Cells and Materials

Volume 7 | Number 2 | Article 7

1997

Image Analysis of Primary Bone-Derived Cells on Different Polystyrene Surfaces

R. M. Shelton
University of Birmingham

G. Landini
University of Birmingham

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

Part of the Biomedical Engineering and Bioengineering Commons

Recommended Citation

Shelton, R. M. and Landini, G. (1997) "Image Analysis of Primary Bone-Derived Cells on Different Polystyrene Surfaces," *Cells and Materials*: Vol. 7 : No. 2 , Article 7.
Available at: https://digitalcommons.usu.edu/cellsandmaterials/vol7/iss2/7

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Cells and Materials by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.

Cells and Materials Vol. 7, No. 2, 1997 (pages 147-159) 1051-6794/97$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

IMAGE ANALYSIS OF PRIMARY BONE-DERIVED CELLS ON DIFFERENT POLYSTYRENE SURFACES

R.M. Shelton[1*] and G. Landini[2]

[1]Biomaterials Unit and [2]Oral Pathology Unit, School of Dentistry, University of Birmingham, UK

(Received for publication July 12, 1997, and in revised form October 23, 1997)

Abstract

The aim of the present study was to examine whether two different cell populations could be discerned using image analysis of a variety of morphological parameters on bacteriological and tissue culture polystyrene surfaces. Rat periosteal and osteoblast cultures were established on both polystyrene petri dishes and examined using phase contrast microscopy after one and two weeks before capturing digital images which were stored on a personal computer. The digital images were processed to identify the cell margins or perimeter, from which seven different morphological parameters were calculated using a program developed (by GL) for both the cell populations on the two polystyrene surfaces. None of the morphological parameters were able to distinguish between all of the culture types, so discriminant analyses were applied using different combinations of the parameters. The best discrimination between the different cell outlines was found after one week using 5 of the parameters combined in a quadratic discriminant analysis, which allocated 94% of the outlines to their correct group and 94% after two weeks using all the parameters. The study demonstrated that it was possible to quantify morphological differences between two cell populations grown on either tissue culture or bacteriological polystyrene petri dishes. It may be possible to develop this image and statistical analysis further to allow non-invasive automatic identification of particular cells in mixed populations *in vitro*.

Key Words: Image analysis, phase contrast microscopy, bacteriological polystyrene, tissue culture polystyrene, osteoblast, periosteal cell, cell morphology, substrata, morphological parameters, *in vitro*.

*Address for correspondence:
R.M. Shelton
Biomaterials Unit, School of Dentistry
University of Birmingham
St Chad's Queensway, Birmingham B4 6NN, UK
Telephone number: 0121 237 2905
FAX number: 0121 237 2932
E-mail: R.M.Shelton@bham.ac.uk

Introduction

There has been a considerable amount of research into the behaviour of cells including cell morphology and adhesion on bacteriological (BAC) and tissue culture (TC) or concentrated sulphuric acid-treated bacteriological polystyrene (ATBAC). Native untreated polystyrene (BAC) does not support good cell attachment, adhesion or spreading. Several authors have demonstrated the preferential adhesion of fibroblasts to wettable acid-treated bacteriological polystyrene as compared with unwettable bacteriological polystyrene (Martin and Rubin, 1974; Maroudas, 1975; Klemperer and Knox 1977; Curtis *et al.*, 1983; Lydon and Clay, 1985). Initially it was proposed that concentrated sulphuric acid treatment of bacteriological polystyrene caused sulphonation of the surface and this made it more adhesive for BHK fibroblasts (Maroudas, 1975). Later evidence was presented that the acid treatment introduced hydroxyl ions onto the surface of polystyrene (Curtis *et al.*, 1983; Thomas *et al.*, 1986). Curtis *et al.* (1986) proposed that hydroxyl ions were required for adhesion of BHK cells which was demonstrated using polymer surfaces with measured densities of hydroxyl ions. When the surface hydroxyl ions were blocked by acetylation with acetic anhydride there was a corresponding decrease in cell adhesion. More recently Callen *et al.* (1993) examined the surfaces of polystyrene dishes treated with either concentrated sulphuric acid or exposed to gamma irradiation using x-ray photoelectron spectroscopy and found that the two treatments introduced different chemical groups onto the polystyrene surfaces, suggesting the acid treatment introduced sulphonic acid groups whilst gamma irradiation introduced hydroxyl groups.

The difference in the ability of cells to colonize BAC and TC polystyrene has been accounted for in different ways. Grinnell and Feld (1981, 1982) suggested that the cell-adhesive protein fibronectin was adsorbed to BAC dishes in a less biologically active conformation than on TC dishes, whilst Curtis and Forrester (1984) proposed that larger quantities of anti-adhesive proteins were adsorbed onto BAC polystyrene than on TC plastic which then resulted in less cell

attachment on BAC polystyrene. Later Curtis *et al.* (1986) suggested that cell adhesion was influenced by the presence of hydroxyl groups, which were present on ATBAC and TC polystyrene but absent on BAC polystyrene.

The above studies commonly utilised cell lines isolated with a proteolytic enzyme such as trypsin which is known to remove cell membrane glycoproteins involved in cell adhesion (Revel *et al.*, 1974) and also interfere with intracellular microtubules (Osunkoya *et al.*, 1969) which are known to contribute to the determination of cell shape. The present study investigated the behaviour of primary non-trypsinized calvarial cells on the different polystyrenes to avoid any trypsin-associated morphological alterations of the cells and undertook a quantitative analysis of seven parameters to define the cell morphology observed on differently treated polystyrene dishes in numerical terms.

Commonly cell morphology has been described in subjective terms such as rounded, flattened, stellate, fibroblastic etc., and whilst this is suitable in many cases to identify certain cells, raises many interesting points such as at which point does a rounded cell become stellate or flattened and in which dimension is the cell rounded? It clearly would be beneficial to have a non-subjective method for characterising cell morphology as Folkman and Moscona (1978) found a strong correlation between untransformed cell morphology and DNA synthesis and growth. It may also be that cell morphology is found to be correlated with gene expression and thereby provide another mechanism for investigating the development of cells in culture. In addition it is important to have indications of cell spreading and morphology which are not purely subjective, to allow meaningful comparisons to be made at different time points and between different cell types in culture.

The objective of the present study was to provide quantitation of the morphological differences previously observed in cells on bacteriological and tissue culture dishes and to identify any particularly useful parameters (or combination of parameters) for defining cell shape on different surfaces. Two cell types were used, in which one was thought to be predominantly osteoblasts whilst the other were a more mixed population derived from periosteum and therefore likely to be fibroblasts and osteoprogenitor cells.

Materials and Methods

Polystyrene Substrata

Polystyrene petri dishes were used as supplied in the two forms of 60 mm diameter bacteriological (Sterilin) and 60 mm diameter tissue culture (Corning) plastic, both supplied by Bibby Sterilin Ltd, Stone, UK.

Cell Culture

The present study used two different cell types established from the calvaria of 1-2 day old albino Wistar rats. Primary osteoblasts (OBs) were derived from the periosteally stripped parietal bones, which were finely minced with scissors and subsequently seeded and grown on the bacteriological (BAC) and tissue culture (TC) petri dishes as explants. Periosteal cell populations were derived from the endo and exo cranial periostea which had been stripped from the parietal bones previously, finely minced with scissors and again grown as explant cultures in the BAC and TC dishes. The medium used for all cultures was Fitton Jackson modified Biggers medium (powder from Sigma, Poole, UK) supplemented with 10% foetal calf serum (Imperial Laboratories, Andover, UK), 1 M HEPES (25 μl/ml), L-glutamine (to a final concentration of 200μl/ml), penicillin and streptomycin (10μl/ml), all from Sigma. Cultures were maintained in 8 ml of medium, replaced every 4 days, for 2 weeks in a Jouan IG150 (Saint Herblain, France) incubator at 37°C in an atmosphere of 5% CO_2.

Image Analysis

Cultures were examined after one and two weeks using phase contrast microscopy (Leitz Fluovert, Leica, Milton Keynes, UK) and this microscope was also used with the same magnification in the image capture process via a C-mount with a Cohu CCD high resolution monochrome camera (San Diego, CA) interfaced to a personal computer. Only cells with well defined margins were used in this study which generally occurred at the periphery of groups of migrating cells, although sometimes adjacent cells were clearly defined and thus it was possible to use cells from more central areas. The images were processed for analysis using Optimas software (Optimas Corp, Bothell, WA) and a macro was used to capture and save a 256 grey scale image, apply a Sobel filter (to assist in edge detection) and then produce and save a final binary image. The resultant binary images were then edited to remove any culture debris and artefacts and saved as individual cell perimeter files from which their perimeter, projected area, feret (longest) diameter (Kaye, 1986), form factor (4π area/perimeter^2) (Payne *et al.*, 1989), shape factor (perimeter^2/area) (Stenkvist and Strande, 1989), PERBAS (perimeter/convex hull length) (Payne *et al.*, 1989) and fractal dimension D (Mandelbrot, 1983) using the box counting algorithm were calculated using a program developed by one of us (G.L.). The entire image capture process was also calibrated using a stage micrometer to determine the dimensions of a pixel.

Form factor and shape factor provide measures of deformation of an outline from a circle (a perfect circle

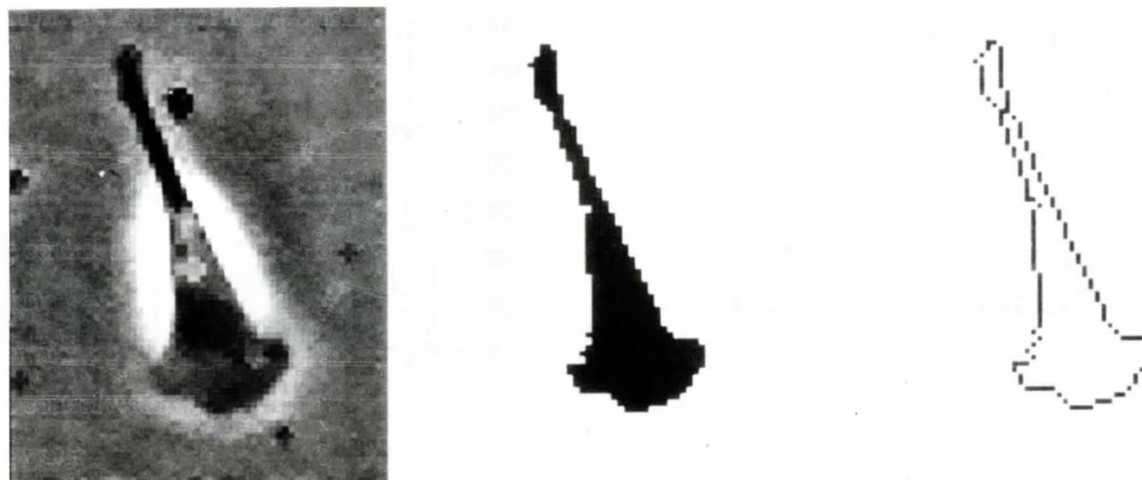

Figure 1. Three digital images captured from the phase contrast microscope of a poorly spread osteoblast cultured on bacteriological polystyrene for 1week, showing from left to right the original 256 grey scale image, the cell shape following edge detection with a Sobel filter and the final binary trace of the cell periphery. Field width of each of 3 images: 146 μm.

Figure 2. Three digital images captured from the phase contrast microscope of poorly spread periosteal cells cultured on bacteriological polystyrene for 1week, showing from left to right the original 256 grey scale image, the cell shape following edge detection with a Sobel filter and the final binary trace of the cell periphery. The poor spreading and therefore increased depth of cells on bacteriological polystyrene unfortunately produced a bright halo around cells which made edge detection more difficult and therefore possibly less accurate. Field width of each of 3 images: 487μm.

---

having a form factor of 1). PERBAS provides a better indication of surface roughness than either form or shape factor but cannot distinguish between circles and ellipses (both having PERBAS values of 1), although is more sensitive to roughness of spherical objects than roughness of ellipsoid shapes (Payne *et al.*, 1989). Consequently using these parameters individually may not provide clear discrimination between different shapes, but in combination can provide precise information regarding circularity and surface roughness. Fractal analysis is a relatively new form of non-Euclidean geometry which is capable of numerically determining the complexity or self similarity of fractal objects (e.g., the branching of trees or vascular networks) which is termed the fractal dimension. The fractal dimension of profile embedded in two dimensional space (such as the cell profiles considered in the present study) varies between 1 and 2, with highly complex shapes having values closer to 2 than more simple shapes such as circles which have values of 1.

Each cell perimeter file was checked to be an accurate representation of the cell margin by superimposing it on the original grey scale image which was also captured at the time of viewing, any images which were not accurate were discarded. This was most common for images of cells seen on bacteriological petri

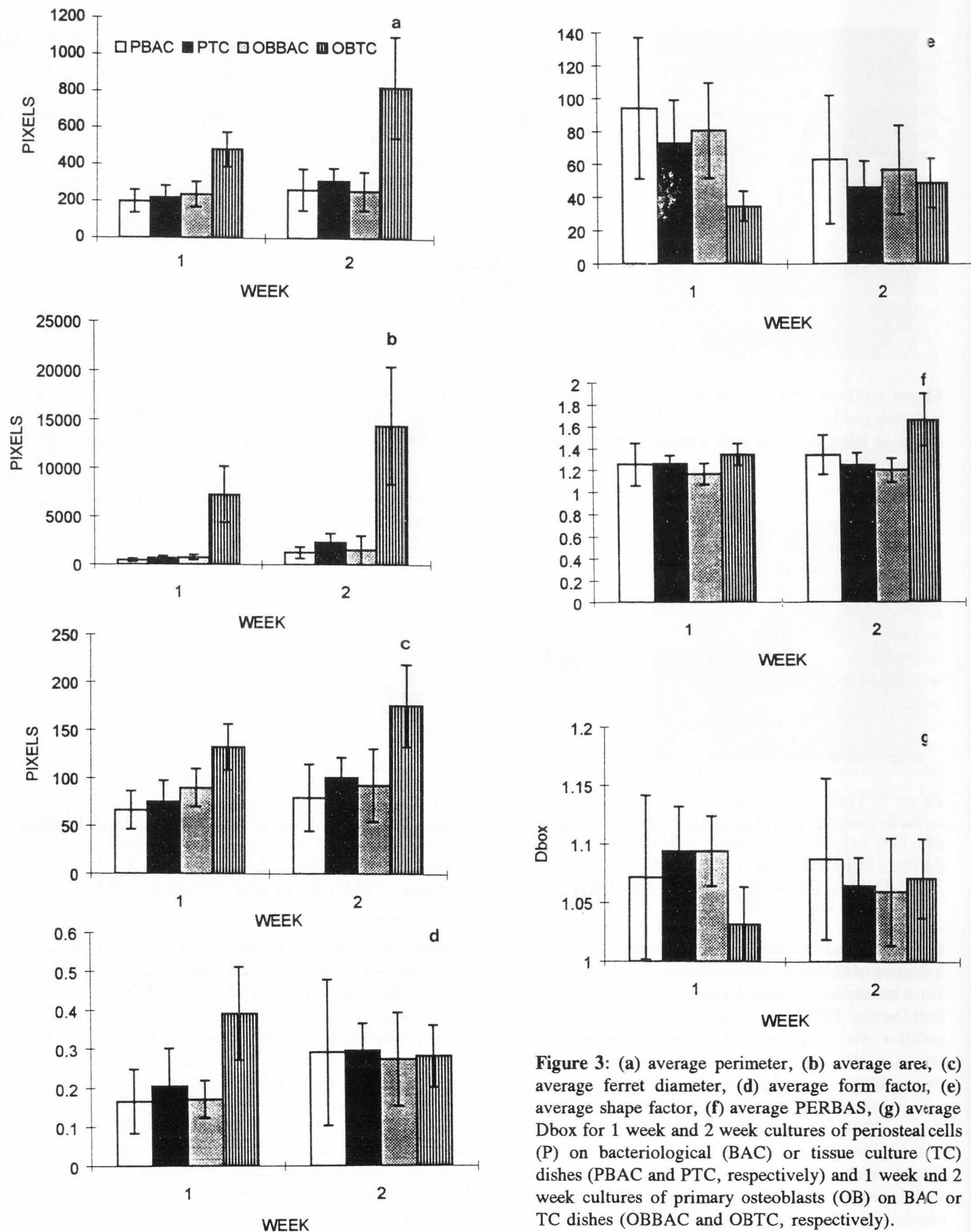

Figure 3: (**a**) average perimeter, (**b**) average area, (**c**) average ferret diameter, (**d**) average form factor, (**e**) average shape factor, (**f**) average PERBAS, (**g**) average Dbox for 1 week and 2 week cultures of periosteal cells (P) on bacteriological (BAC) or tissue culture (TC) dishes (PBAC and PTC, respectively) and 1 week and 2 week cultures of primary osteoblasts (OB) on BAC or TC dishes (OBBAC and OBTC, respectively).

Table 1. Results of the t-tests between the different groups using the morphological parameters.

	Culture		P TC	Ob BAC	Ob TC	P TC	P BAC	Ob TC	Ob BAC
Parameter		Week	1	1	1	2	2	2	2
Perimeter	P BAC	1			**	**		**	
	P TC	1			**			**	
	OB BAC	1			**			**	
	OB TC	1				**	**	**	**
	P TC	2						**	
	P BAC	2						**	
	OB TC	2							**
Area	P BAC	1			**	**	**	**	
	P TC	1			**	**		**	
	OB BAC	1			**	**		**	
	OB TC	1				**	**	**	**
	P TC	2						**	
	P BAC	2						**	
	OB TC	2							**
Feret	P BAC	1			**			**	
	P TC	1			**	**		**	
	OB BAC	1			**			**	
	OB TC	1				**	**	**	**
	P TC	2						**	
	P BAC	2						**	
	OB TC	2							**
Form Factor	P BAC	1			**	**		**	
	P TC	1			**				
	OB BAC	1			**	**		**	
	OB TC	1							
	P TC	2							
	P BAC	2							
	OB TC	2							
Shape Factor	P BAC	1			**	**		**	
	P TC	1			**				
	OB BAC	1			**	**		**	
	OB TC	1						**	**
	P TC	2							
	P BAC	2							
	OB TC	2							
PERBAS	P BAC	1						**	
	P TC	1						**	
	OB BAC	1			**			**	
	OB TC	1						**	**
	P TC	2						**	
	P BAC	2							
	OB TC	2							**
D Box	P BAC	1							
	P TC	1			**				
	OB BAC	1			**				
	OB TC	1				**		**	
	P TC	2							
	P BAC	2							
	OB TC	2							

P: Periosteal cell, OB: Osteoblast, BAC: Bacteriological dish, TC: Tissue culture dish, **: $p<0.01$.

dishes as their roundness tended to create bright areas using phase contrast and thus obscure the cell margin, however 109 images were finally accepted.

Statistical Analysis

The data generated by the image analysis program was analysed statistically using Excel version 5.0 (Microsoft, Redmont, WA) and Minitab version 8.2 (Minitab Ltd, Coventry, UK). T-tests were performed using a confidence level of 95%, were single tailed and assumed equal variances.

Results

Calibration

The stage micrometer viewed using the x10 objective which was used for all cell image capture revealed that each pixel was a square with edge lengths in the captured images equivalent to 0.816 μm.

Bacteriological polystyrene

Cells from both stripped parietal bone fragments (Fig. 1) and minced endo- and exocranial periostea (Fig. 2) migrated onto BAC polystyrene. The cell migration rates were generally slow and subjectively cell spreading appeared poor throughout the culture periods. Very little colonization of the petri dish surface by either cell population was seen until three days had elapsed in culture. At this stage osteoblasts appeared rounded and adopted a mainly ovoid morphology, with very few cytoplasmic processes. The morphologies of both cell populations in bacteriological petri dishes remained as described above for culture periods up to nine days. At no stage did either osteoblast or periosteal cultures reach confluence within 60 mm petri dishes, generally with few cells colonizing the substratum, although occasionally large numbers of cells were seen. The reason for this variation in the degree of colonization of BAC polystyrene was not clear. The cell behaviour may have reflected batch variations in the manufacturers' processing of the polystyrene to produce some more adhesive areas of dishes or possibly lack of colonization occurred as a result of poor contact between the minced bone fragments/periostea and the petri dishes.

Periosteal cells. Periosteal cells increased their spreading on BAC dishes between the first and second weeks indicated by their increase in both average projected area (Fig. 3a) which was statistically significant (Table 1) and perimeter (Fig. 3b) which was not significant (Table 1). All of the other parameters measured between one and two weeks increased apart from shape factor although none of the differences were significant. This decrease in shape factor (perimeter^2/area), in combination with an increase in projected area indicated a spreading associated with a decrease in irregularity of the cell outline/perimeter, or an increasing tendency to roundness with culture time.

Osteoblasts. OBs on BAC dishes spread with increasing culture time in terms of average perimeter (Fig. 3a) and projected area (Fig. 3b), although neither were statistically significant (Table 1) nor clearly discernible (bearing in mind the time between observations). The OBs exhibited similar characteristics to the periosteal cells on BAC dishes in terms of non-significant increases in the other parameters measured apart from shape factor and fractal dimension which decreased and again probably represented an increase in roundness of the cells.

Tissue culture polystyrene

Both OB (Figs. 4 and 5) and periosteal cell (Fig. 6) populations readily colonized TC polystyrene. Initially cells which migrated from their host bone fragments were rounded, yet rapidly spread as they moved further away from the bone. Sometimes colonization was less rapid, probably as a result of poor contact between the minced bone fragments and the petri dishes. Problems of poor contact may also have been exacerbated by movement of the cultures for observation using phase contrast microscopy. The morphology of OB populations appeared typically more spread (Figs. 4 and 5) than the elongated and more stellate appearance of the periosteal cells (Fig. 6).

Periosteal cells. Periosteal cells increased their spreading on TC dishes between the first and second weeks indicated by their increase in both average projected area (Fig. 3a) and feret (Fig. 3c) which were statistically significant (Table 1) and perimeter (Fig. 3b) which was not significant (Table 1). All of the other parameters measured between one and two weeks decreased apart from form factor (Fig. 3d) although none of the differences were significant. Again a decrease in shape factor (perimeter^2/area), in combination with an increase in projected area and an increase in form factor (4π area/perimeter^2) indicated a spreading associated with a decrease in irregularity of the cell outline/ perimeter, or an increasing tendency to roundness with culture time.

Osteoblasts. OBs on TC exhibited the largest increases in spreading between one and two weeks as evidenced by projected area, perimeter and feret (Figs. 3a-c) which were significantly different to each other and the seven other culture types. In contrast with the remaining culture types the OBs on TC dishes exhibited a significant decrease in form factor (Fig. 3d and Table 1) in association with a significant increase in shape factor (Fig. 3e and Table 1). These results may have represented an increased irregularity in the cell outlines although with significant increases in both projected area

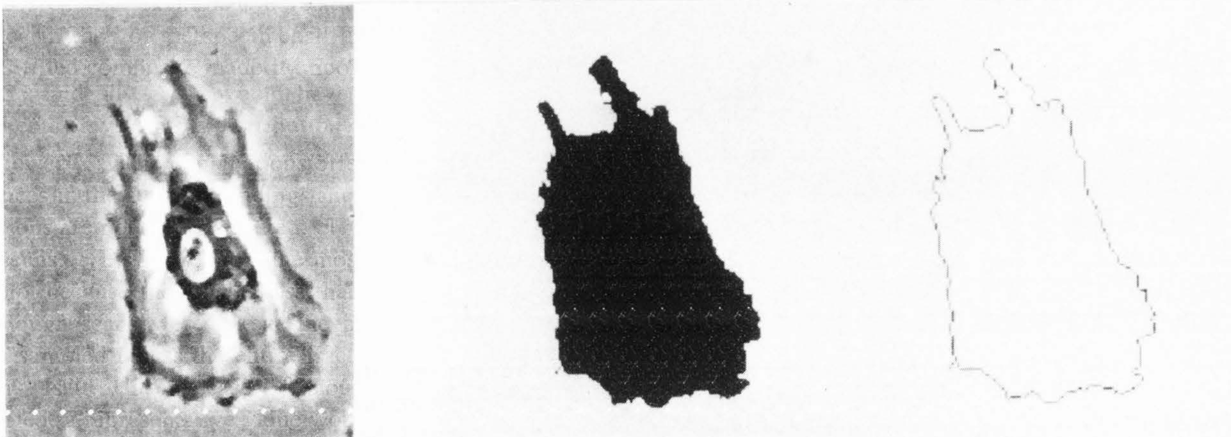

Figure 4. Three digital images captured from the phase contrast microscope of a well spread osteoblast cultured on tissue culture polystyrene for 1week, showing from left to right the original 256 grey scale image, the cell shape following edge detection with a Sobel filter and the final binary trace of the cell periphery. Field width of each of 3 images: 312 μm.

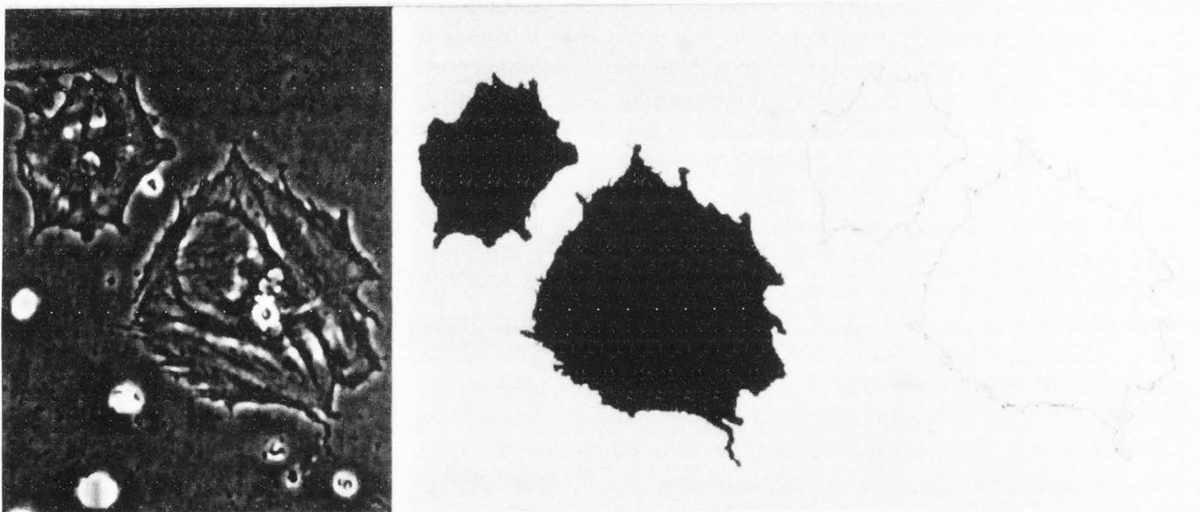

Figure 5. Three digital images captured from the phase contrast microscope of two well spread osteoblasts cultured on tissue culture polystyrene for 2weeks, showing from left to right the original 256 grey scale image, the cell shape following edge detection with a Sobel filter and the final binary trace of the cell periphery. Field width of each of 3 images: 730μm

and perimeter this is not definite. The significant increase in PERBAS (Fig. 3f and Table 1) with increasing culture time seemed to indicate that there was an increase in the number of cytoplasmic extensions from the cells.

Morphological Characterisation

The mean values and standard deviation of the morphological parameters investigated after one and two weeks are shown in Figures 3a-g. The results of the t-tests between the different groups using the morphological parameters are shown in Table 1.

Perimeter. The perimeter of cell outlines allowed the statistically significant differentiation of OBs on TC dishes at both one and two weeks from all of the other culture types. Unfortunately cell perimeters did not allow any other cultures to be distinguished apart from 2 week periosteal cultures on TC dishes and 1 week periosteal cultures on BAC dishes.

Area. The projected area of cells again allowed the statistically significant differentiation between OBs on TC dishes at both one and two weeks from all of the

Figure 6. Three digital images captured from the phase contrast microscope of a reasonably spread periosteal cell cultured on tissue culture polystyrene for 2weeks, showing from left to right the original 256 grey scale image, the cell shape following edge detection with a Sobel filter and the final binary trace of the cell periphery. Field width of each of 3 images: 340μm.

other culture types. In addition 2 week TC periosteal cultures could be distinguished from all 1 week cultures and 2 week BAC periosteal cultures were significantly different to their one week counterparts.

Feret. The longest diameter of cells showed statistically significant differences between OBs on TC dishes at both one and two weeks from all of the other culture types. The only other significant difference occurred between one and two week TC cultures of periosteal cells.

Form factor. Form factor did not appear to allow reliable differentiation between many culture types apart from OBs at 1 week on TC dishes and the remaining one week cultures.

Shape factor. Shape factor again allowed the differentiation between 1 week OBs on TC dishes to be made from all other one week cultures and also from two week osteoblast TC cultures.

PERBAS. Interestingly PERBAS allowed 2 week OB TC cultures to be statistically distinguished from all other cultures apart from 2 week periosteal cultures on BAC dishes which was surprising. Additionally PERBAS allowed OBs at both one and two weeks to be distinguished between TC and BAC dishes.

D Box. Unfortunately the fractal dimension of cell outlines obtained using the box method at this resolution proved an unreliable factor in differentiating between cells, although there were significant differences between one and two week OB cultures on TC dishes and between 1 week OB cultures on TC and BAC dishes and one week periosteal cultures on TC dishes.

Statistical Analyses

Linear Discriminant Analysis. As none of the cell morphological parameters mentioned above were able to distinguish between all of the culture types a linear discriminant analysis was applied using different combinations of the parameters. Discriminant analysis is a statistical technique that minimises the distances between groups based on multivariate samples. Once the minimisation is estimated in the form of a linear function with a constant and a set of coefficients (one for each parameter considered) the function can be used to classify future data.

The best discrimination was found using all the parameters for cell outlines at both one and two weeks to define the groups that the cells belonged to (Tables 2 and 3). With one week cultures the analysis placed 100% of the OB cultures on TC dishes and 71% of OBs on BAC dishes in their correct group, whilst only 33% of periosteal cells on TC dishes and 77% of periosteal cells on BAC dishes were correctly allocated. Out of a total of 52 cell outlines analysed, 41 were correctly assigned by the discriminant analysis to their correct group, giving 79% as an overall proportion correct. Interestingly some of the periosteal cells on BAC and TC dishes in the discriminant analysis were confused with OBs on BAC dishes as shown in Table 3.

After two weeks in culture the overall proportion of cells correctly assigned to their groups remained very much the same as shown in Table 3 (74% of 54 cell outlines analysed) although the individual proportions for the different groups changed. 62% of OBs on BAC dishes and 79% of OBs on TC dishes were correctly assigned, whilst 79% of periosteal cells on TC dishes and 75% of periosteal cells on BAC dishes were correctly placed in their own groups.

After two weeks the ability to allocate periosteal cells improved markedly on TC dishes, although this may have been due to the smaller sample of periosteal cells in the first week.

Interestingly despite the fact that OBs on TC dishes had extremely large perimeters and areas, these cultures were less easily distinguished by the analysis from their

Table 2. Summary of classification of cells grown on different substrates after one week using all morphological parameters and linear discriminant analysis.

Put into Group	....True Groups.... Periosteal Bact.	Periosteal Tiss.	Osteoblasts Bact.	Osteoblasts Tiss.
Periosteal Bact.	*10*	2	1	0
Periosteal Tiss.	3	*2*	3	0
Osteoblasts Bact.	0	2	*10*	0
Osteoblasts Tiss.	0	0	0	*19*
Total N	13	6	14	19
N Correct	10	2	10	19
Proportion	0.769	0.333	0.714	1.000

N = 52; N Correct = 41; Proportion Correct = 0.788

Table 3. Summary of classification of cells grown on different substrates after two weeks using all morphological parameters and linear discriminant analysis.

Put into Group	....True Groups.... Periosteal Bact.	Periosteal Tiss.	Osteoblasts Bact.	Osteoblasts Tiss.
Periosteal Bact.	*6*	0	1	0
Periosteal Tiss.	1	*15*	4	3
Osteoblasts Bact.	1	4	*8*	0
Osteoblasts Tiss.	0	0	0	*11*
Total N	8	19	13	14
N Correct	6	15	8	11
Proportion	0.750	0.789	0.615	0.786

N = 54; N Correct = 40; Proportion Correct = 0.741

Table 4. Summary of classification of cells grown on different substrates after one week using *perimeter*, *area*, *feret*, *form factor* and *D box* parameters and quadratic discriminant analysis.

Put into Group	....True Groups.... Periosteal Bact.	Periosteal Tiss.	Osteoblasts Bact.	Osteoblasts Tiss.
Periosteal Bact.	*11*	0	0	0
Periosteal Tiss.	1	*5*	0	0
Osteoblasts Bact.	1	1	*14*	0
Osteoblasts Tiss.	0	0	0	*19*
Total N	13	6	14	19
N Correct	11	5	14	19
Proportion	0.846	0.833	1.000	1.000

N = 52; N Correct = 49; Proportion Correct = 0.942

Table 5. Summary of classification of cells grown on different substrates after two weeks using all morphological parameters and quadratic discriminant analysis.

	....True Groups....			
Put into Group	Periosteal Bact.	Periosteal Tiss.	Osteoblasts Bact.	Osteoblasts Tiss.
Periosteal Bact.	*7*	0	0	0
Periosteal Tiss.	1	*18*	1	0
Osteoblasts Bact.	0	1	*12*	0
Osteoblasts Tiss.	0	0	0	*14*
Total N	8	19	13	14
N Correct	7	18	12	14
Proport.	0.875	0.947	0.923	1.000

N = 54; N Correct = 51; Proportion Correct = 0.944

counterparts on TC dishes in the first week.

Quadratic Discriminant Analysis. In addition to the linear discriminant analysis, there is an extension of the technique that uses a quadratic formula to minimise the distances between original groups. This technique is known as quadratic discriminant function analysis and has some advantages over the linear analysis because it is not highly dependent on deviations of the samples from normality, or inequalities of the correlation matrices.

The quadratic discriminant analysis revealed better classification of cell outlines in their original groups using perimeter, projected area, feret, form factor and D box for the first week cultures with 94% success (Table 4) Unfortunately the analysis could not be carried out using any further parameters as the statistical program found high correlations between the different parameters/predictors. Again the OBs were the best defined cells with 100% correctly allocated in the first week and slightly less in the second. Although better discriminated than in the linear analysis the periosteal cells on BAC dishes were still the least easy cell types to allocate.

After two weeks the discrimination between the different cell outlines on either of the polystyrene surfaces was still 94% correct utilising all of the parameters measured as the correlations between parameters tended to disappear (Table 5).

Principal Component Analysis. The Principal Component Analysis (PCA) can be used to investigate which variables or parameters account for the highest variability (variance) after normalisation of the variables in a multivariate sample. This technique can be used then to investigate further the parameter that may be a successful predictor or discriminator.

The PCA showed that after one week in culture the parameters that were the most predictive in cell outlines were firstly the projected area, followed by PERBAS, form factor, shape factor, feret diameter, D box and perimeter. The situation changed after two weeks in culture with form factor being the most variable parameter of cell outlines, followed by the projected area, PERBAS, D box, perimeter, feret diameter and shape factor. If the ranking for these parameters is averaged between the two weeks, then projected area appears to be the most variable factor in cells on the polystyrene dishes followed by form factor, PERBAS, D box, shape factor, feret diameter and finally perimeter. Indeed, applying discriminant analyses using the first four parameters in this ranking gave 76% of week one and 67% of week two cultures correctly allocated with the linear analysis and 87% of week one and 90% of week two cultures with the quadratic analysis, which although not better than using all the parameters provided reasonable discrimination.

Discussion

There are a number of problems associated with using image analysis of cells in culture at present and in particular that of edge detection in confluent cultures. This problem was avoided in the present study by analysing cells with clearly defined margins which were predominantly obtained from the outermost areas of cultures where cells had migrated the furthest. A criticism of the present technique was that the majority of cells in this study from outer areas of cultures may not have represented the morphology of cells nearer the centre of cultures. It appears from preliminary investigations that more central individual cells can be discerned using phase contrast microscopy in association with an alkaline phosphatase stain, although this clearly

removes the previously mentioned advantage of the non-invasive nature of phase contrast microscopy alone.

Phase contrast microscopy may not be able to resolve extremely fine cytoplasmic extensions that may be present at the cell surface but despite this the method described in the present study did allow accurate discrimination between two cell populations on two different surfaces from which images were obtained under identical conditions. Utilising microscopes with greater resolving power seems unlikely to reduce the likelihood of being able to discriminate between cell populations or surfaces, indeed it may increase the sensitivity with which cells can be discriminated.

The size of cells found in the present study in terms of projected area (equating to 5389 μm^2 for one week OBs on TC) and feret (equivalent to 113 μm for one week OBs on TC) was surprising given that *in vivo* osteoblasts are typically described as 'cuboidal' being approximately 30 μm wide, high and deep (Rodan and Rodan, 1983). However the OBs were not measured in their third dimension (depth) although this would be expected to be extremely small if the cell volume remained the same *in vitro* as *in vivo*.

It will be of interest to attempt to map out the morphological parameters that change, the sequence of change and precise timing during the process of differentiation of a particular cell type, which may provide further identifiers for example, of the fully differentiated osteoblast and how such cells may be distinguished from fibroblasts and therefore allow a non invasive method of determining or identifying different cell types. Clearly this would be of benefit, particularly in the case of positively identifying osteoblasts which at present have no individual specific marker. At present identification of osteoblasts tends to rely on a combination of phenotypic characteristics, which amongst others include synthesis of type Ia collagen and its subsequent mineralisation, high plasma membrane alkaline phosphatase activity and secretion of osteocalcin and osteonectin (Rodan and Rodan, 1983). However identifying mature osteoblasts in culture which have formed multilayers will clearly produce major difficulties with edge detection, so this method may require adaptation (i.e., alkaline phosphatase staining) which removes the advantage of non-invasiveness, or may only be applicable to shorter term studies.

To date there appears to be little previous work using quantifiable morphological parameters or combinations of those parameters to define cell types and the method utilised in this study describes how discriminant functions may be used in the future to possibly predict cell type on particular substrata using a combination of data obtained from cell profiles. This may then allow the development of semi-automatic or automatic microscopy for the non-invasive identification of cells. With an increased number of cell perimeter profiles then it may be possible to identify specific values of particular or combinations of parameters that define whether a cell is rounded, well spread or stellate and remove the subjective element used to describe cell morphology.

Conclusions

(1) The present study has shown that cell morphology can be quantified using seven parameters which allow differences to be identified between two cell populations established from neonatal rat calvaria when cultured on either bacteriological or tissue culture polystyrene petri dishes, which is important both biologically and methodologically.

(2) The most important distinguishing morphological parameter for the two cell types on BAC and TC polystyrene was projected area after both one and two weeks, followed by PERBAS and form factor.

(3) This preliminary image and statistical analysis may be developed to allow non-invasive automatic identification of particular cell types in mixed populations *in vitro*.

Acknowledgements

Financial support from the West Midlands Regional Health Authority for this project is gratefully acknowledged.

References

Callen BW, Sodhi RNS, Shelton RM, Davies JE (1993) Behavior of primary bone cells on characterized polystyrene surfaces. J Biomed Mater Res **27**: 851-859.

Curtis ASG, Forrester JV, McInnes C, Lawrie F (1983) Adhesion of cells to polystyrene surfaces. J Cell Biol **97**: 1500-1506.

Curtis ASG, Forrester JV, Clark P (1986) Substrate hydroxylation and cell adhesion. J Cell Sci **86**: 9-24.

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

Grinnell F, Feld MK (1981) Adsorption characteristics of plasma fibronectin in relationship to biological activity. J Biomed Mater Res **15**: 363-381.

Grinnell F, Feld MK (1982) Fibronectin adsorption on hydrophilic and hydrophobic surfaces detected by antibody binding and analyzed during cell adhesion in serum-containing medium. J Biol Chem **257**: 4888-4893.

Kaye BH (1986) The description of two-dimensional rugged boundaries in fine particle science by means of fractal dimension. Powder Tech **46**: 245-254.

Klemperer HG, Knox P (1977) Attachment and

growth of BHK and liver cells on polystyrene: effect of surface groups introduced by treatment with chromic acid. Lab Prac **26**: 179-180.

Lydon MJ, Clay CS (1985) Substratum topography and cell traction on sulphuric acid treated bacteriological-grade plastic. Cell Biol Int Rep **9**: 911-921.

Mandelbrot BB (1983) The Fractal Geometry of Nature. W.H. Freeman and Co., New York. pp 25-73.

Maroudas NG (1975) Adhesion and spreading of cells on charged surfaces. J Theor Biol **49**: 417-424.

Martin GR, Rubin H (1974) Effects of cell adhesion to the substratum on the growth of chick embryo fibroblasts. Exp Cell Res **85**: 319-333.

Osunkoya BO, Mottram FC, Isoun MJ (1969) Synthesis and fate of immunological surface receptors on cultured Burkitt lymphoma cells. Int J Cancer **4**: 159-165.

Payne CM, Bjore CG Jr, Cromey DW, Roland F (1989) A comparative mathematical evaluation of contour irregularity using form factor and PERBAS, a new analytical shape factor. Analyt Quant Cytol Histol **11**: 341-352.

Revel JP, Hoch P, Ho D (1974) Adhesion of culture cells to their substratum. Exp Cell Res **84**: 207-218.

Stenkvist B, Strande G (1989) Analysis of machine selected cells with an image analysis system in normal and abnormal cervical specimens. Analyt Cell Path **2**: 1-13.

Thomas KD, Tighe BJ, Lydon MJ (1986) Cellular interactions with synthetic polymer surfaces. II - Surface modification of polystyrene for cell culture. In: Biological and Biomechanical Performance of Biomaterials. Christel P, Meunier A, Lee AJC (eds). Elsevier Science Publishers, Amsterdam, pp 379-383.

Discussion with Reviewers

J.P. Rigaut: Why was phase contrast microscopy used?
Authors: Phase contrast microscopy is used as it is relatively non-invasive, does not require fixation of cells and therefore allows examination of individual cultures over a period of time.

J.P. Rigaut: Were the fractal graphs concave? Is this the reason why a fractal dimension value could not be determined? Or is it because your insistent use of a Sobel filter produces too much smoothing?
Authors: The fractal graphs were not concave because the boxes used in their determination were no smaller than 8 pixels, so the cells fractal dimension was determined, although subsequently found to be a non-discriminating parameter for the identification of particular cells when used alone.

J.P. Rigaut: Does the Sobel filter smooth too much, hence the low values of D?
Authors: No it does not, because as explained above it was calculated from box sizes larger than 8 pixels.

I. ap Gwynn: No comment was made concerning the natural variation in cellular morphology to be expected within populations of a given type. That such variations occur in monolayer cultured cells is well documented - S phase cells for instance, often being spread out to as much as two to three times the area of a G1 cell. Some evidence for this was present in the data, in the form of standard errors. However, no comment was made on this matter. Did the authors consider that this and the fact that populations of different cell types show significantly different distributions of cell cycle phases to be important factors, likely to influence the results?
Authors: These are possibly important factors although the fact that the power of discrimination of the analysis is at the 90% level it seems likely that those factors are not of overriding importance. Certainly it would be interesting in future work to attempt to address the issue of the importance of the cell cycle on the parameters we have measured, possibly by staining cells in S-phase using bromodeoxyuridine for example. One of the main aims of the present study was to examine cells which had not been exposed to any chemical or enzymatic treatment using a non-invasive technique which would be obviated by either identification of the proliferative state of the cells or obtaining all the cells at the same point in their cell cycles.

I. ap Gwynn: In Fig. 1 the image presented seems to show a considerable effect of pixelation contributing to what would probably be a significant increase in perimeter measurement. This effect would be greater on small cells. Did the authors consider this to be an important factor?
Authors: The pixelation is more noticeable in Fig. 1 as it has been subjected to further magnification for publication purposes as the cell was small (note field width). However the pixelation would in theory contribute to a perimeter decrease (as the geometry of the pixels is Euclidean, $D=1$) if the cells have outlines with $D>1$. As the size of the pixels was the same for all cell outlines obtained this inaccuracy would be the same for all outlines measured.

A.S.G. Curtis: The two types of polystyrene are not well characterised by the authors. The bacteriological grade polystyrene slowly undergoes surface oxidation particularly if exposed to fluorescent tube light or to sunlight. Most papers dealing with this sort of comparison of polystyrene nowadays, includes some sort of

characterisation of the surface either in chemical or physical terms.

Authors: We did not chemically characterise the polystyrene dishes used as we did not feel we were going to contribute sufficiently to the knowledge of the chemistry of these surfaces which have been examined and characterised using X-ray photoelectron spectroscopy (Callen *et al.*, 1993). However we did simple measurements of water contact angles of the BAC and TC surfaces which were reproducibly different to each other, although we did not consider these were worth reporting. All of our polystyrene dishes are stored in boxes kept in the dark and are therefore exposed to approximately the same amount of fluorescent tube lighting during the preparation, examination and feeding of cultures. On checking the contact angles of the stored dishes again, there has been no significant change in value.

A.S.G. Curtis: How do the authors really know the cells are osteoblasts?

Authors: Clearly it is not possible to be absolutely definite that all of the cells were osteoblasts in the 'osteoblast' cultures, however in similarly established cultures from either rat or mice calvaria the cells have been shown to synthesise mineralised bone matrix under different conditions (Ecarot-Charrier *et al.*, 1983; Rout *et al.*, 1987; Tarrant and Davies, 1987; Shelton and Davies, 1991) which was only possible if osteoblasts were present.

Additional References

Ecarot-Charrier B, Glorieux FH, van der Rest M, Pereira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol **96**: 639-643.

Rout PGJ, Tarrant SF, Frame JW, Davies JE (1987) Interactions between primary bone cell cultures and biomaterials. Part 3: A comparison of dense and macroporous hydroxyapatite. In: Biomaterials and Clinical Applications. Pizzoferrato A, Marchetti PG, Ravaglioli A, Lee AJC (eds). Elsevier Science Publishers, Amsterdam, pp 591-596.

Shelton RM, Davies JE (1991) Osteoblast reactions to charged polymers. In: The Bone Biomaterial Interface. Davies JE (ed). University of Toronto Press, Buffalo, USA, pp 181-197.

Tarrant SF, Davies JE (1987) Interactions between primary bone cell cultures and biomaterials. Part 2: Osteoblast behaviour. In: Biomaterials and Clinical Applications. Pizzoferrato A, Marchetti PG, Ravaglioli A, Lee AJC (eds). Elsevier Science Publishers. Amsterdam, pp 585-590.