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Aligned nanofibrillar collagen scaffolds – Guiding lymphangiogenesis for treatment of acquired lymphedema

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ABSTRACT

Secondary lymphedema is a common disorder associated with acquired functional impairment of the lymphatic system. The goal of this study was to evaluate the therapeutic efficacy of aligned nanofibrillar collagen scaffolds (BioBridge) positioned across the area of lymphatic obstruction in guiding lymphatic regeneration. In a porcine model of acquired lymphedema, animals were treated with BioBridge scaffolds, alone or in conjunction with autologous lymph node transfer as a source of endogenous lymphatic growth factor. They were compared with a surgical control group and a second control group in which the implanted BioBridge was supplemented with exogenous vascular endothelial growth factor-C (VEGF-C). Three months after implantation, immunofluorescence staining of lymphatic vessels demonstrated a significant increase in lymphatic collectors within close proximity to the scaffolds. To quantify the functional impact of scaffold implantation, bioimpedance was used as an early indicator of extracellular fluid accumulation. In comparison to the levels prior to implantation, the bioimpedance ratio was significantly improved only in the experimental BioBridge recipients with or without lymph node transfer, suggesting restoration of functional lymphatic drainage. These results further correlated with quantifiable lymphatic collectors, as visualized by contrast-enhanced computed tomography. They demonstrate the therapeutic potential of BioBridge scaffolds in secondary lymphedema.

1. Introduction

Purposeful growth of vessels and nerves plays a major role in regenerative processes in mammals. In some conditions this growth is impaired, leading to disease perpetuation. Currently, there is no established method to provide mechanical support for the directed growth of vessels or nerves after initial sprouting. We have previously reported, in the context of vascular disease, that parallel-aligned collagen nanofibrillar scaffolds (BioBridge) provide

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nano-scale spatial guidance cues for alignment and migration of human microvascular blood endothelial cells along the direction of the nanofibrils, as well as induce arteriogenesis in a preclinical model of peripheral arterial disease [1–3].

Acquired (secondary) lymphedema is another pathology directly associated with impaired regeneration of damaged vasculature. This condition leads to accumulation of proteins, cell debris and fluid in the tissues due to the functional impairment of the lymphatic system. It is characterized by chronic swelling that tends to worsen with time [4]. In some cases, it can also be caused by the absence or destruction of lymph nodes. These structures are functionally relevant, as they not only play a fundamental role in immune surveillance but also participate in fluid homeostasis, thereby actively reducing edema [5]. In developed countries, lymphedema often occurs as a consequence of cancer therapeutics. The treatment of many cancers involves surgical extirpation of lymph nodes and/or radiotherapy [6,7]. It is estimated that 20–50% of these cancer patients develop lymphedema [4,7,8]. Present management is palliative and includes lifelong manual lymphatic drainage, compression therapy and, sometimes, wide debulking through liposuction [9]. Secondary lymphedema may occasionally respond to non-palliative surgical interventions. Among several currently employed treatment procedures, free flaps containing lymph nodes seem to provide clinical benefits [10–12]. Engrafted lymph nodes are potent inducers of lymphangiogenesis [13]. However, in some patients with extensive operative scars, irradiated tissues, or late fibrotic stages of lymphedema, the lymphangiogenic response to vital lymph node flaps can sometimes be insufficient to bridge the scar area and produce a clinical improvement [13].

To address this limitation, we examined the effects of the implantation of aligned nanofibrillar collagen scaffolds in a newly developed porcine model for secondary lymphedema. The purpose of the study was to examine the role of BioBridge scaffolds in facilitating the ingrowth of newly formed lymphatic vessels across the zone of scar tissue. Herein we demonstrate that implantation of the scaffolds, with or without simultaneous transplantation of autologous lymph node fragments, significantly improves lymphatic regeneration in comparison with control interventions.

2. Materials and methods

2.1. Fabrication and characterization of aligned nanofibrillar collagen scaffolds

BioBridge resorbable collagen scaffolds (Fibralign Corporation, Union City, CA, USA) were produced according to a process based on the technology developed for liquid crystal display manufacturing [14,15] that is suitable for lyotropic liquid crystal materials, as described previously [2,3]. In brief, purified monomeric bovine type I collagen solution in a liquid crystal state [16,17] was sheared onto a plastic substrate [18] to produce one micron thin membrane formed of about 30 nm diameter parallel-aligned fibrils. The membrane was delaminated from the plastic substrate and converted into a scaffold using liquid-air surface tension [19]. Collagen scaffolds were further cross-linked using 0.25 mg 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and 0.28 mg sulfo-N-hydroxysuccinimide (NHS) per mg collagen scaffold, which corresponded to 1 mg/ml EDC per 1.1 mg/ml NHS working concentration, followed by 4 washes in phosphate-buffered saline (PBS) and 2 washes in deionized water [20]. For quantification of scaffold degradation, the scaffolds were cross-linked at 0.2, 0.5, 0.8 and 1 mg/ml EDC concentrations, with NHS concentration adjusted accordingly. Enzymatic degradation of the scaffolds was performed by incubation in bacterial collagenase (50 U/ml) for 4 h. The degraded collagen in solution was quantified by reacting with 2% ninhydrin. The capillarity of the scaffold was measured for 13-mm long samples of BioBridge placed on hydrophobic teflon surface in a horizontal position. A syringe with 25G needle was used to deliver approximately 0.1 ml of green food coloring dye to the end of the scaffolds. Time points of 2 and 4 min were taken to analyze how far the dye had traveled along each sample. The capillary propagation of the green coloring dye was measured by camera in green channel with the baseline of the initial white color of the dry BioBridge scaffold.

For *in vitro* studies, primary human microdermal lymphatic endothelial cells (LECs, 0.5×10^6 cells/ml, Lonza, Allendale, NJ, USA) were seeded onto the scaffolds after sterilization using 70% ethanol. Characterization and quality control of these thread-like nanofibrillar collagen scaffolds (12–15 cm long and ~0.3 mm in diameter) was performed using conventional atomic force microscopy and scanning electron microscopy, as described previously [2].

2.2. Heparin conjugation of BioBridge scaffolds

To assess the role of exogenous growth factors attached to the collagen scaffolds in the tissues, a number of scaffolds were first cross-linked and conjugated with heparin following a modified procedure of Steffens et al. [21]. Activation of 1 mg heparin was performed using 1 mg of EDC [20] and 1.1 mg NHS (pH 6) during 30 min at room temperature. The pH of this reagent solution was then adjusted to 7.4 with NaOH. It was then added to collagen scaffolds in the proportion of 0.25 mg EDC/NHS reagent solution per 1 mg collagen. Incubation occurred during 4 h at room temperature and under gentle shaking. Thereafter, the heparinized collagen scaffolds were washed four times in 24 h with PBS and five times in 24 h with deionized water. The scaffolds were further sterilized by incubation in 70% ethanol for 1 h at room temperature both for the *in vitro* and for the *in vivo* studies.

2.3. VEGF-C attachment to BioBridge scaffolds

To immobilize VEGF-C to heparin, VEGF-C molecules (R&D Systems, Minneapolis, MN, USA) were exposed to the heparin bound on collagen scaffolds. Sterile heparinized scaffolds were incubated in sterile VEGF-C solution (0.5–1.5 μg/ml in PBS for initial studies, and 1.5 μg/ml was selected for subsequent experiments) for 24 h at 4 °C [21] and then rinsed three times in PBS. To determine VEGF-C release kinetics, scaffolds conjugated with VEGF-C were whether incubated with 200 μl bacterial collagenase solution (1 U/ml) or with PBS at 37 °C. At specific time intervals, the incubation solutions were collected and replaced with fresh aliquots for further incubation. The VEGF-C content in the collected samples was measured using VEGF-C enzyme linked immunosorbent assay (ELISA) according to manufacturer's instructions (R&D Systems).

2.4. Bioactivity of VEGF-C-conjugated BioBridge scaffolds

A modified wound-healing chemotaxis assay was performed to verify the bioactivity of the VEGF-C released from the scaffolds. LECs (Lonza) were cultured to confluence in growth media (EGM-2MV, Lonza) within wound healing chambers having a predefined gap space (Ibidi, Verona, WI, USA), according to our previous studies [22]. Wound healing chambers were removed and the media were changed to basal media (EBM, Lonza) in the presence of 5% fetal bovine serum without growth factor supplement. Sterile 4-cm scaffolds conjugated with VEGF-C (delivering 59.2 ng VEGF-C) or heparin (control) were placed into 400 μl media. Cell migration into the predefined gap was analyzed after 18 h using Image J (open source, National Institutes of Health, Bethesda, USA). A positive assay control consisted of soluble VEGF-C (100 ng/ml, R&D