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Differential in-gel electrophoresis (DIGE) analysis of human bone marrow osteoprogenitor cell contact guidance

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Abstract

We have used a recent comparative proteomics technique, differential in-gel electrophoresis (DIGE), to study osteoprogenitor cell response to contact guidance in grooves. In order to increase protein output from small sample sizes, we used bioreactor culture before protein extraction and gel electrophoresis. Mass spectroscopy was used for protein identification. A number of distinct proteins were observed to exhibit significant changes in expression. These changes in protein expression suggest that the cells respond to tailored grooved topographies, with alterations in their proteome concurrent with changes in osteoprogenitor phenotype.

Keywords: Tissue engineering; Surface topography; Contact guidance; Osteoprogenitor cells; Proteomics

1. Introduction

There is a rapidly growing need to understand how cells are affected by bioactive materials in a more global way. This is especially true when considering a recent description of third-generation biomaterials in *Science* [1] that describes materials influencing cells at a molecular level. We have to go beyond assessing just what we consider to be key proteins, e.g. the cytoskeleton or phenotypical marker proteins, and consider the whole cell response in order to discover which underlying cell pathways are important in processes such as material-related bone differentiation.

Recently, researchers have started taking a genomic approach using microarrays [2,3]. However, array data can be difficult to present due to the very large number of results often achieved and thus the important data can be difficult to dissect out. Also, arrays consider the cell response before protein translation, and there are many steps (e.g. post-translational modification) that can affect the gene regulatory outcome. Thus, a proteomic approach is desirable.

However, until recently, this has been hard to achieve in a reliable comparative (i.e. sample and control) manner. In order to compare two-dimensional (2-D) gels (where extracted proteins are loaded onto an acrylamide gel and then separated first by isoelectric point and then by mass), two gels would have to be run and then compared by eye or by gel-matching software. However, the gels are complex, and slight changes in the running conditions or gel

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preparation can lead to artefactual error. Differential in-gel electrophoresis (DIGE) is a new method [4] whereby the proteins are fluorescently labelled (sample and control with fluores of different wavelength) and run in the same 2-D gel. The technique is sensitive and allows for less than 50 μg of protein to be successfully separated and detected, compared to the 500 μg required for a successful traditional Coomassie blue or Sypro Orange-stained 2-D gel.

Here, in the study of osteoprogenitor response to topography, we have used the most sensitive method available, that of saturation labelling, in which all cysteine residues are fluorescently labelled to maximize the sensitivity of protein detection. As little as 5 μg of total protein can successfully be analysed [4]. For bone-related biomaterials, a highly sensitive technique is required because, as cells differentiate into bone-forming cells in vitro, they tend to form discrete nodules rather than confluent sheets and thus low protein yields are expected. In fact, for most academic-scale biomaterials research, when considering either genomics or proteomics, sensitivity is a key issue as the sample area normally provided is typically only 1–2 cm^2.

In order to generate protein, we have used a tissue engineering approach. In tissue engineering, the aim is to form complex tissues in vitro using bioactive materials to guide cell growth. Ideally the cell choice should be multipotent and available from an autologous source, and the scaffold materials should be bioactive and biodegradable. To help with nutrient delivery over the long-term in cultures required to produce bone in vitro, bioreactor culture can also be used. Here, human bone marrow osteoprogenitor cells have been used which were isolated from trabecular bone marrow. These comprise a mixture of cells, from primitive mesenchymal stem cells to mature osteoblasts, with precursor cells in between at different stages of commitment [5,6]. This heterogenous cell mix has the potential to differentiate into bone-forming cells/osteoblasts under appropriate conditions using selected hormones/growth factors [7–9], vitamins/chemical reagents [10,11] and/or defined surface topographical guidance [12–15].

As a bioactive cue, grooves have been used here as they have previously been shown to alter osteoprogenitor differentiation and allow the formation of bone nodules in vitro without medium supplements [12,14,16]. The grooves were embossed into the biodegradable polymer polycaprolactone (PCL, an FDA-approved biomaterial with good biocompatibility and excellent micro- and nanoreplication characteristics [17,18]). In order to facilitate improved cell growth to obtain higher protein yields, a flow system bioreactor was used to perfuse the basal medium [19]. It is important to use a basal medium so that we can access directly the bone-inducing potential of the material rather than using stimulants such as dexamethasone or ascorbate.

Here we will detail the methodology and discuss the results obtained from application of DIGE to biomaterials research.

2. Materials and methods

2.1. Material fabrication

Quartz slides were cleaned in seven parts sulphuric acid and one part hydrogen peroxide for 5 min. The slides were then spin-coated with AZ primer at 4000 rpm for 30 s. Shipley S1818 photoresist was next added, and the slides were spun for a further 30 s. After spinning, the slides were soft baked at 90 °C for 30 min. The samples were then exposed to ultraviolet light through a chrome mask (Hoya), patterned with a 12.5 μm wide line pattern. The exposed resist was developed using 1:1 (v/v) Shipley AZ developer:water. The slides were next etched to produce 2 μm deep grooves in a Plasma Technology RIE80 unit (trichloromethane environment, pressure of 15 mTorr, R.F. power of 100 W, giving an etch rate of 25 nm min^{-1}). The mastering resist was then removed and the whole slide was etched for a further minute to produce a uniform chemistry.

For embossing, a PCL (Sigma–Aldrich) sheet was cut into 2 cm^2 squares before being cleaned with 75% ethanol followed by deionized water and blow-dried with cool air. The PCL substrates were heated by light until they started to melt. Either the grooved or planar slides were embossed onto the PCL substrates. PCL substrates were cooled down and the glass slides were removed. Planar controls were checked by atomic force microscopy and had a mean Ra of 28.406 nm (Nanoscope IIIA) (Fig. 1).

2.2. Cell culture

Human bone marrow osteoprogenitors were obtained from hematologically normal patients undergoing routine surgery. Only tissue that would have been discarded was used with the approval of the Southampton & South West Hants Local Research Ethics Committee. Primary cultures of bone marrow cells were established as described previously [20].

Osteoprogenitor cells were cultured in 75 cm^2 tissue culture flasks at passage 2. Culture was maintained in basal medium (α-MEM containing 10% foetal bovine serum (FBS) and 2% antibiotics) at 37 °C, supplemented with 5% CO_2. Confluent cell sheets were trypsinized and 1×10^5 cells were seeded onto grooved and flat control PCL sheets. The experiment was separated into two groups. The first group was maintained in a static culture for 4 weeks; the second group was maintained in static culture for 1 week prior to culture in the flow system for 3 weeks. Medium was changed twice a week for static culture conditions.

The bioreactor consisted of a medium reservoir connected to three 'tissue container' cassettes with continuous medium flow controlled by a peristaltic pump. Basal medium was supplied to osteoprogenitor cells at a flow-rate of 0.5 ml min^{-1}. Five hundred millilitres of basal medium was reused for 1 week.

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