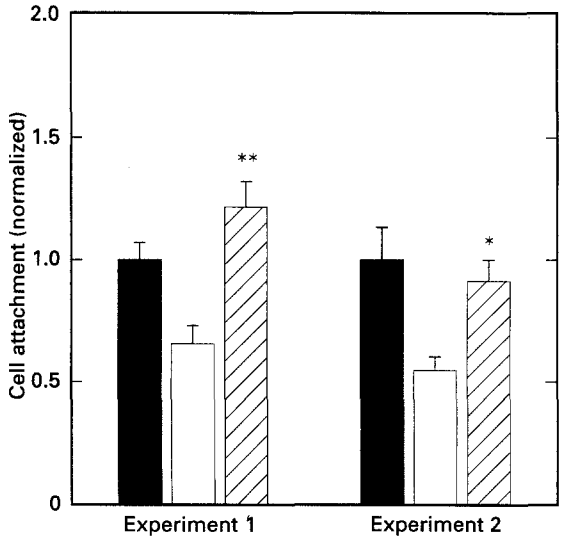

Figure 4 Histogram of results from two representative experiments determining the relative amounts of metabolically labelled HBDC attached to uniformly implanted (Mg)–(Al_2O_3) as compared to Al_2O_3 and expressed as a proportion of that to PET during 90 min culture in media containing 10% (v/v) FBS. Mean ± SEM of triplicate determinations in each experiment (■ PET; □ Al_2O_3; ▨ (Mg)–(Al_2O_3)).

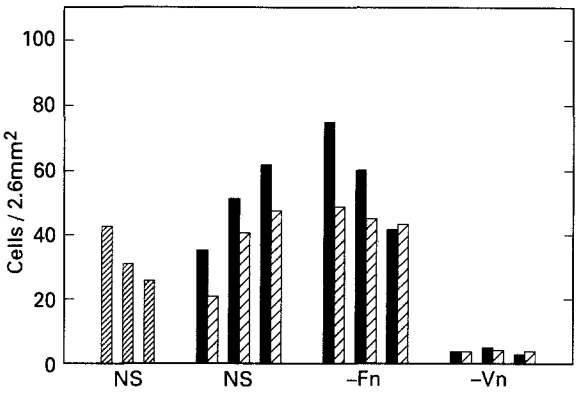

Figure 5 Histogram highlighting the requirement for serum Vn and Fn for cell attachment to patterned implanted discs for both the (Mg)–(Al_2O_3) treated (solid bars) and the Al_2O_3 control zone (diagonal stripe). In addition there are cell counts for three native Al_2O_3 discs (Al_2O_3). HBDC were seeded in culture medium containing intact FBS (NS), FBS depleted of Fn(– Fn) or FBS depleted of Vn(– Vn) onto discs that were ion implanted in a patterned manner and cultured for 24 h, fixed and then counted visually. The three columns for each treatment are counts of all cells on each of three different discs of each treatment, in a representative experiment. In the experiments with normal serum there was a significant difference between implanted and unimplanted areas, $p < 0.01$. Fewer cells were present in experiments with vitronectin-depleted serum ($p < 0.0001$). There was no significant difference between numbers of adherent cells on unimplanted zones of the patterned disc and those adherent to the native control discs (▨ Al_2O_3; ▨ (Mg)–(Al_2O_3) unimplanted area; ■ (Mg)–(Al_2O_3) implanted area).

were conspicuously larger in all dimensions and none were disc-shaped. The number of HBDC that attached and spread on the (Mg)–(Al_2O_3) regions was approx 155% of that to Al_2O_3 ($p < 0.01$). At 24 h all cells on the (Mg)–(Al_2O_3) substratum were elongated, large and spread over a greater area than those on the Al_2O_3. In addition, at least one-third of the cells on Al_2O_3 were spread in a circular pattern and were distinctly smaller and flatter. The radioactive attachment assay designed for initial attachment of 90 min was continued for 24 h and although there was a significant difference ($p < 0.06$, Fig. 5) between the (Mg)–(Al_2O_3) discs and unimplanted Al_2O_3, it was not as striking as the visual cellular counts. As the cells adhered to (Mg)–(Al_2O_3) substrata appeared voluminous, it is possible that these cells were involved in considerable synthetic and metabolic activity and therefore much of the *L*-[^{35}S]-methionine was incorporated into proteins and excreted as either functional products of the cell or released as protein turnover during the extended culture assay period. Hence more emphasis should be given to the physical counts at 24 h and these results together with those from the 90 min assay point to the conclusion that HBDC preferentially adhere to (Mg)–(Al_2O_3) over the Al_2O_3 native surface.

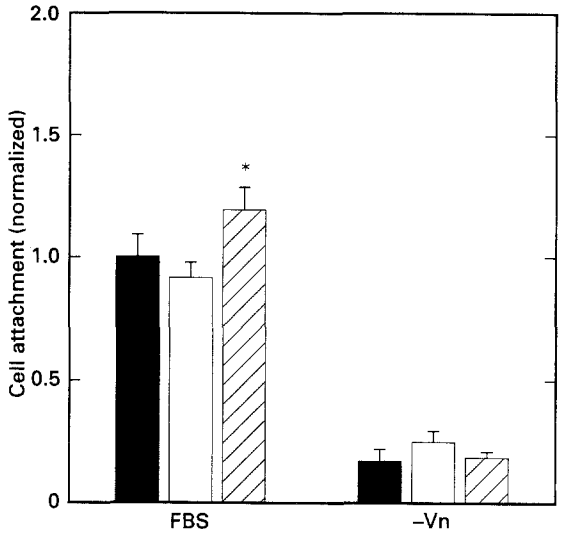

Figure 6 Histogram showing the requirement for serum Vn and Fn for cell attachment to (Mg)–(Al_2O_3) and Al_2O_3 surfaces of metabolically labelled HBDC, expressed as a proportion of that to PET. HBDC were seeded in culture medium containing intact FBS (FBS), or FBS depleted of Vn (– Vn). Cell attachment in these depleted media over a 24 h culture period is expressed as a percentage of those measured for the same surface, with the medium containing FBS. Mean ± SEM in a representative experiment (■PET; □ Al_2O_3; ▨ (Mg)–Al_2O_3)).

3.2. Mechanism of cellular adhesion to alumina implanted with magnesium

3.2.1. Effect of depletion of Fn and Vn from the FBS

The relative contribution of Fn and Vn to the initial attachment and spreading of HBDC on (Mg)–(Al_2O_3) was determined by selectively stripping these components from the FBS prior to addition to the culture medium. Figs 5 and 6 show the effect of selective removal of Fn or Vn upon the attachment of HBDC during the first 24 h of culture. For each of the Al_2O_3 and (Mg)–(Al_2O_3) surfaces, the attachment was dependent upon Vn within the FBS (Figs. 5 and 6). Selective removal of Vn from the 10% (v/v) FBS virtually abolished the attachment and spreading of

HBDC on Al_2O_3 and on (Mg)–(Al_2O_3). By comparison, the removal of Fn from the FBS did not reduce cell adhesion to either surface (Fig. 5). These results clearly show that it is Vn from the FBS that is the principal attachment factor for the initial adhesion of HBDC onto the (Mg)–(Al_2O_3) surface, and confirm the same finding for Al_2O_3, as reported previously [9].

3.2.2. Adsorption of Vn onto the surfaces

From the enhanced attachment of HBDC to the (Mg)–(Al_2O_3) surface as compared to that to Al_2O_3 during the first 90 min of culture, together with the Vn-dependence of cell attachment to both of these surfaces, we proposed as a hypothesis that the mechanism of enhanced cell attachment to (Mg)–(Al_2O_3) was due to an increased adsorption of Vn onto that surface during the period of initial cell attachment to the (Mg)–(Al_2O_3) surface. The surfaces were compared for their adsorption of Vn from culture medium containing FBS during a 90 min incubation period, using ^{125}I-Vn. The amount of ^{125}I on the surfaces was determined by two methods, the first involving an *in situ* assay with the ^{125}I being detected with a phosphor image system, with the ^{125}I then being eluted and counted directly. Fig. 7 shows that, surprisingly, the (Mg)–(Al_2O_3) surface adsorbed 1.6 to 2.5-fold less Vn than Al_2O_3 during the 90 min incubation. This was seen with both detection methods, ruling out any possibility that the ^{125}I-Vn was more tightly adsorbed onto the (Mg)–(Al_2O_3) surface and as a consequence was not effectively desorbed by the treatment with trypsin, alkali and detergent. Furthermore, the amounts of Vn that adsorbed onto the Al_2O_3 surface from FBS solution of 5–10% (v/v) were in the range of 4.5–7 ng Vn/cm^2 and (Mg)–(Al_2O_3) adsorbed 2.5–3 ng Vn/cm^2. These levels are markedly less than the Vn that adsorbs onto TCPS, which were 19–27 ng Vn/cm^2. Thus although the depletion experiment showed that HBDC adhesion to (Mg)–(Al_2O_3) was dependent upon the surface adsorbing Vn from the FBS, this surface does not show increased adsorption of Vn as compared to the Al_2O_3 surface.

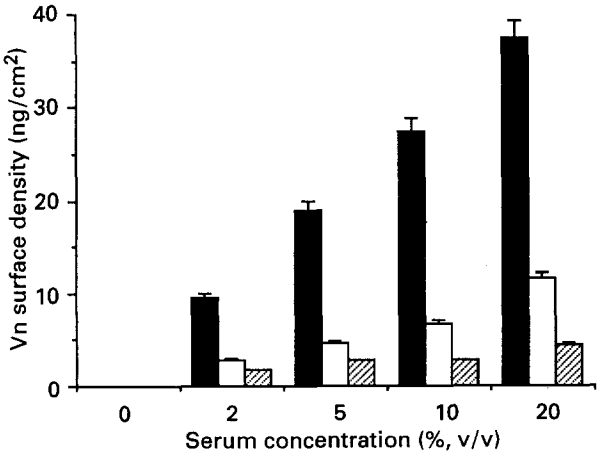

Figure 7 Histogram comparing the adsorption of Vn to the (Mg)–(Al_2O_3) (▨), Al_2O_3 (□) and TCPS (■) surfaces from culture media containing various concentrations of FBS. Dilution series were made from sera containing 50 μg/ml Vn, then the amounts of Vn adsorbed onto the surfaces was determined following incubation for 90 min by determination of the ^{125}I content by radioactive counting. Mean ± SEM of triplicate determinations in a representative experiment.

4. Discussion

4.1. Introduction

Despite the practical advances in forming and modifying the surface topography of a prosthesis for tissue fixation [20–27], much is still to be understood about controlling cellular adhesion and subsequent tissue differentiation by selectively modifying the surface chemistry of the biomaterial. There is an unmet need for surfaces with a designed topography and chemistry that will allow and enhance differentiated tissue to interlock into them, thereby effectively bonding a device to the skeleton promptly and permanently. It is generally agreed that anchorage dependent cells must adequately attach to a substratum in order to spread and thereby multiply [28, 29]. Later a differentiated tissue will form and may be influenced by the biomaterial [30, 31]. Since cellular attachment is primarily a molecular occurrence dependent upon the chemical and topographical characteristics of the substrata within a few nanometres of the surface, and the topography of the surface, it would be preferred that the surface layers be altered in a controlled manner. Ion beam implantation is one such technology that can achieve controlled modification of surface atomic layers. In this study we have evaluated the effect of ion beam implantation of Al_2O_3 with Mg for possible improvement of the attachment of HBDC.

4.2. Effect of implantation with magnesium upon cell adhesion to alumina

In this study Al_2O_3 discs were modified by ion beam implantation in either a diffuse or patterned manner. The latter modification allowed examination of the cellular morphology, at the interface between implanted and unimplanted regions, by Nomarski microscopy. It was calculated from Rutherford backscatter results that Mg had been implanted in the surface atomic layers and penetrating beyond 25 nm from the surface of these Al_2O_3 discs.

The number of HBDC that adhered to the (Mg)–(Al_2O_3) discs at 90 min and 24 h culture period was significantly increased above that adhered to the unmodified Al_2O_3 surface. This occurred in both the diffusely implanted surfaces, and also more effectively with Al_2O_3 where the ion implantation was applied in a patterned format. Indeed, there was quite a marked difference between number and morphology of HBDC on the implanted zones as compared to those on control zones of these pattern-treated discs. That is, (Mg)–(Al_2O_3) regions had approximately 50% more cells present after 24 h culture than the Al_2O_3 regions. Moreover, the morphology of these cells was strikingly different, being more voluminous and larger with their cytoplasmic processes being quite extensive and conspicuous. The higher cell density could indicate that HBDC are being recruited or preferentially at-

tached to the (Mg)–(Al_2O_3) surface. Although, less likely given that the cell assay period was for 24 h, it is possible that a difference in mitotic rate of the HBDC could underlie the difference in cell numbers and distribution but a full growth study would be needed to determine whether the surfaces differ in their support of mitotic activity. The difference between implanted and control regions in HBDC adhesion seen on the patterned implanted discs is similar to that reported by Lee and colleagues with bovine aortic endothelial cells, using patterned implantation of Na^+ and Ne^+ into a polymeric substratum [5].

The enhanced attachment of HBDC to (Mg)–(Al_2O_3) demonstrated in this study persisted for at least the first 24 h of culture, and in this respect was superior to the results obtained in a previous study where oxygen was implanted to a dose of $1 \times 10^{16}/cm^2$ into silicon wafers [6]. However, the present results with (Mg)–(Al_2O_3) are consistent with those obtained when Mg was implanted into silicon wafers. Such a treatment supported improved colonization with HBDC over a 14-day culture period, with the cells packing in a more orderly fashion than occurred with HBDC grown on silicon wafers implanted with either silicon or titanium [2]. Thus is would appear that the Mg *per se* is having an effect on the cellular behaviour of HBDC.

The morphological change in HBDC on (Mg)–(Al_2O_3) detailed above, as compared to those cells on Al_2O_3, could indicate that HBDC on (Mg)–(Al_2O_3) are more metabolically active than those residing on untreated zones or native Al_2O_3 discs. Similar morphological differences in bone cells on different substrata were observed by Vrouwenvelder *et al.* [32] in their comparison of bioactive and non-reactive glasses, and in that study the plumper and larger cells produced more type 1 collagen and osteocalcin.

Such obvious differences in morphology of HBDC were linked to the different streaming potentials measured from silicon wafers when ion beam implanted with different elements [2]. Furthermore, ion concentration has been recorded as affecting the streaming and zeta potentials of bone [33]. These bioelectrical potentials are considered to be involved in generation, repair and remodelling of bone [34, 35]. Certainly Shelton and Davies [7] considered that charge enhanced *in vitro* osteoblastic colonization and *in vivo* osteogenesis. In considering the effect of implantation with Mg, it should be noted that we cannot exclude the possibility that some of the Mg^{+2} implanted into the surface is mobile, and therefore some may be released realizing an effect at the interface. Divalent cations, including Mg^{+2}, are known to be active in cell adhesion mechanisms [36] and it will be of interest in future studies to determine whether there is significant release of Mg^{+2} from the implanted surface. However, the diminished cellular attachment and spreading of HBDC on Al_2O_3 zones close to the boundary of (Mg)–(Al_2O_3) zones in the patterned implanted discs suggest that any such effects, due to possible Mg^{+2} release, are either extremely localized or do not occur.

4.3. Mechanism of cell adhesion to alumina implanted with magnesium

When cellular colonization of biomaterial surfaces occurs in a culture assay using medium which contains FBS, the attachment of cells is concurrent to adsorption onto the substratum of serum glycoproteins. Understanding which glycoproteins of FBS are supportive or inhibitory to the attachment of HBDC to a surface could provide a mechanistic basis for the choice of surface chemistry to be used in an implant. For this reason, we sought to determine the role of Fn and Vn from FBS in the colonization of HBDC onto the (Mg)–(Al_2O_3) surface. Previously we have shown that the initial attachment and spreading of HBDC onto metallic and polymeric surfaces, as well as to Al_2O_3, is as a result of adsorption of serum Vn [9]. In the present study, we similarly found that for each of the Al_2O_3 and (Mg)–(Al_2O_3) surfaces, the attachment was dependent upon Vn within the FBS. Selective removal of Vn from the 10% (v/v) FBS virtually abolished the attachment and spreading of HBDC on Al_2O_3 and on (Mg)–(Al_2O_3). By comparison, the removal of Fn from the FBS did not reduce cell adhesion to either surface. These results demonstrate that a similar mechanism of cellular attachment occurs with the (Mg)–(Al_2O_3) surface as previously reported for Al_2O_3 [9]. The initial adhesion of HBDC to tissue culture plastic polystyrene (TCPS) was previously shown to be superior to that of hydrophobic polystyrene, a difference attributed to enhanced adsorption of Vn to TCPS [8, 14].

The enhanced attachment of the HBDC to the (Mg)–(Al_2O_3) surface as compared to unmodified Al_2O_3, together with the Vn-dependence of HBDC attachment to both surfaces, raised the hypothesis that the enhanced attachment of (Mg)–(Al_2O_3) resulted from higher levels of Vn adsorbing onto (Mg)–Al_2O_3) substratum. The amount of Vn adsorbed was measured using an *in situ* assay, then by elution and direct radioactive counting. These equivalent results showed that the effect was upon adsorption of Vn rather than merely being a difference between (Mg)–(Al_2O_3) in the elutability of the Vn from the surface. The adsorption of ^{125}I Vn onto (Mg)–(Al_2O_3) and Al_2O_3 was determined in an incubation that mimicked the conditions during the first 90 min of incubation with culture medium containing different levels of serum. Curiously, the (Mg)–(Al_2O_3) substratum showed markedly lower adsorption of Vn from media containing from 2–20% (v/v) FBS than did Al_2O_3. Furthermore, the amounts of Vn that adsorbed onto the Al_2O_3 surface was lower than that to TCPS. These findings, taken together, suggest that the surface density of Vn required for HBDC adhesion to these ceramic surfaces could be markedly lower than that for the polymeric TCPS surface. Regardless of the fact that Vn adsorption was significantly less than for the native alumina, it is apparent that HBDC adhesion to (Mg)–(Al_2O_3) was dependent upon the surface adsorbing sufficient Vn as to support cell attachment and that this (Mg)–(Al_2O_3) ceramic had more adherent cells.

It will be of interest in future investigations, to study the relationship between HBDC adhesion and surface

density of adsorbed Vn, comparing the following substrata of (Mg)–(Al_2O_3), Al_2O_3 and TCP.

5. Conclusions

The attachment and spreading of cultured human bone derived cells onto (Mg)–(Al_2O_3) was significantly enhanced, as compared to Al_2O_3. HBDC on (Mg)–(Al_2O_3) had markedly different morphology, being better spread and more voluminous, suggestive of higher metabolic and synthetic activity. The cellular adhesion to alumina and the (Mg)–(Al_2O_3) surfaces in the presence of FBS was dependent upon serum Vn.

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