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Use of magnetic particles to apply mechanical forces for bone tissue engineering purposes

S H Cartmell, A Keramane, G R Kirkham, S B Verschueren, J L Magnay, A J El Haj and J Dobson
Institute of Science and Technology in Medicine, University of Keele, Thornburrow Drive, Hartshill, Stoke-on-Trent, Staffordshire ST4 7QB, UK

E-mail: s.h.cartmell@keele.ac.uk

Abstract It is possible to influence osteoblast activity by the application of mechanical forces. There is potential in using these forces for tissue engineering applications in that cell matrix production may be upregulated, resulting in a functional tissue engineered construct created in a shorter culture time. We have been developing a novel technique for applying mechanical forces directly to the cell with the use of magnetic particles. Particles attached to the cell membrane can be manipulated using an external magnetic field thus applying forces in the piconewton range. We have previously demonstrated that primary human osteoblasts respond to this type of stimulus by upregulating bone related gene expression and producing mineralized matrix at early time points. In this paper we discuss the optimization of this technique by presenting data on the effects of this type of force on osteoblast proliferation, phagocytosis and also the potential use of this technique in developing 3D tissue engineered constructs.

1. Introduction

Mechanical stimulation has been proposed for preconditioning of bone and stem cell seeded constructs and ultimately to produce tissue engineered bone. It is possible to use mechanical forces, if they are applied within the correct parameters, to influence bone cell differentiation and ultimately increase the amount of mineralized matrix produced by these cells. In this way, the length of time required to grow a tissue-engineered product *in vitro* prior to patient implantation could potentially be significantly reduced using mechanical stimulation.

We are studying a novel method of applying mechanical forces, in the piconewton range, directly to the cell over a long-term incubation period. The novel technology employs magnetic microparticles which have been attached to primary human bone cells via specific membrane receptors which are then exposed to an oscillating magnetic field either in 2D monolayer culture or seeded onto 3D constructs. As this cyclical magnetic field is applied, the magnetic particles respond with the cell experiencing a force as a result of the permanent magnet movement. By applying forces directly to the cell, a variety of non-load-bearing matrices/scaffolds can be used. This technology was first developed to study mechanotransduction effects on single cells [1-3]. We have designed a magnetic force bioreactor which enables us to establish varying load regimes e.g. duration, frequency and magnitude of force applied on human bone cell seeded constructs and utilises this novel way of applying mechanical forces to cells for tissue engineering purposes [4-6]. In this paper, we describe optimization studies demonstrating the effects of magnetic particle presence and force application on cell proliferation. The monitoring of varying sizes of magnetic particles attached to osteoblasts is

presented over a time course from 30 minutes up to 1 week. We also discuss the effects of this type of mechanical conditioning on human bone cells in both 2D and 3D environments.

2. Methods

2.1 Cell Proliferation studies

Fifty thousand primary human osteoblast cells were seeded into separate T75 flasks. Experiments were repeated using two different patient sources. RGD coated magnetic particles (4.4 μm CrO_2 particles, CFM-40-10, Spherotech Inc.) were adhered to the osteoblast membrane at a concentration of two particles per cell via integrin receptors. An external magnetic field was applied using a magnetic force bioreactor (figure 1). Rare earth (NdFeB) magnets moving at a 2 Hz frequency applied magnetic fields varying from 0 – 2000 G. In this way, a force of approximately 50 pN per particle was applied to the cells. Control groups of cells only, cells plus particles only (no magnetic field applied) and cells with magnetic field only (no particles) were also examined (N = 5 for all groups). A force was applied for one hour per day over four days. After four days, when the cells were approaching confluency, all cells were removed from monolayer culture using trypsin and pelleted using centrifugation. An equal and small aliquot was removed from each sample for a cell count using a haemocytometer. Three hundred μls of a TritonX based lysis buffer was added to the remainder of each sample. The DNA content of each sample was then measured using a picogreen assay (Molecular Probes). A student's t-test assuming unequal variance was used to test for differences in cell proliferation.

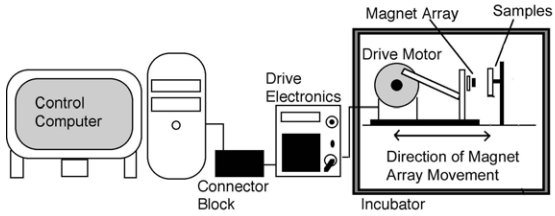

Figure 1. Setup of constructs exposed to magnetic field.

2.2 Particle internalisation studies

Magnetic particles (4.4μm, chromium dioxide, CFM-40-10, Spherotech Inc.) were coated with RGD (SIGMA) using a passive absorption technique. The particles were then attached to 20,000 monolayer primary human osteoblast cells seeded in 6 well plates at a concentration of one particle per cell. Control groups of the same magnetic particle samples with no RGD coating were also included. At time points of 30 minutes, 1 hour, 24 hours and 1 week the samples were fixed using 2.5 % glutareldehyde (SIGMA). The samples were then serially dehydrated using ethanol and a critical point dryer, gold coated and then viewed using a scanning electron microscope (SEM) (Hitachi). Normal surface morphology information was obtained using 1.5 kV in addition to back scattered imaging using a voltage of 15 kV in order to image internalized particles.

2.3 3D study

RGD-coated fluorescent magnetic particles (2.8 μm magnetite, UMC3F, Bangs Labs Inc.) were attached at a density of approximately four particles per cell to primary human osteoblasts. Five hundred thousand cells with attached particles were then seeded onto 88±2 % porous PLLA scaffolds. The PLLA scaffolds were manufactured in our laboratory using a standard salt leaching technique to create 4 mm height x 10 mm diameter cylinders with a pore size of 250-350 μm. The cell/particle/scaffold (construct) was placed into a vial and cultured with 5 mls alphaMEM containing 10 % FCS and osteogenic supplements of dexamethasone (10^{-8} M), ascorbic acid (50μgrams/ml) and betaglycerophosphate (10 mM). After one week, calcein (Molcular Probes) was used to stain the live cells in the construct. Confocal microscopy was then used to image the location of cells/particles on the scaffolds and also determine the cell viability.

These studies were carried out in accordance with ethical approval from The North Staffordshire Local Research Ethics Committee, project no. 03/16.

3. Results

No statistical difference in cell proliferation could be determined between the different experimental groups as can be seen in a typical graph of results in figure 2. Both the cell count and the picogreen DNA assay consistently confirmed this through all the experiments performed and with the primary osteoblasts. The 3D study performed showed the cells with and without attached magnetic particles were all viable and cells were adhered to the PLLA porous scaffold (figure 3). The majority of the cells (both with and without particles) were located on the periphery of the scaffold, with only a few cells in the centre.

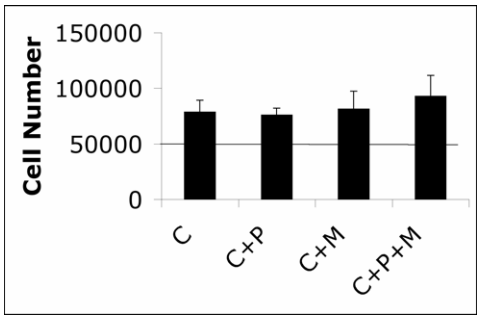

Figure 2. Cell proliferation study – graph demonstrates the cell number as quantified by a pico green assay. C - cells only, C+P – cells plus magnetic particles only, C+M – cells plus magnetic field only, C+P+M - cells plus particles plus magnetic field (mechanical force group). Black line indicates initial cell seeding number. No significant differences between any of the samples.

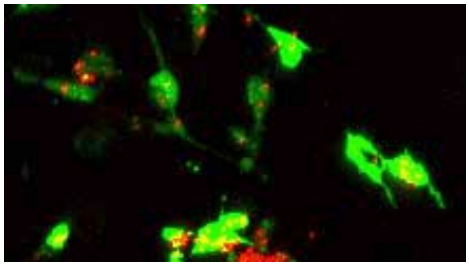

Figure 3. 3D Study - Confocal microscope image; green = live cells (calcein); red = magnetic particles. Image taken of cell/particles adhered onto PLLA scaffolds after 1 week of culture.

The SEM analysis showed that very little, to no particles were found on the cells of both uncoated and RGD coated particle group that were left to adhere for only 30minutes. It seems that the particles were neither internalised nor attached to the cells after such a short time. For both the RGD and uncoated particles that were left to adhere to cells for 3hours, the particles attached were located on the cell-edges (see figure 4A). Backscattered electron analysis revealed no internalised particles. For an adherence time of 24 hours, internalised particles were clearly seen. Backscattered electron imaging showed internalised particles that would otherwise would have been difficult to identify (see figure 4B and 4C). After one week of particle adherence to cells, a much higher proportion of the RGD and uncoated particles were seen to be internalised. Little difference was seen between RGD and uncoated 4.4μm magnetic particles in the SEM analysis with regards to internalisation. Particles adhere to cell edges after 3hours of adherence, and have begun to be phagocytosed after 4 hours and are fully internalised by 1week.

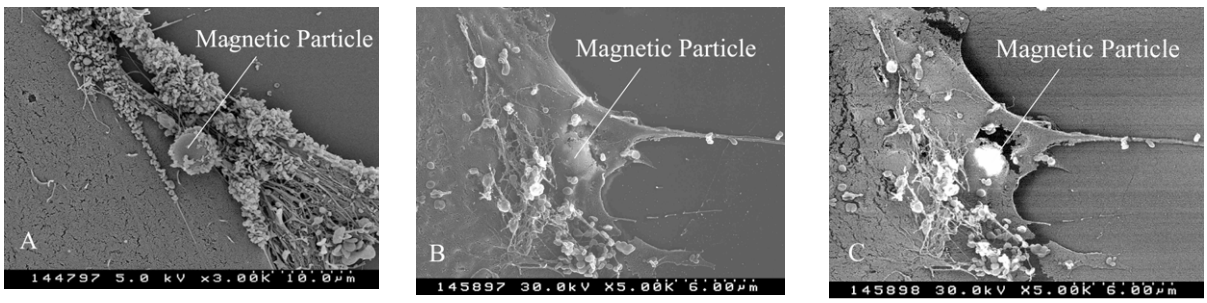

Figure 4. SEM images: **A.** Magnetic particle attached to primary human osteoblast lamellapodia after 3 hours adherence time. **B.** Magnetic particle internalised by a primary human osteoblast after 24 hours adherence time. 1.5kV gives surface morphology only. **C.** Image same as 4B but using back scattered electron imaging at 15kV clearly highlighting the internalised chromium dioxide particle.

4. Discussion

Magnetic micro- and nanoparticle-based techniques have the potential to be used for the application of mechanical forces to osteoblasts in culture, thus improving the mechanical strength and shortening the *in vitro* preparation time of bone tissue engineered constructs. We have previously demonstrated upregulation of bone related gene expression and early matrix mineralization using this technique which is extremely encouraging [4-5]. This study has added further important information to the development of this technique. Neither magnetic particles, magnetic field not force application affected cell proliferation. This is a promising result with regards to tissue engineering as a decrease in cell proliferation in response to this stimuli would have been detrimental. As we already know that this technique upregulates bone-related gene expression, it is interesting to note that these effects do not also extend to proliferation. It has been shown previously that osteoblast proliferation is significantly reduced (but not viability) with the higher the number of particulate that is phagocytosed (data from up to 40-60 engulfed metal/polymer particles) [7]. However, it appears from our studies that with the lower limit of two particles per cell that proliferation is not significantly affected.

SEM analysis has shown that the surface coating of RGD on the magnetic particles does not appear to affect the rate of phagocytosis. The length of time that the particles were left to adhere to the osteoblasts was important with a minimum time of thirty minutes for particles to adhere. These studies do, however, demonstrate that the particles that adhere are phagocytosed within 1 week. Further experiments are underway to determine the internal cell structures to which the phagocytosed particles are attached and which structures are influenced when they are manipulated by magnetic fields.

The 3D study demonstrated the non-cytotoxicity of these magnetic particles, with cells remaining viable after 1 week of *in vitro* culture. Concentration of cells at the periphery of the 3D scaffold is often seen in standard cell seeding techniques. In addition to applying mechanical forces, future applications of the magnetic particles may also include manipulating the distribution of cell seeding by applying magnetic fields to guide cells into the centre of constructs.

This enabling technology has major advantages over existing methods of *ex vivo* mechanical conditioning, such as: (i) unprecedented control of physical parameters during culture, (ii) the forces are applied directly to the cell membrane enabling the use mechanically weak scaffold materials, (iii) no interruption of nutrient perfusion during stimulation, (iv) there is no infection route into the culture system, as the particles are remotely coupled to the applied fields. These advantages overcome the major problems of simulation of the *in vivo* stress environment *ex vivo*.

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