Application of Confocal Laser Scanning Microscopy to Cytocompatibility Testing of Potential Orthopaedic Materials in Immortalised Osteoblast-Like Cell Lines

R. Macnair
University of Strathclyde

R. Wilkinson
University of Strathclyde

C. MacDonald
University of Paisley

I. Goldie
Karolinska Hospital

D. B. Jones
Philipps Universitat

Follow this and additional works at: https://digitalcommons.usu.edu/cellsandmaterials

See next page for additional authors

Part of the Biomedical Engineering and Bioengineering Commons

Recommended Citation

Utah State University
MERRILL-CAZIER LIBRARY

Application of Confocal Laser Scanning Microscopy to Cytocompatibility Testing of Potential Orthopaedic Materials in Immortalised Osteoblast-Like Cell Lines

Authors

R. Macnair, R. Wilkinson, C. MacDonald, I. Goldie, D. B. Jones, and M. H. Grant

APPLICATION OF CONFOCAL LASER SCANNING MICROSCOPY TO CYTOCOMPATIBILITY TESTING OF POTENTIAL ORTHOPAEDIC MATERIALS IN IMMORTALISED OSTEOBLAST-LIKE CELL LINES

R. Macnair[1], R. Wilkinson[1], C. MacDonald[2], I. Goldie[3], D.B. Jones[4] and M.H. Grant[1]*

[1]Bioengineering Unit, University of Strathclyde, 106 Rottenrow, Glasgow G4 0NW, U.K.
[2]Dept. Biological Sciences, Univ. Paisley, Paisley, U.K.; [3]Dept. Orthopaedics, Karolinska Hospital, Stockholm, Sweden; [4]Dept. Experimental Orthopaedics, Philipps Universitat, D-35033 Marburg, Germany

Abstract

Confocal laser scanning microscopy (CLSM) was used in conjunction with *in vitro* cell culture to investigate cellular interactions with orthopaedic biomaterials. Transfected rat and human osteoblasts were seeded on two potential isoelastic hip prosthesis materials, carbon fibre reinforced polyetheretherketone (PEEK) and epoxy. Titanium 318 alloy was employed as a control. Determination of the material surface contour, an important factor influencing cellular adhesion, proliferation and function, was performed using the industry standard Talysurf® and compared to analogous results obtained using the CLSM. The latter technique consistently gave higher values of material roughness but offers the advantage that it can be used to correlate roughness with cell distribution on the same samples, whereas Talysurf® measurement of roughness requires clean rigid samples. Image analysis and processing, performed on cells after attachment and culture on the materials for 48 hours, provided cell morphology data. Cells cultured on titanium were larger, with a higher percentage of cytoplasm, than those grown on either of the other materials. The macroscopic surface of epoxy resulted in smaller cells with altered morphology, which orientated themselves along carbon fibres. In conclusion, we believe CLSM offers great potential for investigating the cellular interactions of biomaterials involving minimal sample preparation, non-invasive optical sectioning of samples and minimal opportunity for generation of cellular deformation and sample preparation artefacts.

*Address for correspondence:
M.H. Grant
Bioengineering Unit, University of Strathclyde,
106 Rottenrow, Glasgow G4 0NW, U.K.
            Telephone number: (44) 141 552 4400 ext. 3438
                        FAX number: (44) 141 552 6098

Introduction

Prior to use in humans, biomaterials are routinely implanted in laboratory animals to assess long term biocompatibility (Riccio *et al.*, 1994). The cytocompatibility of these materials is screened *in vitro*, usually using material extracts in conjunction with cultured cells representative of the implantation site. However, this method does not allow prediction of the direct contact interaction between the materials and cells. Surface topography can influence a number of cellular processes both *in vitro* and *in vivo* (Curtis and Varde, 1964). Cells respond to the substrata through contact guidance (Dunn, 1982; Brunette, 1986; Curtis and Clark, 1992) and through surface chemistry and energy (Ratner, 1987).

We examined the response of two immortalised osteoblast lines to direct contact with biomaterials. FFC cells, originating from neonatal rat calvaria and THO cells, from human adult jaw bone, were grown on titanium-6Al-4V (Ti-6Al-4V) and two potential orthopaedic biomaterials, carbon fibre reinforced polyetheretherketone (PEEK) and epoxy. Examination of the initial cellular interaction during direct contact between osteoblasts and materials may provide more significant and relevant data than a multitude of biochemical extraction tests. Cellular adhesion influences every subsequent response (Sinha *et al.*, 1994) such as spreading, proliferation and biosynthetic behaviour.

The availability of immortalised differentiated stable osteoblast cell lines for testing the biocompatibility of orthopaedic materials will be of considerable value. Their use avoids the requirement to isolate and characterise primary cultures for each set of biomaterial evaluations, and enables us to evaluate and compare the reactions of several materials on cells from the same donor. We have previously compared the responses of primary and immortalised rat osteoblasts and primary human osteoblasts to the toxicity of nickel chloride, and to exposure to extracts of both carbon fibre reinforced PEEK and epoxy (Macnair *et al.*, 1996). We found that immortalised osteoblasts provided a good model system for biocompatibility studies. The immortalised cells

contain stable alkaline phosphatase activity which is inducible by 1,25-dihydroxy vitamin D_3 and by dexamethasone to a similar extent as is the activity in primary cells. The immortalised cells also secrete collagen I (Macnair, unpublished data). Based on these observations, the immortalised cells appear to have retained and to express osteoblast phenotype, and their use in the field of biocompatibility and regulation of osteoblast function is likely to expand.

The concept behind cementless total hip arthroplasty (THR) is of direct anchorage between bone and the prosthesis. The long term stability of the implant is determined by the initial interaction between the material and bone cells. Fibrous ingrowth, which leads to stable fixation, is necessary for this union to occur, therefore, material properties which affect cellular adhesion and proliferation will be of vital importance in determining the long term stability of the implant in the host bone.

Although conventional optical microscopy of cells grown on materials has been performed previously (Puleo and Bizios, 1992; Martin *et al.*, 1995), confocal laser scanning microscopy (CLSM) can offer considerable advantages (Piattelli *et al.*, 1993, 1994). Cells and materials can be examined with water immersion lenses, and CLSM allows non-invasive serial optical sectioning unlike analysis with transmission electron microscopy (TEM), which requires specimens to be mounted in methyl-methacrylate prior to sectioning using an ultramicrotome. Furthermore, sample preparation for CLSM is simple, requires less time, causes fewer artefacts and reduces out of focus blur in images compared to conventional light microscopy. These advantages, along with powerful image analysis and processing capabilities, render CLSM a useful additional imaging technique for determining initial cellular adhesion on, and interaction with biomaterials.

Materials and Methods

Isolation and culture of cells

Primary rat osteoblasts were obtained from the calvaria of 24-hour-old neonatal rats by a modification of a published method (Binderman *et al.*, 1974). Briefly, frontal, parietal and occipital regions of the calvariae were excised, washed with Tris buffered saline (TBS), minced and incubated for 90 minutes with a 1:1 mixture of 0.25% (weight/volume, w/v) trypsin and 0.02% (w/v) versene (both from Gibco BRL Life Technologies, Paisley, Scotland). Cells were harvested at 700 g for 8 minutes, suspended in Dulbecco's modification of Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) foetal calf serum (FCS), and seeded in 35 mm Petri dishes at a density of 2 x 10^4 cells/cm^2.

Primary human osteoblasts derived from adult jaw bone were provided by Dr. David B. Jones, Philipps Univ., Marburg, Germany. These were routinely grown in Ham's F-10 medium supplemented with 10% FCS and 50 mg/l L-ascorbic acid (Sigma, Poole, Dorset, U.K.).

Transfection procedure

Transfection was carried out on both cell types at passage 4. The plasmid used was pUK42 (10.9 kb) which consists of the complete simian virus 40 (SV40) genome, except for a 6 bp deletion at the origin of replication, ligated to pRSVneo, which contains the gene coding for neomycin resistance.

A confluent 75 cm^2 flask was split 1:3 one day prior to transfection. Plasmid at a concentration of 100 $\mu g/10^6$ cells was introduced using calcium phosphate precipitation (Graham and van der Eb, 1973). Primary rat and human osteoblasts were incubated with pUK42 for 16 and 4 hours, respectively. The monolayer was washed with serum free medium to remove the precipitate before growth medium was replaced. After 4 further passages, the presence of SV40 large T antigen was detected by immunofluorescence, proving that the cells were expressing the SV40 DNA. After transfection, the immortalised rat osteoblasts were called FFC cells, and the immortalised human cells THO cells.

Material preparation

All materials were examined as discs 16 mm in diameter and 3 mm thick. The control material, Ti-6Al-4V (from IMI Titanium Ltd., Birmingham, U.K.) was machined in the Bioengineering Unit and presented to the cells with the original fine machined surface. The test materials, carbon fibre reinforced PEEK and epoxy, were supplied by the Department of Aeronautical Structures and Materials, The Royal Institute of Technology, Stockholm, Sweden. Titanium was cleaned in an ultrasonic cleaner for 30 minutes in 10% Decon 90 (Decon Laboratories, Hove, Sussex, U.K.). All materials were sterilised with 70% ethanol for 18 hours, followed by treatment with phosphate buffer saline (PBS) containing 100 units/ml penicillin and 100 $\mu g/ml$ streptomycin for 24 hours. The discs were washed in sterile medium (DMEM or Ham's F-10) and placed in a 9 cm diameter Petri dish. Twenty-five milliliter cell suspension at 2.5 x 10^4 cells/ml was introduced, and the dish was incubated for 48 hours at 37°C, in 95% air, 5% CO_2. Discs were removed, washed for 10 minutes in PBS with supplements as before and fixed overnight with 10% buffered formalin, prior to staining with 0.1% w/v ethidium bromide (Sigma) in PBS for 5 minutes. Examination was immediately carried out using the CLSM.
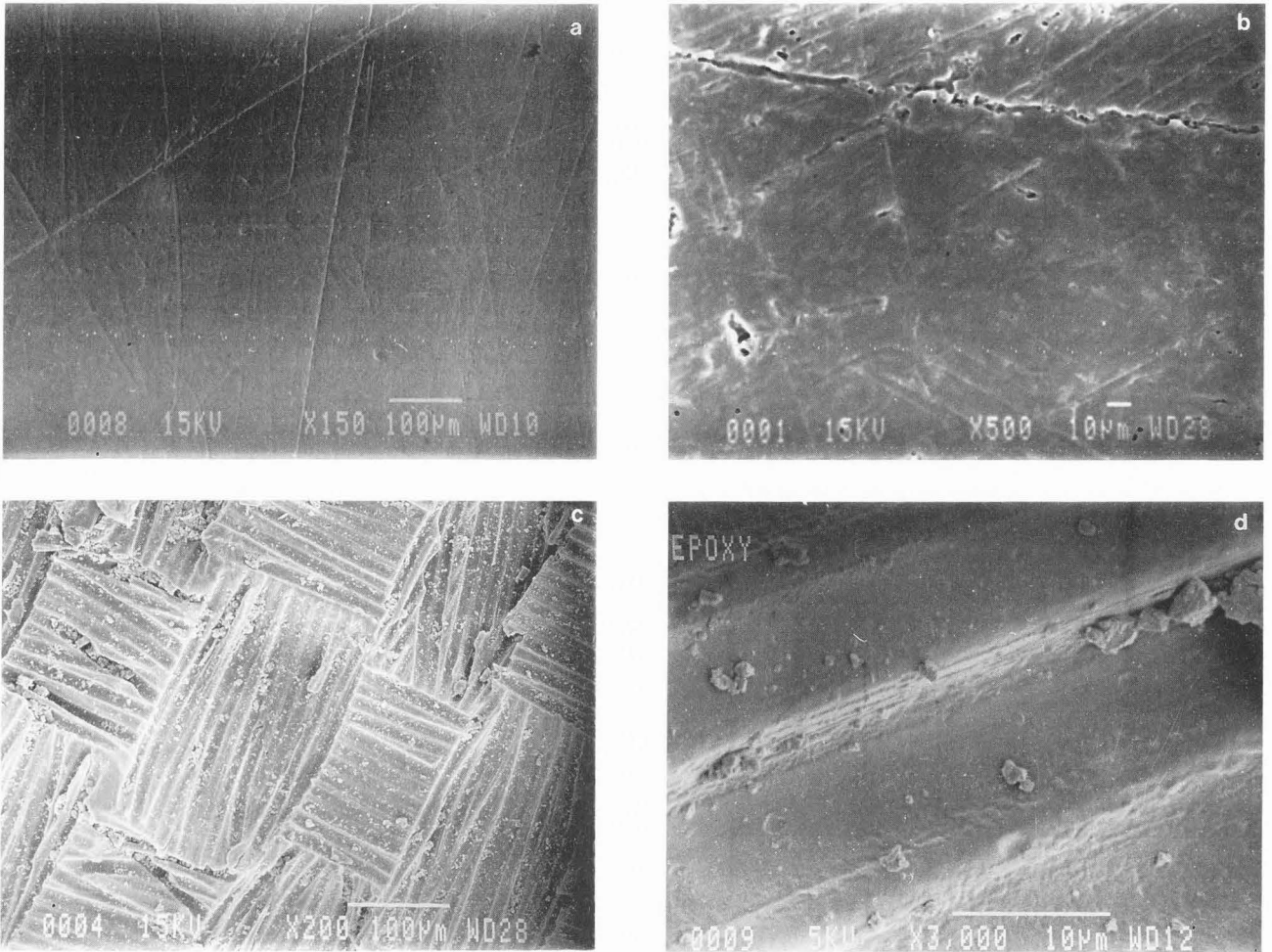
Figure 1. Scanning electron micrographs of the biomaterials. (**a**) control material Ti-6Al-4V; (**b**) polyetheretherketone (PEEK); (**c** and **d**) epoxy at different magnifications. Bars = 100 μm (in **a** and **c**) and 10 μm (in **b** and **d**).

Microscopic evaluation

Scanning electron microscopy (SEM) examination. Examination of blank discs was carried out with a scanning electron microscope (JEOL 840A, Tokyo, Japan). Both PEEK and epoxy were gold coated prior to examination. Titanium was simply attached to a specimen holder using conductive tape and carbon paint.

CLSM examination. After staining with ethidium bromide for 5 min, individual discs were attached to a 60 mm Petri dish with medical grade adhesive tape. Distilled water was added to submerge the discs, preventing cellular dehydration, prior to examination with a (x25/0.75 N.A.) water immersion lens and the 488 nm excitation line from an argon ion laser. Two dichroic mirrors, one centred at 510 nm, which rejects reflected incident light, and the other centred at 580 nm, were used in conjunction with 2 photomultipliers and 2 long pass filters (514 and 590 nm) to split fluorescent emis-sion.

Fields were randomly selected for morphological examination and image analysis. Image field depth was determined before collection of images at 1 μm intervals using both channels. After data collection, processing was carried out using the dedicated computer. Separate stacks were first reconstructed then superimposed to produce a single image with extended focal depth consisting of a pseudocolour image from each channel.

Discs without cells were imaged in reflection using the 514 nm line and a single dichroic mirror set at 580 nm. Using channel 2, which accepts wavelengths below 580 nm, and a 514 nm long pass filter, most of the fluorescence would be rejected. The photomultipliers were set to give full range, i.e., 0-255 over the image stack and a "TOPO" (CLSM Software Reference Manual, Leica Lasertechnik GmbH, Heidelberg, Germany) reconstruction performed which expresses height as brightness. A further subroutine gave values of surface

roughness; these were compared to values obtained from Talysurf® (Rank, Taylor & Hobson, Leicester, U.K.) a standard mechanical surface roughness measuring device. Talysurf® measurements were carried out by technicians in the Metrology Laboratory, Strathclyde University. Basically, surface roughness is determined using a pointer which follows surface contour (similar to the stylus of a record player).

Image analysis measurements were obtained through use of a random window subroutine which enabled outlining of cells or nuclei with a trackerball, before determination of the enclosed area in arbitrary units. The area of the nucleus was visualised by the ethidium bromide staining, and the area of the cytoplasm both by ethidium bromide staining and autofluorescence. Measurement of the cell compartment areas were reproducible to within 5%.

Results

Material examination

Figure 1 presents the SEM observations of material surfaces; Ti-6Al-4V alloy (Fig. 1a) exhibited a flat surface although multiple scratches were present. These scratches were analysed using a Talysurf® and also estimated by CLSM (Table 1). They appeared much deeper with CLSM than Talysurf®. Irrespective of which method was used to estimate the surface, titanium was much smoother than PEEK, where pits, cracks and other anomalies were observed (Fig. 1b). Epoxy, observed under low power magnification (Fig. 1c), had a basket weave surface caused by orientation of the carbon fibre reinforcement. Estimated depth of the troughs was approximately 9 μm (Table 1). This was approximately 14 times rougher than the surface of PEEK and 60 times that of titanium. At higher magnification (Fig. 1d), the epoxy coating over individual carbon fibres could be visualised. The gap between fibres was estimated using CLSM to be approximately 15 μm.

Surface roughness

Surface roughness data, compiled by Talysurf® and CLSM for measurement of square average peak to trough height, are shown in Table 1. With PEEK and epoxy, CLSM roughness estimates were approximately 25% higher than those of Talysurf®. Analysis of titanium presented very different results with CLSM estimation around two and a half times higher than Talysurf®.

Cell material interaction

CLSM examination of FFC cells and THO cells grown on the three materials for 48 hours are presented in Figures 2 and 3, respectively. With each material, green fluorescence was observed from the upper layers of the materials along with some autofluorescence from

Figure 2. Confocal laser scanning micrographs of FFC osteoblasts grown on titanium (**a**), PEEK (**b**) and epoxy (**c**). Bar = 10 μm.

**Figure 3**. Confocal laser scanning micrographs of THO osteoblasts grown on titanium (**a**), PEEK (**b**) and epoxy (**c**). Bar = 10 μm.

--

the cells. Cells grown on titanium (Figs. 2a and 3a) were well spread and demonstrated no particular orientation. However, a number of giant cells were noted in preparations from both rat and human cells. Cellular interaction with PEEK (Figs. 2b and 3b) exhibited interesting characteristics. Instead of spreading, FFC cells clumped together, forming discrete colonies. Again, there was no evidence of cellular orientation, and FFC cells were observed growing across carbon fibres (Fig. 2b). On the final material, epoxy, which has characteristic macroscopic surface morphology, both cell types aligned along the carbon fibres (Figs. 2c and 3c).

Image analysis

Data concerning the sizes of the nuclear and cytoplasmic compartments of 50 THO cells were collected from each material (Table 2) and analysed using Mann-Whitney's Confidence Interval and Test to establish statistical significance (Minitab for Windows Software, version 10.1). This compares a specified area, i.e., nucleus or cytoplasm, on every material with every other material. Results of the statistical comparison are summarised in Table 3. Nuclear areas were significantly different ($p < 0.05$) on all combinations of material with the exception of PEEK/titanium. Cytoplasmic area assessment proved significantly different ($p < 0.05$) on every material. A similar analysis was attempted using FFC cells, however, after the 48 hour incubation period on materials, the osteoblasts were in direct contact with each other making image analysis impossible.

Discussion

Criteria for successful prosthetic hip implantation include material biocompatibility, intimate apposition and functional dissipation of forces from load on the prosthesis to the underlying bone. This principle of optimal co-existence between implant and bone is defined as osseointegration and has been widely accepted for a long time. The surfaces of orthopaedic implants, as discussed by Ratner in 1987, govern their interactions with osteoblasts, which in turn, determine the stability of the bone/implant interface via their influence on the production of an osteoid matrix. This is particularly important for the development and improvement of cementless orthopaedic
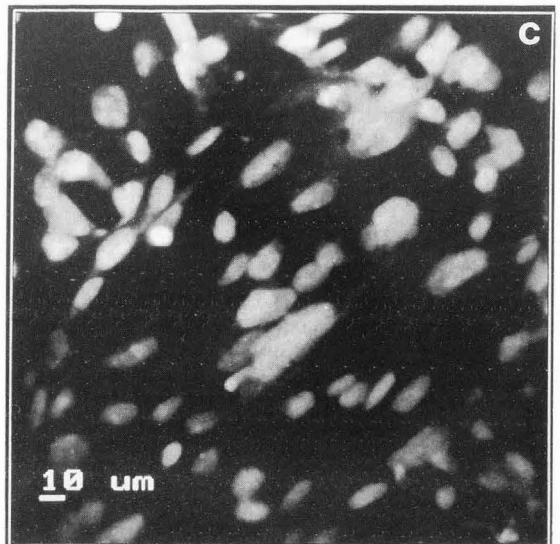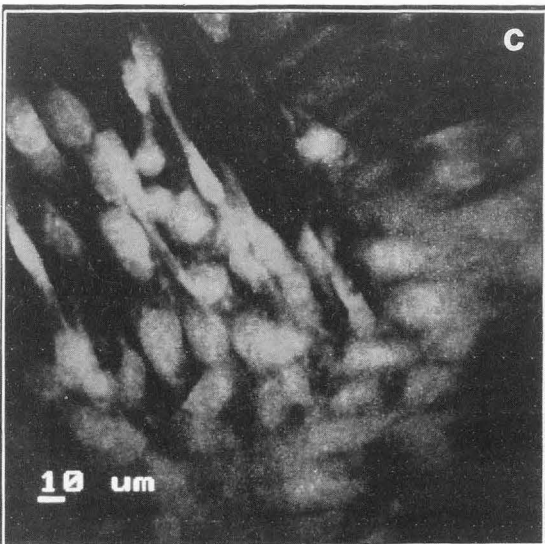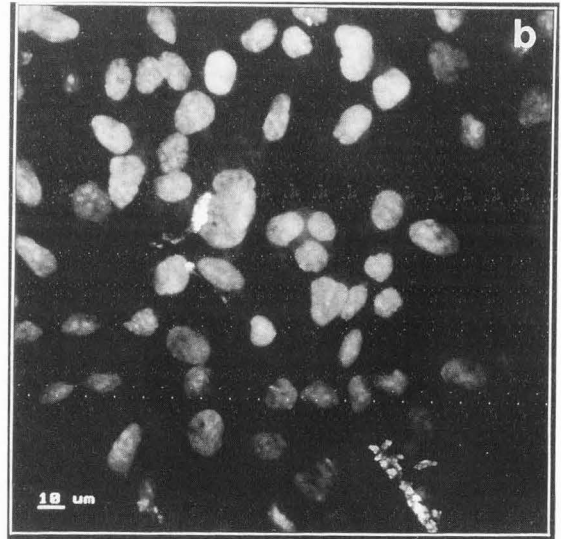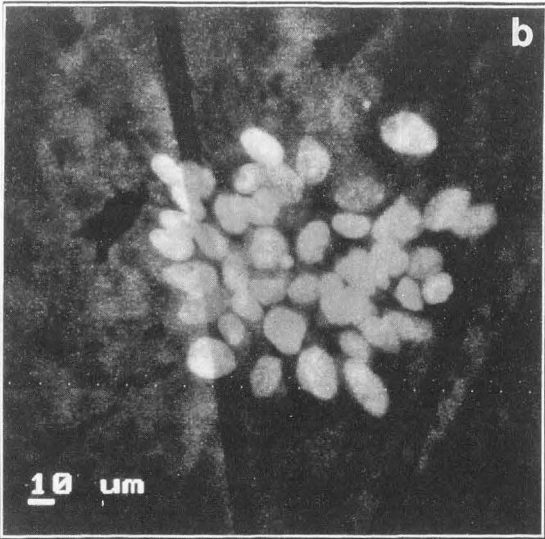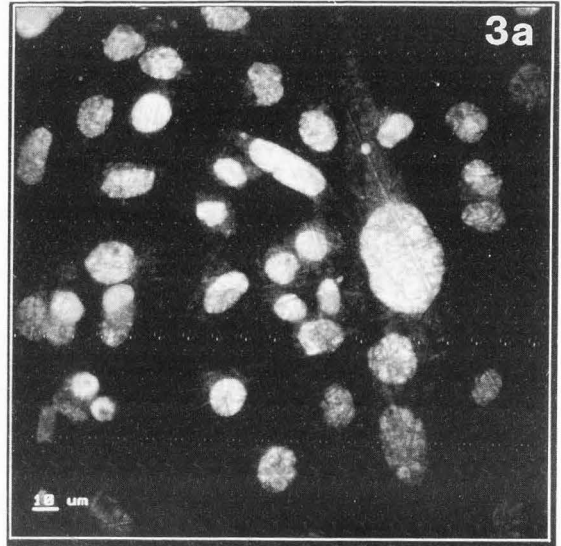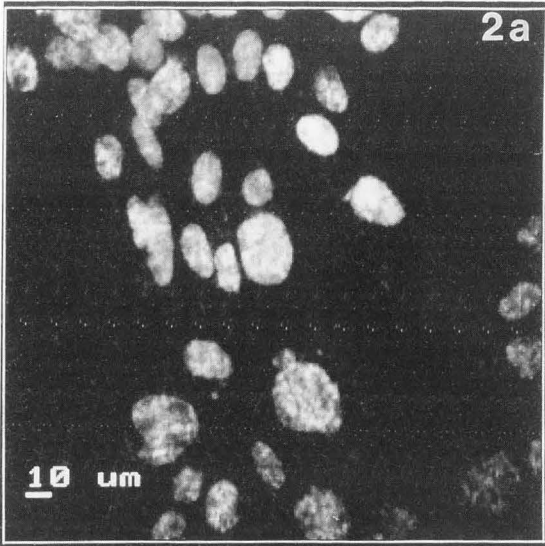
Table 1. Data obtained by Talysurf® and CLSM analysis of material surfaces.

| Material | Analysis Method | | CLSM %age diff. |
	Talysurf® (μm)	CLSM (μm)	
Titanium	0.153 ± 0.021	0.547 ± 0.021	+257.5
PEEK	0.617 ± 0.061	0.795 ± 0.074	+28.9
Epoxy	9.067 ± 0.330	11.180 ± 1.602	+23.3

Values are mean ± standard deviation of n = 6 CLSM, n = 3 Talysurf®. Measurements represent Ra (average peak to trough height).

Table 2. Data from image analysis of THO cells grown on the various materials.

Material	Area	Mean ± SD[1]	Mean ratio[2]
Titanium	Nucleus	535.3 ± 362.4	
Titanium	Cytoplasm	1329.1 ± 568.5	0.403
PEEK	Nucleus	443.8 ± 129.6	
PEEK	Cytoplasm	978.4 ± 363.3	0.454
Epoxy	Nucleus	333.2 ± 123.1	
Epoxy	Cytoplasm	587.3 ± 281.9	0.567

[1]Values (arbitrary units) are mean ± SD, where n = 50.
[2]Mean nuclear/cytoplasm ratio.

Table 3. Statistical comparison of image analysis results.

Material/Compartment	Significant Difference
Nucleus	
Titanium versus PEEK	No
Titanium versus Epoxy	Yes
PEEK versus Epoxy	Yes
Cytoplasm	
Titanium versus PEEK	Yes
Titanium versus Epoxy	Yes
PEEK versus Epoxy	Yes

Data are from THO cells. Statistical comparison was by ANOVA (analysis of variance) followed by Mann-Whitney; $p < 0.05$.

implants as discussed earlier. Surface factors contributing to this biological response include implant material chemistry, energy and topography. Many aspects of surface topography are important in the biological performance of materials, e.g., wettability, electric charges, and the effects of cleaning and sterilisation procedures. In addition, cellular adhesion and spreading *in vitro* are influenced by interactions between the materials and adsorbed medium and serum components. This is also the case *in vivo*. Polymers are currently being examined because their material properties can be specifically altered to improve both biocompatibility and biofunctionality.

We used CLSM to provide data on surface micro/macrostructure and to examine cellular response on three distinct materials. With regard to effects on cellular response, two distinct variables are associated with the materials: differences in chemical composition and surface structure. It has been shown previously that micromachined surfaces can control cell orientation and locomotion *in vitro* (Brunette *et al.*, 1983), and enhance or inhibit cell migration *in vivo* (Chehroudi *et al.*, 1989).

When FFC and THO immortalized cell lines were cultured on the control material, Ti-6Al-4V, they were significantly larger than when cultured on either of the other materials. Sinha *et al.* (1994) cultured primary human osteoblasts on the same material and also found large cells which were better spread than on other materials examined, which included tissue culture polystyrene and CoCrMo. This increased cell size may mean that cytoskeletal organisation is at a more advanced stage on this material, possibly due to conditioning biomolecules present in the serum being attracted to titanium alloys inherent in the surface oxide layer (Keller *et al.*, 1990). Alternately, the larger cells could be a cell type other than osteoblasts, which are present in the population and adhere well to the titanium.

We found that both FFC and THO immortalized osteoblasts cultured on the surface of epoxy mimicked a phenomenon also seen with epithelial cells (Brunette, 1986), fibroblasts and polymorphonuclear leukocytes (Wilkinson *et al.*, 1982) on a variety of micromachined materials. Although the cell numbers were not quantified, proliferation appeared to be improved when compared to the other materials, possibly as a result of increased cellular attachment, as discussed by Bowers *et al.* (1992). The 9 μm deep and 12 μm wide troughs present on the epoxy surface caused an alteration in cellular morphology. This may have resulted in increased proliferation, as there is evidence to suggest cell shape is involved both in regulating cellular functions such as matrix protein production and in proliferation (Folkman and Moscona, 1978). It is possible that contact guidance on micromachined surfaces increases the chance of stable implant integration, as surface topog-

raphy can affect mineralisation (Brunette, 1988). However, the affect of topography and roughness appears dependent on cell type. Work performed by Rich and Harris (1981) showed that fibroblasts preferred smooth surfaces unlike macrophages, which preferentially colonised rough areas. More detailed experiments must be performed on epoxy and other materials, under varying roughness/topological conditions, in order to determine whether the material itself or its surface properties cause these characteristic cellular responses.

When FFC cells divided on PEEK, they formed a discrete colony, an observation sometimes made on tissue culture polystyrene. THO cells also spread and proliferated on the PEEK disc as if growing on tissue culture polystyrene. Similar cellular responses have been observed on tissue culture plastic and PEEK previously when UMR 106.01 osteoblast-like cells, isolated from a rat osteogenic sarcoma, were allowed to attach before analysis by SEM (Hunter *et al.*, 1995).

Our material investigations were aimed at complementing existing *in vitro* cell culture techniques for biocompatibility testing recommended by ISO 10993-5, *in vitro* method (ISO, 1991). CLSM has tremendous potential for investigating cell morphology on materials and infiltration into materials; using immunofluorescence, the synthetic behaviour of cells on materials can be readily assessed, too. Availability of powerful image analysis software will enable quantification of cell volume, in addition to areas of intracellular compartments.

CLSM overestimated PEEK and epoxy material surface roughness by about 25% when compared to Talysurf® analysis, but the titanium results were overestimated by 250%. We believe this occured because not all of the signal from the CLSM was coming from reflection. Some was coming from a fluorescence component, and this was unexpected especially from titanium. Narrow pass filters were not available for the photomultipliers to minimise this. However, if CLSM could estimate surface roughness accurately then its non-contacting nature would provide several important benefits. Talysurf® requires a pointer to track over material surfaces. This method works well on clean and rigid material but has serious limitations when either a soft material such as polythene is under test, or when a solid material such as PEEK has biological material present on its surface. Instead of the needle passing across the surface and determining contour, it would rip the material if it was too soft, or become clogged with biological material. Both scenarios would produce erroneous results. CLSM, however, because of its non-contacting nature can easily determine contour of soft materials. Roughness of solid samples can also be quantified after cell growth and proliferation has occured, and this would be useful to correlate surface properties of materials directly to their biocompatibility.

References

Binderman I, Duksin D, Harell A, Katzir E, Sachs L (1974) Formation of bone tissue in culture from isolated bone cells. J. Cell Biol. **61**: 427-439.

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

Brunette DM (1986) Fibroblasts on micromachined substrata orient hierarchically to grooves of different dimensions. Exp. Cell Res. **164**: 11-26.

Brunette DM (1988) The effects of implant surface topography on the behaviour of cells. Int. J. Oral Maxillofac. Implants **3**: 231-246.

Brunette DM, Kenner GS, Gould TRL (1983) Grooved titanium surfaces orient growth and migration of cells from human gingival explants. J. Dent. Res. **62**: 1045-1048.

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.

Curtis ASG, Clark P (1992) The effects of topographic and mechanical properties of materials on cell behaviour. Crit. Rev. Biocomp. **5**: 343-362.

Curtis ASG, Varde M (1964) Control of cell behaviour: Topological factors. J. Nat. Cancer Res. Inst. **33**: 15-26.

Dunn GA (1982) Contact guidance of cultured tissue cells: A survey of potentially relevant properties of the substratum. In: Cell Behaviour. Bellairs R, Curtis ASG, Dunn GA (eds.). Cambridge University Press. p. 247.

Folkman J, Moscona A (1978) Role of cell shape in growth control. Nature **273**: 345-349.

Graham FL, van der Eb A (1973) Transfection by calcium phosphate precipitation. Virology **52**: 456-466.

Hunter A, Archer CW, Walker PS, Blunn GW (1995) Attachment and proliferation of osteoblasts and

fibroblasts on biomaterials for orthopaedic use. Biomaterials **16**: 287-295.

ISO (1991) Biological Testing of Medical Devices-Part 5: Tests for Cytotoxicity. Draft International Standard 10993-5. ISO, Geneva, Switzerland.

Keller JC, Draughn RA, Wightman JP, Dougherty WJ, Meletiou SD (1990) Characterization of sterilized cp titanium implant surfaces. Int. J. Oral Maxillofac. Implants **5**: 360-367.

Macnair R, Rodgers EH, MacDonald C, Wykman A, Goldie I, Grant MH (1996) The response of primary rat and human osteoblasts and an immortalized rat osteoblast cell line to orthopaedic materials: Comparative sensitivity of several toxicity indices. J. Matl. Sci. Matl. Med., in press,

Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J Jr, Dean 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. Mater. Res. **29**: 389-401.

Piattelli A, Trisi P, Emauelli M (1993) Bone reactions to hydroxyapatite coated dental implants in man: An histological study by means of SEM, light microscope and laser scanning microscope. Int. J. Oral Maxillofac. Implants **8**: 69-74.

Piatteli A, Trisi P, Passi P, Cordioli GP (1994) Histochemical and confocal laser scanning microscopy study of the bone-titanium interface: An experimental study in rabbits. Biomaterials **15**: 194-200.

Puleo DA, Bizios R (1992) Formation of focal contacts by osteoblasts cultured on orthopaedic biomaterials. J. Biomed. Mater. Res. **26**: 291-301.

Ratner BD (1987) Biomaterial surfaces. J. Biomed. Mater. Res. Appl. Biomater. **21**: 59-90.

Riccio V, Ragione FD, Marrone G, Paulumbo R, Guida G, Olivia A (1994) Cultures of human embryonic osteoblasts. Clin. Orthop. Rel. Res. **308**: 73-78.

Rich A, Harris AK (1981) Anomalous preferences of cultured macrophages for hydrophobic and roughened substrata. J. Cell Sci. **50**: 1-7.

Sinha RK, Morris F, Shah SA, Tuan RS (1994) Surface composition of orthopaedic implant metals regulates cell attachment, spreading, and cytoskeletal organization of primary human osteoblasts *in vitro*. Clin. Orthop. Rel. Res. **305**: 258-272.

Wilkinson PC, Shields JM, Haston WS (1982) Contact guidance of human neutrophil leukocytes. Exp. Cell. Res. **140**: 55-62.

Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers**.