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The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs

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ABSTRACT

One of the key challenges in tissue engineering is to understand the host response to scaffolds and engineered constructs. We present a study in which two collagen-based scaffolds developed for bone repair: a collagen–glycosaminoglycan (CG) and biomimetic collagen-calcium phosphate (CCP) scaffold, are evaluated in rat cranial defects, both cell-free and when cultured with MSCs prior to implantation. The results demonstrate that both cell-free scaffolds showed excellent healing relative to the empty defect controls and somewhat surprisingly, to the tissue engineered (MSC-seeded) constructs. Immunological analysis of the healing response showed higher M1 macrophage activity in the cell-seeded scaffolds. However, when the M2 macrophage response was analysed, both groups (MSC-seeded and non-seeded scaffolds) showed significant activity of these cells which are associated with an immunomodulatory and tissue remodelling response. Interestingly, the location of this response was confined to the construct periphery, where a capsule had formed, in the MSC-seeded groups as opposed to areas of new bone formation in the non-seeded groups. This suggests that matrix deposited by MSCs during *in vitro* culture may adversely affect healing by acting as a barrier to macrophage-led remodelling when implanted *in vivo*. This study thus improves our understanding of host response in bone tissue engineering.

1. Introduction

Every year over 2 million bone replacement procedures are performed worldwide which require the use of bone graft materials, making bone second only to blood on the list of transplanted materials. With demand escalating and increasing limitations with traditional bone graft supply, techniques are being developed to provide alternatives with properties suitable for clinical use [1–4].

Cell-free scaffolds as tissue graft substitutes have been used in the human clinical setting since Yannas et al. developed the collagen–glycosaminoglycan (CG) scaffold for skin grafting almost three decades ago [5–7]. Since then a considerable amount of research has been carried out to enhance the mechanical properties of this scaffold to make it suitable as a bone graft substitute. In a series of recent studies from our laboratory, we have developed a collagen–glycosaminoglycan (CG) scaffold with improved mechanical strength, porosity and biological properties for bone regeneration [8,9]. Based on the CG scaffold initially developed and FDA approved for skin regeneration but by increasing the collagen composition relative to the standard CG scaffold and cross-linking the collagen fibres with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDAC), the compressive stiffness is increased 7-fold over the standard skin regeneration materials which are crosslinked using a dehydrothermal process; however, the high porosity of the scaffolds (>98%) is maintained [8]. Furthermore, the modified scaffolds showed increased osteogenic response compared to the standard material when seeded with osteoblasts and maintained in culture [9]. This modified CG scaffold serves as the first scaffold biomaterial in this study.

The second scaffold used in this study is a biomimetic collagen–calcium phosphate (CCP) scaffold. This consists of a collagen scaffold, produced by the same lyophilisation (freeze-drying) technique as the CG scaffolds, and subsequently mineralised

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using a bi-phasic immersion technique first in phosphate and then in calcium [10]. These scaffolds have a compressive modulus which shows a greater than 25-fold increase on the skin regeneration CG scaffold and a 4-fold increase on the optimized CG scaffold while maintaining a porosity as high as 96% [10]. This suggests that this scaffold has an advantage over previously developed composite scaffolds which have failed to combine collagen and calcium phosphate effectively without compromising either porosity or strength [11,12]. Furthermore, this scaffold has already demonstrated *in vitro* osteogenic activity comparable to the CG scaffolds [13].

The potential of cell-free scaffolds to be used as an *off-the-shelf* product presents an ideal clinical solution to the limitations of traditional bone graft; however the principal strategies of bone tissue engineering suggest the use of cells seeded onto scaffolds prior to implantation. Recent investigations have focused on the use of stem cells instead of mature differentiated cell types due to their expansion potential and ease of access. In particular, mesenchymal stem cells (MSCs) have shown promising results in a number of tissue engineering areas, e.g. tendon [14,15], cartilage [16], fat [17] and bone [17–19]. The *in vitro* osteogenic differentiation of MSCs has been studied extensively showing the existence of marrow-derived progenitors that give rise to bone [19]. Previously, we have demonstrated the potential of the collagen-based scaffolds developed in our laboratory to support *in vitro* osteogenesis by MSCs [20–22].

In this study, we evaluate the *in vivo* host response to both cell-free scaffolds and cell-seeded tissue engineered (TE) constructs i.e. the same scaffolds but pre-cultured with MSCs in osteogenic media for 4 weeks prior to implantation. In addition, utilizing immunohistochemical techniques, we evaluate the complex macrophage-led host response to the cell-free scaffolds and TE constructs.

The specific aims of this study were threefold: (1) to assess the effects of *in vitro* culture on cell proliferation, matrix deposition and resultant mechanical properties. (2) To compare the ability of collagen–glycosaminoglycan and collagen–calcium phosphate scaffolds, both cell-free and as tissue engineered constructs seeded with mesenchymal stem cells, to heal critical-sized bone defects using an established rat cranial defect *in vivo* model. (3) To determine the relationship between the host macrophage-led immune response and the overall defect healing. Healing rates were assessed by measuring new bone formation within the defect using micro-computed tomography and histomorphometric techniques. Immunohistochemistry was carried out to analyze the host cell response to the TE constructs and the cell-free scaffolds.

2. Materials and methods

2.1. CG and CCP scaffold fabrication

CG scaffolds were fabricated as previously described using a freeze-drying technique [9,23,24]. CG suspension was produced in a solution of 0.05 M acetic acid using 1% w/v type I collagen derived from bovine tendon (Integra Lifesciences, Plainsboro, NJ) combined with 0.044% w/v chondroitin-6-sulphate (Sigma–Aldrich, Germany). The slurry was freeze-dried at −40 °C using a constant cooling technique [23]. The scaffolds were finally crosslinked by immersion in a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and mM N-Hydroxysuccinimide (NHS) for 2 h (Sigma–Aldrich, Germany) using standard protocols from this laboratory [25].

CCP scaffolds were fabricated as previously described using the same freeze-drying technique [23] followed a bi-phasic calcium phosphate immersion process [10]. A pure collagen suspension was prepared using 0.5% w/v microfibrillar type I collagen derived from bovine tendon (Integra Lifesciences, Plainsboro, NJ) and freeze-dried at −40 °C using a constant cooling technique [23]. 7 mm samples with a height of 3 mm were cut from the collagen sheets and pre-hydrated in PBS. The scaffolds were then mineralised using stepwise immersions in solutions of ammonium sodium hydrogen phosphate 0.1 M ($NaNH_4HPO_4$) and calcium chloride ($CaCl_2$) as previously described [10]. The scaffolds were immersed in $NaNH_4HPO_4$ for 22 h, followed by 22 h in $CaCl_2$. This procedure was then repeated once.

2.2. MSC harvesting, seeding and culturing of the scaffolds

Using techniques established in our laboratory [20–22] MSCs were harvested from bone marrow extracted from the femora and tibiae of young-adult Wistar rats and cultured in standard tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Germany) supplemented with 10% foetal bovine serum, 100 U/mL penicillin/streptomycin, 2 mM Glutamax, 1 mM L-glutamine, and 1% non-essential amino acids. Media were changed every 4 days and cells were removed from flasks using trypsin–EDTA. Cell number was calculated using a haemocytometer.

CG and CCP scaffolds were seeded with 200 μL of 2.5×10^6 cell/mL cell suspension using established techniques in our laboratory [20–22] and maintained in DMEM supplemented with 8 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid for 28 days in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

2.3. In vitro characterisation of tissue engineered constructs (TE-CG and TE-CCP)

Following culture for 28 days, the tissue engineered constructs (TE-CG and TE-CCP) were removed and the number of cells remaining on the scaffolds was quantified using a fluorescent DNA assay (Hoechst 33258) following standard protocol [26]. Briefly, DNA was released from the scaffolds using 1 mL QIAzol lysis reagent (QIAGEN, Germany) per scaffold. 30 μL of the digested scaffolds were mixed with 600 μL of a working dye solution (Hoechst 33258; Sigma–Aldrich, Germany). The fluorescence of the samples was measured at 460 nm after excitation at 355 nm in a multilabel counter (Wallac Victor2™ 1420, Perkin–Elmer Life Sciences, Finland) and compared to a standard curve to give cell number.

Collagen and cell distribution in the scaffolds were determined by hematoxylin and eosin (H&E) staining as previously described [8]. After cell culture, the scaffold samples were embedded in paraffin wax with an automatic tissue processor (ASP300, Leica, Germany). All constructs were sectioned longitudinally at a thickness of 10 μm throughout the depth of the sample using a rotary microtome (Leica microtome, Leica, Germany). Each sample was subsequently washed through a graded series of ethanol from 100 to 70% (v/v), and sections were stained in either hematoxylin & eosin, or in alizarin red to evaluate spatial distribution of cells or calcium phosphate, respectively. The slides were then dehydrated and mounted in DPX for observation under light microscopy (data not shown).

2.4. Mechanical testing

Mechanical characterisation of the constructs was performed following 1, 7, 14 and 28 days *in vitro* cultivation using a uniaxial testing system (Zwick Z005 with a 5 N load cell). The mechanical tests were carried out by measuring the diameter of each construct using a digital camera and the image editing software ImageJ. The scaffolds immersed in phosphate buffered saline (PBS) were fixed between two platens in the setup. After the initial contact between the construct and the platens, a pre-load of 0.002 N was applied and compression to 10% strain was performed using a loading rate of 10%/minute. The modulus was calculated as the slope of a linear fit to the stress-strain curve over 2–5% strain.

2.5. Animal model: experimental design

This study was approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, and an animal license was granted by the Irish Government Department of Health (Ref. B100/3967). A total of 90 young-adult male Wistar rats (mean weight 375 g, range 360–395 g) were divided into 5 groups of 18 rats. The individual steps are described in greater detail below but briefly after creation of a 7 mm transosseous calvarial defect, the defect in each group was implanted with either the CG scaffold, TE-CG construct, CCP scaffold, TE-CCP construct, or with no scaffold (empty defect control), and sutured closed. Rats were returned to their cages with half of each group selected at random for sacrifice at either 4 or 8 weeks by carbon dioxide insufflation. All specimens were harvested and subjected to micro-CT before decalcification and histological, histomorphometric and immunohistochemical analysis. Qualitative and quantitative comparisons were made between the tissue engineered construct groups as well as the cell-free and negative control groups.

2.6. Surgical procedure

Under Irish Government license (Ref. B100/3967), all rats were prepared for the surgical procedure by the designated veterinary surgeon. Anaesthesia was induced with intra-peritoneal mixed xylazine hydrochloride 75 mg/kg and ketamine hydrochloride 10 mg/kg and maintained with isofluorane and oxygen delivered through a facemask. The rat was then positioned supine on the operating table and the skin over the head shaved and prepared with 10% aqueous iodine skin solution before draping. A 1.5 cm midline sagittal incision was made over the rat calvarium exposing the perisosteum which was incised and reflected. Using a dental trephine burr drill (Dentalfarm, Torino, Italy), a 7 mm circular transosseous defect was created in the rat parietal calvarium lateral to the sagittal suture under constant