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Porcine bone marrow stromal cell differentiation on heparin-adsorbed poly(e-caprolactone)–tricalcium phosphate–collagen scaffolds

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Abstract

We evaluate the potential of heparin as a substrate component for the fabrication of bone tissue engineering constructs using poly(e-caprolactone)–tricalcium phosphate–collagen type I (PCL–TCP–Col) three-dimensional (3-D) scaffolds. First we explored the ability of porcine bone marrow precursor cells (MPCs) to differentiate down both the adipogenic and osteogenic pathways within 2-D culture systems, with positive results confirmed by Oil-Red-O and Alizarin Red staining, respectively. Secondly, we examined the influence of heparin on the interaction and behaviour of MPCs when seeded onto PCL–TCP–Col 3-D scaffolds, followed by their induction into the osteogenic lineage. Our 3-D findings suggest that cell metabolism and proliferation increased between days 1 and 14, with deposition of extracellular matrix also observed up to 28 days. However, no noticeable difference could be detected in the extent of osteogenesis for PCL–TCP–Col scaffolds groups with the addition of heparin compared to identical control scaffolds without the addition of heparin.

Keywords: Scaffold; PCL–Collagen; Cell differentiation; Heparin

1. Introduction

Mesenchymal progenitor cells are the principal cells contributing to bone formation by virtue of their ability to differentiate into osteoblasts [1]. The multi-step transformation of progenitor cells into bone-forming cells involves the complex interaction of a variety of growth factors and cell signalling molecules. The majority of extracellular signals that guide cells through these types of phenotypic progressions bind to the particular class of carbohydrates that is prominent in the extracellular matrix of growing and repairing tissues. Heparan sulfate (HS) and heparin are members of the glycosaminoglycan (GAG) family of polysaccharides, and the importance of HS to bone is best explained by its ability to bind and bioactivate most of the growth and adhesive factors involved in regulating bone cell metabolism and osteoblast lineage progression [2,3]. Although constituted by the same disaccharide building blocks (though in different proportions), compared to heparin, HS is less sulfated, more heterogeneous and present on the surface of most cell types and in the extracellular matrix (ECM), while the hypersulfated anticoagulant heparin is usually sequestered in mast cells [4]. The ECM-resident HS acts to concentrate growth factors close to cell surfaces, protecting them from extracellular proteases, and facilitating binding to their specific receptors in an active form [3]. Heparin, both commercially and readily available, is often used to approximate HS action (albeit with reduced specificity), and has been shown clinically to interfere with the ligand-binding activities of HS by competitively inhibiting the susceptible factors needed by osteoblasts [5,6], leading to an osteoporotic-like reduction in bone formation [7–9]. Various molecular mechanisms have been proposed for these observations, including the enhancement of interleukin-11 signalling through an up-regulation of the MAPK

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pathway [10]. However, a recent two-dimensional (2-D) cell culture study has shown that although high concentrations of heparin can lead to a reduction in cell numbers with inhibited matrix deposition and mineralization, almost certainly by nonspecific competition with endogenous HS, low concentrations of heparin (5–500 ng ml^{-1}) actually promote ECM deposition and mineralization in osteoblast-like Saos-2 cells [11]. Thus heparin may exert biphasic effects, and it appears that low doses might actually be beneficial for bone formation by potentiating local growth factors. However, the aforementioned study has two disadvantages: it was performed with an osteosarcoma cell line, and on a 2-D surface. Biomedicine has become increasingly aware of the limitations of conventional 2-D cell culture. This has greatly intensified the pace at which 3-D cell culture systems have been developed; this environment might be considered to occupy the space between a Petri dish and a mouse. Although traditional culture dishes have had an enormous impact on modern biology, the Petri dish and its ancillaries, including multiwell plates and glass coverslips, are less than ideal for studying cells and tissues. The Petri dish surface is rigid and inert without coating, as opposed to the in vivo "soft" environment where cells interact intimately with the ECM and with each other in three dimensions. 2-D and 3-D intracellular transport phenomena are drastically different. In 2-D culture systems, cytokines, chemokines and growth factors quickly diffuse in the media across the culture dish, whereas the in vivo environment benefits from chemical and biological gradient diffusion systems, which play a vital role in signal transduction, cell–cell communication and development.

The use of mesenchymal progenitor cells (MPCs) for preclinical in vivo studies, wherein they are implanted into critical-sized bone defects, is the subject of much interest [12], particularly when they employ large-animal, load-bearing immunocompetent models [13]. The characterization of MPCs within this context is of great importance, especially considering the potential availability of large numbers of immune-privileged, harvestable cells and the similarity in the mechanical features of the bone biology in comparison to humans. Although studies have been published on the characterization of porcine bone marrow mesenchymal stem/stromal cells [14], the specific biochemical cues influencing their osteogenic differentiation in 3-D culture systems and on specific substrates have been explored to a much lesser extent [15].

The aims of the present study were twofold. First, we sought to explore the ability of porcine MPCs to differentiate into either the osteogenic or adipogenic lineages within a 2-D tissue culture plate system. Secondly, we sought to determine the effect of heparin on porcine MPCs seeded onto poly(e-caprolactone)–tricalcium phosphate–collagen type I (PCL–TCP–Col) 3-D scaffolds.

The study thus aimed to evaluate the potential use of 3-D PCL–TCP–Col scaffolds for future in vivo applications. It also aimed to investigate whether the inclusion of heparin as a substrate would improve this system, particularly pertaining to bone tissue engineering by investigating its effect on the bone-forming potential of porcine MPCs.

2. Materials and methods

2.1. Progenitor cell extraction, culture conditions and cell seeding

Bone marrow precursor cells (MPCs) were isolated from 10–15 ml of marrow aspirated from 16-week old Duroc/Yorkshire cross pigs using strict aseptic techniques. Progenitor cells were isolated by plating the freshly aspirated bone marrow onto polystyrene T-150 culture flasks (approx 5 ml of aspirate per flask) (Nalgen-Nunc, Denmark) in 30 ml control medium consisting of Dulbecco's Modified Eagle's Medium (DMEM: low glucose), 10% fetal bovine serum and 1% penicillin–streptomycin, and incubating the cells at 37 °C in a humidified atmosphere containing 5% CO_2 (Binder, Tuttlingen Germany). Non-adherent cells were removed at day 4 and the culture media changed every 3–4 days; this was continued until the cells attained 70% confluence. MPCs were then utilized either in 2-D studies (for osteogenic and adipogenic assays), or seeded onto 3-D PCL–TCP–Col scaffolds (with and without the addition of heparin) and observed for their bone tissue engineering capabilities.

2.2. Two-dimensional studies: adipogenic and osteogenic induction

For adipogenic induction, cells in their third passage were plated in control media at a density of 5000 cells cm^{-2} and on the second day of culture treated with adipo-induction media (control medium containing 1 mM dexamethasone (1 mM stock in 100% ethanol), 0.2 mM indomethacin (100 mM stock in 100% ethanol), 0.01 mg ml^{-1} insulin, 0.5 mM 3-isobutyl-1-methylxanthine (500 mM stock in DMSO)). The adipo-induction media was replaced every 3–4 days and was continued for 6 weeks. The adipogenic differentiation was visually monitored by phase-contrast microscopy and the formation of lipid-filled vacuoles confirmed by Oil-Red-O staining. For staining, cells were fixed with 4% (w/v) formaldehyde/1% (w/v) calcium, washed with 70% ethanol and incubated with 2% (w/v) Oil-Red-O (Sigma Aldrich, St Louis, MO, USA) for 5 min at room temperature. Excess stain was then removed by further washing with 70% ethanol then distilled H_2O). Counterstaining was performed with Meyer's hematoxylin (H&E; Sigma Aldrich). For quantitation, 10 randomly chosen, nonoverlapping, low-power micrographs of each sample were taken; the positive cells were counted and compared with the total cell number.

For osteogenic induction, cells in their third passage were plated in control medium at a density of 3000 cells cm^{-2} and on the second day of culture treated with osteo-induction media (control medium containing 0.1 mM ascorbic acid 2-phosphate, 10 mM β-glycerophos-

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