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Comparison of bone marrow cell growth on 2D and 3D alginate hydrogels

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Calcium cross-linked sodium alginate hydrogels have several advantageous features making them potentially suitable as tissue engineering scaffolds and this material has been previously used in many biomedical applications. 3D cell culture systems are often very different from 2D petri dish type cultures. In this study the effect of alginate hydrogel architecture was investigated by comparing rat bone marrow cell proliferation and differentiation on calcium cross linked sodium alginate discs and 1mm internal diameter tubes. It was found that bone marrow cell proliferation was diminished as the concentration of alginate in the 2D hydrogel substrates increased, yet proliferation was extensive on tubular alginate constructs with high alginate contents. Alginate gel thickness was found to be an important parameter in determining cell behaviour and the different geometries did not generate significant alterations in BMC differentiation profiles.

1. Introduction

Tissue engineering is a rapidly emerging interdisciplinary field that applies methods from both engineering and life sciences to create tissue-scaffold constructs or to direct tissue regeneration [1]. Currently no synthetic bone graft offers the combination of osteogenicity, osteoconduction and osteoinduction that may be provided by bone autografts, which are limited in availability. Hence tissue engineering of bone using mesenchymal stem cells potentially offers a means to generate autograft type tissue without the need for a graft harvesting procedure. Formation of three-dimensional scaffolds, which serve as a physical support for growth of progenitor cells that can be differentiated into specific cell types is therefore the topic of considerable research activity [2]. Pre-implantation investigations have been performed on a variety of mainly ceramic and polymeric biomaterials assessing both osteoblastic and osteoblast progenitor cell proliferation and differentiation. Among the possible candidate materials for bone tissue engineering scaffolds, calcium cross-linked sodium alginate hydrogels have several potentially attractive features namely long history of clinical use, non-toxicity, biodegradability, ability to encapsulate living cells, ability to be cross linked *in situ* and the potential to form 3D constructs in a variety of shapes from aqueous solutions [3, 4].

It is a naturally derived polysaccharide, which has been used in many biomedical applications, including cell transplantation, wound healing and drug delivery etc. [5, 6]. Sodium alginate is soluble in aqueous solutions, and forms stable gels in the presence of divalent cations (e.g., Ca^{2+} Ba^{2+}) through the ionic interaction between the ions and guluronate (G) groups in the polymer. Although using alginate hydrogels have been extensively studied as cell immobilisation matrices there are few studies on alginate gels as cell growth substrates.

Previously we have demonstrated that sodium alginate polymers with a higher proportion of G residues than mannuronic (M) residues provide better substrates for colonisation and differentiation of rat bone marrow cells (rBMC) [7]. Recently, we have found that the attachment and proliferation behaviour observed for rat bone marrow cells on alginate gels could not be replicated with human bone marrow cells without the addition of tricalcium phosphate particles and collagen type I to the alginate [8]. Recent interest in 3D culture systems has revealed significant differences between cells grown on flat 2D substrates and those grown as spheroidal aggregates or on scaffolds [9, 10] which brings into question the relevance of cell culture studies on flat biomaterial surfaces to tissue engineering applications. In this study the effect of alginate

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hydrogel architecture was investigated by comparing rat bone marrow cell proliferation and differentiation on calcium cross linked sodium alginate discs and 1mm internal diameter tubes. The expression of osteoblastic markers, osteocalcin (OC), osteopontin (OPN), alkaline phosphatase (ALP), osteonectin and type I collagen (COLL) were analysed.

2. Materials and methods

Investigations into the effect of alginate concentration as well as geometry were instigated as a consequence of two observations in preliminary experiments. Firstly tubes made of 3% sodium alginate gels were extremely fragile and often collapsed during handling. Secondly *in vivo* degradation experiments indicated that 3% alginate gels degraded rapidly. To conduct this experiment diffusion chambers (Millipore PR000/401) containing 160 μl of calcium sulphate crosslinked 3% or 8% MVG alginate (Novamatrix, FMC Biopolymer, Norway) gels were implanted subcutaneously into BALB/C male mice. After 2 and 4 weeks gels were removed from the chambers and dissociated in 55 mM sodium citrate solution for 1 h at room temperature. The uronic acid content of the gels was measured using a colorimetric method [11]. Briefly, aliquots (200 μl) of each test sample or galacturonic acid standards (0–100 μg) were added to borosilicate tubes and 3 ml sulphuric acid-borate reagent was added followed by 0.1% (w/v) carbazole reagent (100 μl). Tubes were then incubated at 60 °C for 1 h, cooled to room temperature and the absorbance read at wavelength 530 nm on a DU 640 spectrophotometer (Beckman, USA).

2.1. Alginate scaffolds

3, 5, 8 and 10% alginate solutions with high (70%) guluronic acid content (MVG, Novamatrix, Norway) were prepared in PBS (Sigma). Modified alginate solutions were made with the addition of collagen I (200 μl/ml) (Sigma) and β-tricalcium phosphate (1.2 g/ml) (TCP, Plasma Biotal Limited). In order to make different thickness alginate discs, either 1.2 ml or 0.3 ml of 8% alginate solution was added to each well in a 12-well plate. Sterilised 100 mM $CaCl_2$ (Sigma) was used to cross-link the alginate solution to form the hydrogel discs. Alginate tubes were made using 1mm diameter glass rods to dip into alginate solutions before using 100 mM $CaCl_2$ to cross-link the alginate coating and then the tubes were removed from the glass rods and cut into 3 mm in length. 60–65 tubes were added to each 12-well plate. After the gels were formed, they were stored in 2 ml sα-MEM medium per well and then stored in a fridge at 4 °C for 72 h.

2.2. Cell culture

rBMC were isolated and cultured according to the method described by Maniatopoulos *et al.* [12]. Briefly, femora from mature albino Wistar rats (around 120 g in weight) were dissected out removing as much as adherent soft tissue as possible. The epiphyses were removed and the femora were repeatedly flushed with minimum essential medium (α-MEM) (Sigma) containing 10% foetal bovine serum (FBS), 2.5% HEPES, 10% penicillin/streptomycin (P/S) and 1% amphotericin. Cells were then incubated in a 75 ml plastic flask using supplemented α-MEM medium (Sα-MEM) containing 10% FBS, 2.5% HEPES, 1% penicillin/streptomycin in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. After 7 days of primary culture, cells were detached using trypsin-EDTA (0.25% (w/v) trypsin-0.02% EDTA; Sigma). The cells were concentrated using centrifugation at 1500 rpm (400 g) for 5 min and resuspended in a 75 ml flask (5×10^5) for 7 days. During incubation, the medium was changed every 2 or 3 days. Passages 2 or 3 of rBMC were used to seed on the tissue culture dishes and alginate scaffolds.

2.3. Cell seeding and culture on alginate scaffolds

rBMC suspensions (4×10^4 cells) were seeded on each type of alginate scaffold, either with SαMEM or the osteogenic differentiation medium that consisted of SαMEM containing dexamethasone (10^{-8} M), ascorbic acid (0.05 mg/ml), and sodium β-glycerophosphate (0.01 M) (DexSαMEM). Seeding was performed on alginate discs or tubes or on TCP or in a 12-well plate either with DexSαMEM or SαMEM. All cultures were placed in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. During incubation, the medium was changed every 2 or 3 days.

2.4. Cell proliferation

The MTT assay was used to count rBMC after being cultured on the alginate discs or tubes for periods of 0, 3, 6, 9 and 12 days. Cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance of the resulting solution was determined at 570 nm using a plate reader (BP 800, Biohit, Finland). Cell number was determined from the calibration data, (not shown linear fit, six points, $n = 3$, $R^2 = 0.97$) obtained using the same cell type.

2.5. Isolation of total RNA

Total RNA was extracted from cells grown on tissue culture plastic wells and alginate discs or tubes using Rneasy® Mini Kit (Cat. No. 74104, Qiagen, UK). Cells were collected on day 0, 7 and 14 directly from the 12 well plate using RLT buffer (supplied), cells growth on alginate discs or tubes on 12 well plates were treated with trypsin to separate the cells from hydrogels before using the lysis buffer. RNase-Free DNase set (Promega) was used to degrade genomic DNA contamination within the RNeasy mini column, used as the manufacturers instructions. The integrity of isolated total RNA was checked both by spectrophotometer (BioPhotometer, Eppendorf, UK) and agarose gel electrophoresis.

The first strand cDNA was reverse transcribed from 2 μg of total cellular RNA in 20 μl solution using an Omniscript RT Kit (QIAGEN). RNA products with 1 μg oligo (dT_{15}) were heated at 85 °C for 10 min before cooling the reaction tube immediately on ice for 5 min

TABLE I Sequences of RT-PCR primers for analysis of osteogenic differentiation

Gene		Primer sequence (5' to 3')
Osteopontin	Sense	AAG CCT GAC CCA TCT CAG AA
	Antisense	GCA ACT GGG ATG ACC TTG AT
Collagen 1	Sense	TAA AGG GTC ATC GTG GCT TC
	Antisense	ACT CTC CGC TCT TCC AGT CA
Alkaline phosphatase	Sense	CTC CGG ATC CTG ACA AAG AA
	Antisense	ACG TGG GGG ATG TAG TTC TG
Osteocalcin	Sense	TCC GCT AGC TCG TCA CAA TTG G
	Antisense	CCT GAC TGC ATT CTG CCT CTC T
Osteonectin	Sense	AAA CAT GGC AAG GTG TGT GA
	Antisense	AGG TGA CCA GGA CGT TTT TG
Bone sialophospho protein	Sense	ATG GAG ATG GCG ATA GTT CG
	Antisense	TCC ACT TCT GCT TCT TCG TTC
GAPDHF	Sense	CGA TCC CGC TAA CAT CAA AT
	Antisense	GGA TGC AGG GAT GAT GTT CT

before 2 μl 10× reaction buffer, 40 units Rnasin Ribonuclease inhibitor, 2 μl PCR nucleotide mix (5 mM) and 200 units reverse transcriptase were added to the tube. The reaction was stopped at 37 °C for 60 min. Amplification primer pairs for collagen 1a, osteocalcin, osteopontin, osteonectin, bone sialophosphoprotein and alkaline phosphatase were selected (Table I) to examine the mRNA expression of these marker genes, and expression of the house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), was monitored as a control for RNA loading of samples.

For a standard PCR reaction 100 ng of cDNA products were amplified using 5 μl of 10 × Bioline buffer, 1 μl of 10 mM dNTP, 1.5 μl of 50 mM $MgCl_2$, 2 μl of 25 mM each primer and 0.2 μl of Bioline Taq. DNA polymerase, and cycled 20–32 times at 94 °C for 20 s, 62.5 °C for 20 s and a final extension of 10 min at 72 °C. The PCR products were separated electrophoretically using an agarose gel (Promega) containing ethidium bromide. Images of resolved products were analysed by an EDAS system (Kodak, UK).

3. Results

3.1. *In vivo* degradation

3% gels were found to degrade rapidly and disintegrated upon handling after 4 weeks subcutaneous implantation. In contrast the 8% gels retained their original appearance, despite evidence of considerable degradation from uronic acid assay measurements (Fig. 1).

3.2. rBMC proliferation on alginate discs and tubes

In 2D cultures a clear relationship was apparent between rBMC proliferation and the concentration of alginate discs with the highest proliferative rate seen on 3% MVG discs, followed by successively lower rates on 5, 8 and 10% discs (Fig. 2).

The addition of either collagen alone or collagen with TCP to 8% or 3% MVG alginate discs did not alter the relative proliferative capabilities of rBMCs that had colonised the surfaces, with greater proliferation still observed on the 3% alginate discs compared with the 8% hydrogels (Fig. 3). Increasing the thickness of 8%MVG alginate discs from 0.3 mm to more than 1mm was found to inhibit rBMC proliferation (Fig. 4).

Altering the geometry of the alginate substrates influenced the proliferative behaviour of rBMCs with greater cell numbers per unit area measured on MVG alginate tubes, compared with MVG alginate discs

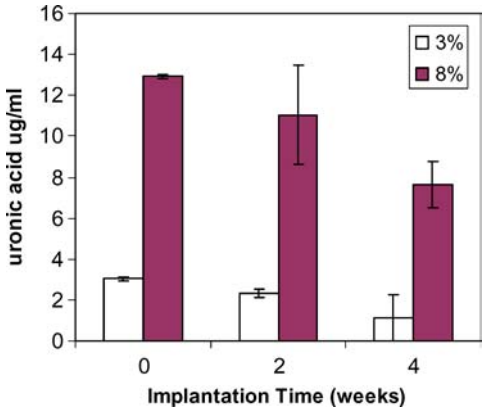

Figure 1 *In vivo* degradation profile of 3 and 8 wt% alginate gels.

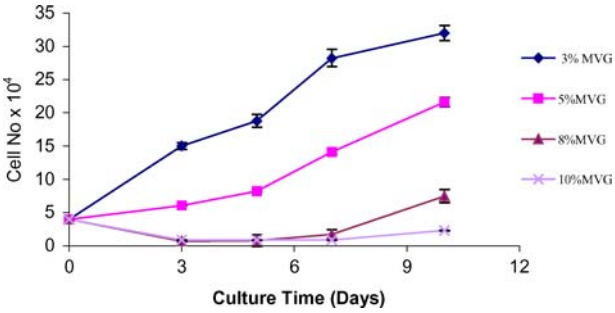

Figure 2 Proliferation of rBMC grown on different concentrations of MVG alginate discs.

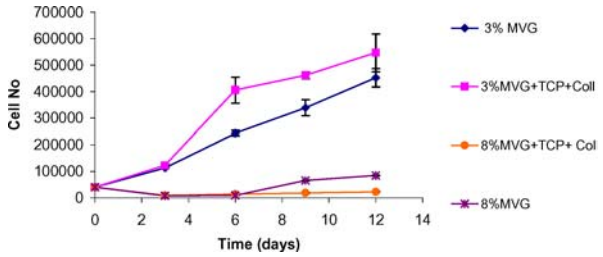

Figure 3 Proliferation of rBMC on MVG alginate and modified discs.

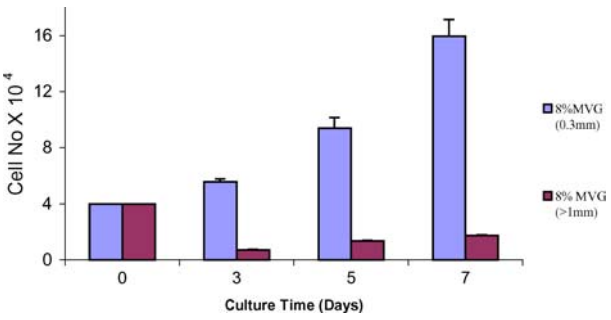

Figure 4 rBMC growth on different thickness 8% MVG alginate discs.

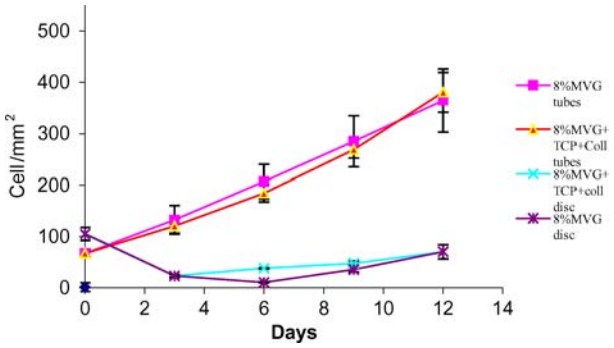

Figure 5 Proliferation of rBMC grown on 8% alginate discs and tubes.

(Fig. 5). Addition of TCP and collagen to the alginate discs and tubes did not appear to influence rBMC proliferation.

3.3. RT-PCR

The expression of osteoblastic markers osteocalcin (OC), osteopontin (OPN), alkaline phosphatase (ALP), osteonectin (ON), type I collagen (COLL) and bone sialophosphoprotein (BSP) were analysed after 7 and 14 days (Fig. 6a and b respectively). Generally, there were similar patterns of expression of the osteoblastic markers at both time points. The mRNA levels of OC, OPN and BSP expressed by rBMCs did not appear to be affected by the 2-D disc or 3-D tube geometry of alginate hydrogels, although the levels of expression were generally higher on both than found on the tissue culture plastic controls.

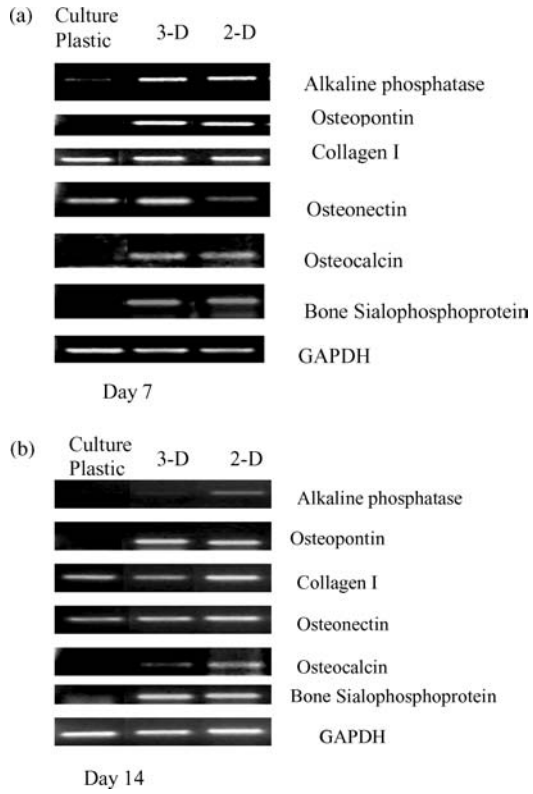

Figure 6 (a) Comparison of mRNA expression by rBMC cultured on tissue culture plastic, 3D 8% alginate tubes and 2D 3% alginate discs after 7 days culture and (b) 14 days.

4. Discussion

4.1. rBMC proliferation on alginate hydrogels

Improved rates of rBMC colonisation of discs with progressively lower alginate concentrations (Fig. 2) was unexpected, as surfaces demonstrating greater flexibility have previously been associated with less motile cells [13]. It is possible that increasing concentrations of MVG alginate released a constituent at levels that inhibited rBMC proliferation, although further growth curves over longer culture periods would be necessary to determine this. However this possibility was supported by the finding that there was significant inhibition of rBMC growth on 8%MVG discs greater than 1 mm in thickness compared with discs 0.3 mm thick (which may release less of any inhibitory constituent).

Addition of collagen and TCP to alginate discs did not significantly change the rBMC colonisation profiles from plain alginate discs nor influence the fact that lower concentration alginates continued to provide better substrates for colonisation than higher concentrations.

The marked differences between rBMC growth on 8% alginate tubes or discs may be attributed to the different thickness of alginate gels (approx 200–300 μm) which had already been shown to influence proliferation on discs (Fig. 4).

Three dimensional alginate scaffolds have been made previously e.g. Eiselt *et al.* [14] yet effects of geometry were not compared to a 2D control. In that study porous beads made from 1.75 wt% MVG alginate were investigated, however the pore size (50–200 μm) was smaller than the diameter of the tubes investigated in this report than the and pore walls were relatively thicker.

4.2. Analysis of gene expression levels in 2-D and 3-D alginate scaffolds

The expression of osteoblastic genes were analysed at 7 and 14 days to investigate the impact of alginate tubes and discs on cell differentiation. Generally, in a population of bone cells, an increase in the specific activity of ALP reflects an early shift to an increased level of differentiation and is thought to have an important role in the initiation of matrix mineralisation. Raised levels of ALP expression at day 7 in the present study for both the 2-D discs and 3-D tubes as compared with the control suggested that these cells were actively differentiating into osteoblasts. The study also showed that the expression levels of OC, OPN and BSP were not greatly affected by the 2 or 3-D nature of the alginate. Furthermore, OC, a late marker of osteoblast differentiation, was present both from cells in tubes and discs at day 7 and 14, suggesting that differentiation had occurred. This was surprising as recently Hishikawa *et al*. demonstrated up regulation of 72% of genes in a microarray in 3-D collagen gels compared with 2-D cultures [15].

5. Conclusions

rBMC proliferation was inversely related to the concentration of MVG alginate substrates and was independent of addition of either collagen or collagen and TCP to the alginate. rBMC colonisation and proliferation on

alginate 2D discs and 3-D tubes was very different with tubes much better able to support growth of BMCs, although the different geometries did not generate significant alterations in BMC differentiation. The findings of this study suggested that investigations in 2D might be inaccurate indicators of cell behaviour on 3D alginate scaffolds however altering the geometry also altered the gel thickness and this too was found to be an important parameter in determining cell behaviour. Further study may elucidate the mechanism of these observations.

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