Image and fractal analysis of osteoblastic cells in viscous media

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The aim of the present study was to determine whether osteoblastic morphology was affected by the viscosity of culture media and whether any morphological differences could be readily quantified. A cytochemical stain was used for alkaline phosphatase and a combination of image and fractal analysis, utilising seven morphological parameters to assess the morphology of osteoblasts. Using regression analysis it was determined that as the viscosity of the culture media increased the area of the cells significantly decreased and the fractal dimension of the cell profiles significantly increased. A discriminant function analysis was used to examine whether cell populations could be classified according to culturing time or the viscosity of culturing media based on the seven morphological parameters. It was determined that the cells could be classified up to 93% correct according to the viscosity of the media they were cultured in and up to 93.5% correct according to the culturing time. This study demonstrated that viscous media affects the morphology of osteoblastic cells and that discriminant function analysis can be used to classify these cells based on their morphological parameters.

1. Introduction

The hypothesis that the extracellular culturing environment can affect both cell morphology and cell function is supported by a number of studies. When examining neurones [1] it was demonstrated that the morphology of the cells was affected by culturing in a methyl cellulose (MeC) supplemented media. When liver cells were cultured in media with increased viscosity [2–4] through the addition of various macromolecules (including MeC), it was noted that the secretion of lipoproteins and lysosomal enzymes was decreased as a function of the viscosity of the media.

With the advent of fractal analysis [5] and the increasing accessibility of high-resolution image analysis due to the increase in the availability of personal computers, there has been a rapid increase in the biological subjects to which image and fractal analysis have been applied. Among the topics examined have been the quantification of cell and tissue morphology [6–8], the quantification of biological structures [9, 10] and the classification of cell populations [11].

In the present study we used a number of image analysis techniques to examine the morphology of osteoblastic cells grown in culture media supplemented with MeC to vary the viscosity. This was conducted to determine if this variation of the extracellular environment would affect the morphology of the cells in a manner that could be quantified. Also given the previous success of discriminant function analysis (DFA) in separating populations of fibroblastic and osteoblastic cells [11], a DFA was used here to attempt to correctly classify cells based on their culturing time and their culturing viscosity.

2. Materials and methods

2.1. Establishing primary cultures

Fitton Jackson modified Biggers media (BGJ_b) was prepared according to the manufacturer's instructions (Sigma, Poole, UK) and supplemented with either 0.25, 0.5, 1% by weight methyl cellulose (MeC) (Sigma, Poole, UK) or left without methyl cellulose. The BGJ_b media was subsequently supplemented with 10% fetal calf serum (Sigma, Poole, UK), 2.5% HEPES (1 M) (Sigma, Poole, UK) and 1% penicillin/streptomycin (5000 units penicillin and 5 mg streptomycin per ml in 0.9% sodium chloride) (Sigma, Poole, UK) immediately

prior to use. Squares of parietal bone were dissected from the calvaria of 1–2-day-old neonate Wistar rats, and used to establish cultures in the various viscous media on 60 × 15 mm tissue culture petri dishes (Corning, New York, USA). These cultures were kept for 10, 15 and 20 days, replacing the media with fresh media every 5 days.

2.2. Determination of viscosity of methyl cellulose supplemented culture media

Viscosity was determined using a constant pressure apparatus. This consisted of a container with an overflow pipe running from below the top of the container through the sealed base. Culture media was siphoned into the container at 38 °C and allowed to form a pressure head above a capillary tube running horizontally through the side of the container, the distance between the pressure head and the horizontal tube was h. The volume of media issuing from the capillary tube was collected in a beaker over ten seconds and used to determine the volume issued per second.

From Poiseuilles's formula [12]

$$v = \frac{\pi h \rho g a^4}{8 \eta l} \tag{1}$$

where: h = height of pressure head over capillary tube (m); ρ = density(kg m^{-3}); g = gravity(m s^{-2}); a = radius of capillary tube (m); η = viscosity (N s m^{-2}); l = length of capillary tube (m); v = volume per second (m^3).

Viscosity can be calculated as

$$\eta = \frac{\pi h \rho g a^4}{8 v l} \tag{2}$$

2.3. Alkaline phosphatase staining

At each specified time period (10, 15 or 20 days) the cultures were fixed in 3 ml of 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by a wash in PBS. The staining solution was made up by dissolving 10 mg Fast Red TR (Sigma, Poole, UK) and 4 mg Napthol AS MX phosphate (Sigma, Poole, UK) in 10 ml of Tris–HCl (0.1 M pH 8.2) [13]. Three milliliters of staining solution was added to each dish, left for 15 min and washed in PBS. Coverslips were mounted over cell colonies in glycerine jelly.

2.4. Isolation and image analysis of the alkaline phosphatase positive osteoblastic cells

The alkaline phosphatase positive cells, stained red, were examined using a 10 × objective lens (NA of 0.25) on an Ortholux Leitz microscope (Germany) with a green filter to maximize the image contrast, using a COHU (San Diego, USA) charge-coupled device (CCD) digital camera. The image analysis software Optimas (version 4.0) (BioScan, Edmonds, USA) was used to capture the images. The border of furthest migration of the stained cells was located and a cell was chosen at random to examine. Image noise was reduced by averaging the image frame eight times, background illumination was corrected by dividing the final averaged image by the brightfield (an image captured with the light source on, but with no specimen) minus the darkfield (an image captured with no light source – to compensate for bias on the camera sensor). This gray-scale image was thresholded and binarized to separate the image of the cell (foreground) from the background. This process was repeated on every third cell around the periphery of the cultures in order to randomize the sampling. The binarized images were converted to a single pixel width trace using the image analysis program IMAN (developed by G.L.) and saved as chain encoded profiles [14, 15]. Using Optimas the circularity, rectangularity, perimeter, breadth, major axis length (MAL) and area (see Table I for definitions) were extracted from the chain encoded profiles and exported to Excel (Microsoft, Redmond, WA, USA) for statistical analysis. Using the calibration from a slide micrometer (1 pixel = 0.629 μm) all measurements were converted from pixels into μm. The fractal dimension was determined using the box-counting method from the IMAN program (see below).

2.5. Determination of fractal dimension (D)

The box-counting method for determining fractal dimension can be used for an object embedded in a two dimensional plane. First, a grid of specified box length (ε) was superimposed over the image to be studied. The minimum number of boxes out of 32 random positions of the grid that contained part of the image were counted ($N(\varepsilon)$). Next a grid with box side length $\varepsilon - x$ (where x was positive and smaller than ε) was superimposed over the image and the number of boxes containing part of the image were again counted; this was repeated for different sizes of ε.

This method was conducted on a personal computer using box sizes from 5 to 184 pixels. The fractal dimension was estimated from the negative slope of the linear region of the $\log(N(\varepsilon))$ on $\log(\varepsilon)$ slope (Equation 3)

$$D(box) = -\frac{\log(N(\varepsilon))}{\log(\varepsilon)} \quad [5] \tag{3}$$

TABLE I Definitions of the shape descriptors from the Optimas image acquisition software used to characterize the cell profiles

Measurement	Definition [22, 23]
Circularity	The ratio of between the perimeter length squared and the area of an object
Rectangularity	The area divided by the area of an enclosing bounding box orientated along the major axis of an object
Perimeter	The total boundary length in pixels
Breadth	The maximum distance to the boundary from either side of the major axis in pixels
Major axis length (MAL)	The length of the major axis in pixels
Area	The bounded area in pixels squared

TABLE II Mean cell depth for each culture medium and time period examined

% MeC by weight	Mean depth of cell (μm)		
	10 day	15 day	20 day
0	6.6	7.5	7.7
0.25	6.9	7.9	8.5
0.5	7.0	8.9	8.5
1	7.7	6.8	8.0

2.6. Determination of cell depth

Five randomly selected cells cultured in each viscous medium (0% MeC, 0.25% MeC, 0.5% MeC and 1% MeC) and time period (10, 15 and 20 days) were chosen for analysis. Cell depth was determined by using the calibrated focus controls of the microscope: the range of movement of the focus controls was noted and, using a calibrated gauge, converted into μm depth for each cell examined. The results were averaged to give a mean cell depth (Table II).

2.7. Statistical analysis

All statistical tests were considered significant at the 0.01 confidence level. Regression analyses were carried out on the data to determine if there were any significant trends in the seven parameters (circularity, rectangularity, perimeter, breadth, MAL, area and fractal dimension) with changes in culturing time or viscosity of the culturing media.

A quadratic discriminant function analysis (DFA) was conducted on the seven morphological parameters to determine if these parameters could be used to classify the cells based on either their culturing time or the viscosity of the culturing media.

3. Results

The viscosities of the culture media were calculated using Poiseuilles' formula and the figures obtained from the constant pressure apparatus, presented in Table III, showing increases in the viscosity of the culture media with the addition of MeC.

The means for each of the seven morphological measurements at each time period and viscosity can be found in Table IV, these results show the morphological differences that occur with increasing culturing time and increasing culturing viscosity. Showing general trends for increases in perimeter, breadth, MAL and area with increased culturing time (apart for cells cultured in 1% MeC supplemented media).

When regression analysis was used to examine cells cultured in 0% MeC media it was determined that perimeter, breadth, MAL and area all significantly increased ($p < 0.01$) as culturing time increased. Circularity, rectangularity and fractal dimension showed no change as the culturing time increased. When regression analysis was utilized to examine the cells cultured in the viscous media it was demonstrated that as the viscosity of the culturing media increased perimeter, breadth and area all significantly decreased ($p < 0.01$). Fractal dimension significantly increased ($p < 0.01$) as the viscosity of the media increased.

Regression analysis also showed that for cells cultured in viscous media, perimeter, breadth and area all significantly increased ($p < 0.01$) as the culturing time increased.

Discriminant function analysis was conducted to determine if it was possible to classify cells according to their culturing time or the viscosity of the culturing media based on the cells morphological parameters (Figs 1 and 2). The most accurate classification between viscosities was 93% correct between 0% MeC and 0.5% MeC for the 10-day-old cells; the least accurate was 63.6% correct between 0.25% MeC and 0.5% MeC for the 15-day-old cells. Between time periods the most accurate was 93.5% correct between 10 and 20 days for 0.25% MeC; the least accurate was 64.1% correct between 15 and 20 days for 0.5% MeC.

Cell depth was examined and it was determined that mean cell depth increased as the viscosity of the culture media increased, apart from media containing 1% MeC by weight for 15 and 20 days. Also as culturing time increased the mean cell depth increased, apart from media containing 1% by weight MeC, which showed a decreased cell depth from 10 to 15 days.

TABLE III Viscosity of each culture media according to the percentage by weight of MeC content

% MeC by weight	Viscosity ($\mathrm{N\,s\,m^{-2}}$)
0	1.69×10^{-3}
0.25	1.98×10^{-3}
0.5	2.48×10^{-3}
1	4.62×10^{-3}

4. Discussion

Regression analysis showed that as culturing time increased the area of the cells increased significantly (through increases in perimeter, breadth and area). Only one osteoblast phenotypic marker was utilized in the present study (i.e. alkaline phosphatase); however, this type of culture system has previously been shown to mineralize the extracellular environment [16], demonstrating the presence of osteoblast cells. The quantified changes identified in the present study may represent previously described alterations of the osteoblastic morphology in culture [17, 18] as well as responses to variations in the viscosity of the media.

Discriminant function analysis determined that it was possible to correctly classify between any two culturing times independent of the viscosity of the media. The most correctly classified groups of cells were between 10

TABLE IV Means of all the measurements at each of the four viscosity ratings (one non-methyl cellulose supplemented and three supplemented with methyl cellulose) and three time periods examined and the number of cells examined at each of these periods

Time	Number of cells examined	% MeC	Means of measurements (standard deviation)						
			Circularity	Rectangularity	Perimeter (μm)	Breadth (μm)	MAL (μm)	Area (μm^2)	***D***
10 days	93	0	36.94	0.58	533.0	103.2	135.1	13985.3	1.16
			(14.18)	(0.09)	(184.4)	(33.0)	(37.4)	(8079.5)	(0.05)
	78	0.25	39.59	0.52	462.9	86.7	128.9	9822.3	1.17
			(14.01)	(0.08)	(141.8)	(28.4)	(34.1)	(6369.9)	(0.06)
	144	0.5	28.63	0.58	406.9	86.1	120.3	10095.8	1.21
			(6.66)	(0.06)	(110.7)	(24.1)	(33.7)	(5583.7)	(0.05)
	42	1	40.58	0.52	463.8	83.9	131.4	9075.5	1.22
			(14.98)	(0.09)	(96.6)	(21.7)	(28.1)	(3385.7)	(0.04)
15 days	72	0	37.99	0.57	644.1	119.5	167.5	19864.6	1.15
			(14.78)	(0.09)	(245.9)	(41.1)	(51.8)	(12116.2)	(0.05)
	65	0.25	38.18	0.56	599.9	111.9	158.5	17335.0	1.19
			(13.28)	(0.10)	(212.2)	(39.6)	(46.5)	(11816.1)	(0.05)
	38	0.5	36.21	0.58	607.8	112.4	167.2	17791.0	1.17
			(17.41)	(0.09)	(181.8)	(30.4)	(44.0)	(8167.4)	(0.04)
	38	1	34.52	0.55	420.5	83.4	113.3	8494.8	1.23
			(8.55)	(0.07)	(101.8)	(18.6)	(22.3)	(3393.4)	(0.04)
20 days	65	0	38.75	0.59	784.4	144.7	191.6	27189.2	1.15
			(11.90)	(0.07)	(215.0)	(39.4)	(43.4)	(12495.3)	(0.04)
	33	0.25	33.08	0.59	748.5	145.0	203.3	29181.8	1.15
			(9.85)	(0.08)	(233.5)	(45.4)	(55.5)	(15383.3)	(0.04)
	48	0.5	35.86	0.57	651.2	122.0	174.4	20250.4	1.18
			(12.92)	(0.08)	(237.7)	(37.6)	(44.6)	(11310.9)	(0.04)
	36	1	38.56	0.53	538.6	103.4	143.7	12477.8	1.20
			(9.53)	(0.08)	(94.0)	(22.1)	(23.8)	(3694.6)	(0.04)

and 20 days for the 0.25% MeC culture media (93.5% correct), possibly due to the fact that these cells had the greatest morphological differences. It was also determined that DFA could correctly classify between any two culturing viscosities.

When classifying according to culturing time or viscosity, no single time period (e.g. between 10 and 20 days) or viscosity (e.g. between 0.25 and 0.5% MeC) was consistently highly classified. This lack of pattern is confusing; it could be inferred that the morphological differences that cause cells to be classified well, for example between 10 and 20 days for the 0.25% MeC media, would exist for each culturing media. However it appears there may be an interplay between culturing time and the viscosity of the media that affects cellular morphology in a manner that does not allow consistent classification using DFA.

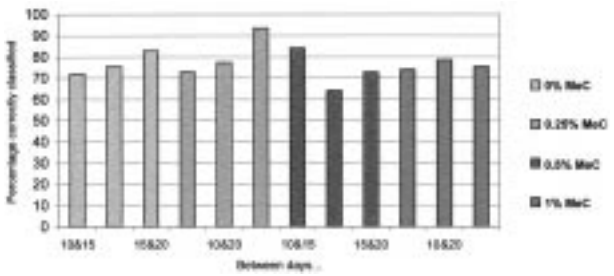

Figure 1 Percentage of cells correctly classified (y) between specified time periods (x) for each MeC-supplemented viscous medium, using quadratic DFA (50% represents a random probability of correct classification).

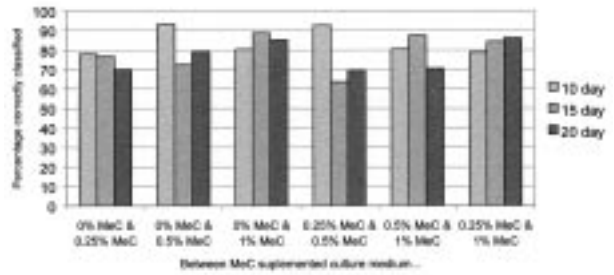

Figure 2 Percentage of cells correctly classified between two specified MeC-supplemented media using quadratic DFA (50% represents a random probability of correct classification).

Increasing the viscosity of the culturing media caused the cells to decrease significantly in area (through significant decreases in perimeter, breadth and area) and increase in cell depth. This suggests the cells were adopting a ''rounded up'' morphology as opposed to the more ''flattened'' morphology typically seen in culture. Regression analysis determined that the fractal dimension of the osteoblastic cell profiles increased with increasing viscosity, but not with increases in culturing times. The reason for the increase in fractal dimension is unclear, it may be related to the ''rounding up'' of the cells that occurred in the viscous media. When a cell ''rounds up'' it effectively reduces its surface area and therefore lowers the amount of nutrients it can absorb [19]. In response to this reduced absorption the cells may put forward cytoplasmic extensions into the media to increase the available surface area for absorption [19]. These extensions may account for the increased fractal dimension of the profiles of the cells in viscous media. The increased fractal dimension may also result from the excess membrane that is converted into surface projections and irregularities due to the transformation from a flattened to ''rounded up'' morphology [19, 20]. The

increased fractal dimension may be due to an osmotic effect caused by the addition of methyl cellulose to the media. Osmolarity of the media, if greater than the osmolarity of the cytoplasm, would cause water to leave the cytoplasm, causing cell shrinkage, resulting in the decreased cell size. The shrinkage of the cytoplasm while retaining some focal contacts [21], would create a more ''roughened'' profile, resulting in a higher fractal dimension.

As well as the morphological changes being wholly attributable to either the effect of the viscous media or the osmotic effects of MeC, an interplay between these two effects may have caused the shape and profile ''roughness'' changes.

5. Conclusions

The present study has demonstrated that the morphological changes that occur in osteoblastic cells when cultured in media of various viscosities can be quantified using image analysis. These morphological parameters can also be used to classify cells based on culturing time or viscosity of the culturing media using discriminant function analysis.

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