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The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen–glycosaminoglycan scaffold in vivo

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ABSTRACT

This paper demonstrates a method to engineer, in vitro, a nascent microvasculature within a collagen–glycosaminoglycan scaffold with a view to overcoming the major issue of graft failure due to avascular necrosis of tissue-engineered constructs. Human umbilical vein endothelial cells (ECs) were cultured alone and in various co-culture combinations with human mesenchymal stem cells (MSCs) to determine their vasculogenic abilities in vitro. Results demonstrated that the delayed addition of MSCs to pre-formed EC networks, whereby MSCs act as pericytes to the nascent vessels, resulted in the best developed vasculature. The results also demonstrate that the crosstalk between ECs and MSCs during microvessel formation occurs in a highly regulated, spatio-temporal fashion, whereby the initial seeding of ECs results in platelet derived growth factor (PDGF) release; the subsequent addition of MSCs 3 days later leads to a cessation in PDGF production, coinciding with increased vascular endothelial cell growth factor expression and enhanced vessel formation. Functional assessment of these pre-engineered constructs in a subcutaneous rat implant model demonstrated anastomosis between the in vitro engineered vessels and the host vasculature, with significantly increased vascularization occurring in the co-culture group. This study has thus provided new information on the process of in vitro vasculogenesis within a three-dimensional porous scaffold for tissue engineering and demonstrates the potential for using these vascularized scaffolds in the repair of critical sized bone defects.

1. Introduction

The survival and successful engraftment of tissue-engineered (TE) constructs post-implantation relies on the rapid formation of a stable and functional vascular supply [1]. One of the major limiting factors in the field of tissue engineering is insufficient vascularization of TE constructs post-implantation. Early vascularization is particularly important for bone regeneration as bone is a highly vascularized tissue whose regeneration occurs in close spatial and temporal association with a vascular network [2]. In most tissues, cells typically can survive a distance of 200 μm from the nearest capillary network whereby they rely on diffusion for a supply of nutrients and oxygen and for the removal of waste products [3]. However, diffusion is not sufficient in the regeneration of thicker tissues such as bone and thus TE constructs rely on the ingrowth of host vessels for implant success [4]. Blood vessel invasion from the host is thought to occur at a rate of ~5 μm h^{-1} and endothelial cell migration and vessel infiltration into TE constructs is often limited to a depth of several hundred micrometres from the implant surface [2,5]. Even if vessels eventually infiltrate thicker constructs, vascular ingrowth is typically too slow for cells within the interior to survive.

Several strategies for promoting vascularization of TE constructs have been investigated with varying degrees of success. Multimodal approaches are often used to overcome the vascularization problem. These may involve combining gene therapies with cellular therapies, for example transfecting cells to over-express key angiogenic factors such as Ephrin-B2 or vascular endothelial

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growth factor (VEGF) in order to target host cells and enhance neovascularization [2,6]. Other approaches have attempted to combine biomaterials with pre-encapsulated microparticles to allow controlled delivery of pro-angiogenic growth factors [7–9]. These approaches aim to produce a pro-angiogenic construct that can activate and encourage endogenous cells to vascularize the defect site and enhance repair. A disadvantage with this approach is that it relies primarily on an efficient host response, and vessel ingrowth may not occur in time to allow TE construct survival. Another potential approach is to produce a pre-vascularized TE construct in vitro, which will allow faster host integration post-implantation. A significant advantage of this method is that it does not rely on host vessel ingrowth to supply the full construct; it merely requires the anastomosis of host vessels with the pre-engineered vascular structures in the scaffold [4,10–13]. The mechanism by which this anastomosis occurs between implanted vessels and host vasculature has recently been demonstrated [14]. The implanted vessels have been shown to surround nearby host vessels and disrupt underlying host endothelium, leading to the formation of a connected, functional vasculature that links the two networks. This approach of pre-vascularization may therefore be more conducive to both the functionality of the TE construct and to the survival of implanted cells post-implantation. As a result, this approach was selected for investigation within this current study.

Another consideration regarding the vascularization of TE constructs is that the majority of this work has been investigated using primarily hydrogel materials [8,12,13,15–17]. This is a less complex process than vascularizing three-dimensional (3-D) porous scaffolds, as hydrogels provide an environment that is easily manipulated by vascular forming cells. In addition, hydrogels lack the suitable structural properties required for the repair of large, load-bearing bone defects, thus limiting their overall clinical applicability. In contrast, collagen–glycosaminoglycan (CG) scaffolds, which were originally developed for skin regeneration [18], have now been optimized for bone tissue regeneration [18–27]. CG scaffolds have displayed great potential for bone tissue repair due to their ability to promote cell growth and tissue development [20,21,28,29] and we have demonstrated their capacity to repair bone defects in a number of animal models [28,30–32]. However, as with many TE constructs, these too would benefit from improved angiogenic capability in order to facilitate vascularization and successful healing of very large defects post-implantation.

The aim of this study, therefore, was to develop a novel method to engineer, in vitro, a nascent microvasculature within a CG scaffold with a view to overcoming the major issue of graft failure due to avascular necrosis of TE constructs. The approach used within this study employed a co-culture system consisting of human umbilical vein endothelial cells (ECs) and human mesenchymal stem cells (MSCs). Endothelial cells are the main pro-angiogenic cell type and cellular crosstalk with perivascular cells is often crucial for the establishment of functional vasculature that can mature and become stabilized [11,33]. Early studies on the multilineage potential of MSCs demonstrated their ability to differentiate down an endothelial cell lineage [34,35]; however, previous work within our laboratory demonstrated that rat MSCs did not fully differentiate into a mature endothelial cell phenotype and thus were not the ideal cells to vascularize the CG scaffold [36]. More recent evidence strongly supports a perivascular origin for MSCs [37–45] and previous results have demonstrated that MSCs act as perivascular cells to stabilize and maintain vascular structures in vivo [12,38,46,47]. Therefore, in this current study, MSCs were employed in the role of the perivascular cell in co-culture with ECs in order to observe their ability to further promote vessel formation within the CG scaffold. Various co-culture combinations were first investigated in vitro to determine the optimal conditions, e.g. delayed addition of MSCs to EC-seeded scaffolds, which attempts to replicate what occurs in vivo, whereby endothelial cells form the initial vascular structures followed by recruitment of perivascular cells in order to stabilize newly formed vessels. Furthermore, this study aimed to provide additional insight into the crosstalk that occurs between ECs and MSCs during microvessel formation within a 3-D porous scaffold. Finally, the optimal vascularized scaffolds were investigated using a subcutaneous implant model in immunocompromised rats to observe vessel formation, anastomosis of pre-engineered vessels with host vessels and successful vascular perfusion 4 weeks post-implantation.

2. Materials and methods

2.1. CG scaffold fabrication

A CG suspension was produced by blending micro-fibrillar type I bovine tendon collagen (Integra Life Sciences, Plainsboro, NJ) with chondroitin-6-sulphate, isolated from shark cartilage (Sigma–Aldrich, Germany) in 0.05 M acetic acid. This suspension was maintained at 4 °C and blended in a reaction vessel. The CG suspension was lyophilized using a previously described method [22]. Briefly, the suspension was degassed at room temperature. Scaffolds were then freeze-dried at a final freezing temperature of −10 °C and were held constant for 60 min. The ice phase was then sublimated under vacuum (100 mTorr) at 0 °C for 17 h to produce the porous CG scaffold. A final scaffold mean pore size of 325 μm was achieved using this freezing protocol [19,21]. After freezing, scaffolds were dehydrothermally crosslinked at 105 °C under a vacuum of 50 mTorr for 25 h. Prior to use, discs of 4 mm × 6.35 mm were cut using a biopsy punch and chemically crosslinked using 1-ethyl-2-(3-dimethylaminopropyl)carbodiimide (EDAC) in combination with *N*-hydroxysuccinimide (NHS) as previously described [23]. Scaffolds are then stored in sterile phosphate buffered saline (PBS) prior to seeding.

2.2. Cell culture

All culture incubations were carried out at 37 °C with 5% CO_2 and 95% relative humidity. Bone marrow aspirates were obtained from the iliac crest of normal human donors; all procedures were performed with informed consent and approved by the Research Ethics Committee of the National University of Ireland Galway and the Clinical Research Ethical Committee at University College Hospital, Galway [48]. Human bone marrow derived MSCs, isolated by direct plating [48], were cultured in standard tissue culture flasks using low glucose Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma–Aldrich, Ireland). Human umbilical vein ECs were purchased from Lonza, Wokingham, Ltd. and cultured in EGM-2 media (Lonza, UK). Medium was replaced every 3 days and upon reaching 80–90% confluency, cells were passaged using trypsin–EDTA solution. MSCs and ECs were each culture-expanded to passage four.

2.3. Labelling with fluorescent dyes

Red or green fluorescent linker kits (PKH26 and PKH2, respectively, Sigma–Aldrich) were used to facilitate morphological observations within the scaffold. ECs were labelled red and MSCs were labelled green. Labelling was carried out according to the manufacturer's instructions: per 4×10^6 cells, the initial wash (serum-free medium) was in 10 ml, labelling (4 μM dye) was in 1 ml for 5 min and terminated by mixing in an equal volume (1 ml) of serum then 10 ml complete (10% serum) medium; after pelleting,

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