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In Vivo evaluation of adipogenic induction in fibrous and honeycomb-structured atelocollagen scaffolds

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ABSTRACT

Nowadays, soft tissue restoration techniques are mainly focused on volume regeneration instead of function recovering. So far, autologous fat transplant has been the most popular method although its multiple reported problems like volume and function loss. Adipose tissue engineering therefore emerges as a solution for development of biological substitutes for soft tissue which promotes not only volume regeneration but also function restoration with minimal consequences. Here we tested fibrous-structured atelocollagen (FSA) scaffolds and honeycomb atelocollagen (HCA) scaffolds for their ability to induce adipogenesis *in vivo*. Implants were subjected to histological and immunohistochemical assessment after 1, 2, and 4 weeks of implantation. Our studies showed that FSA scaffolds induced *in vivo* a markedly adipogenic response, whereas an acute inflammatory process was observed at HCA scaffolds without tissue regeneration detected within them. Our histological findings concerning FSA scaffolds clearly showed the presence of adipose-like tissue surprisingly composed by a mixture of brown-like and white-like adipocytes at week 2 whereas only white-like adipocytes at week 4. Subsequent positive Pax7 immunostaining at weeks 1 and 2 suggested the existence of a common myogenic progenitor shared by brown-like and white-like adipocytes observed. Then, in this work we present FSA scaffolds as a promising structure for brown and white adipose tissue engineering.

1. Introduction

Contour defects caused by tumor resection, trauma or congenital abnormalities have represented an interesting challenge for health sciences. Until recently, restoration strategies of natural tissue were not often the primary goal in reconstruction; rather, restoration of soft tissue aesthetic function was targeted in order to minimize anxiety and negative psychological feelings associated with disfigurement [1,2].

Autologous fat tissue has been so far the most used repairing material for soft tissue defects. Although its use is logical in its approach, this method has not been consistently successful in patients. When autologous fat tissue is transplanted from one location to the defect site, the common occurrence is significant resorption of transplanted tissue over time, resulting in 40–60% of graft volume loss [3–5]. Then, only small defects can be corrected with this repairing material, and even these limited applications require repeated procedures to maintain the desired volume [3]. Adipose Tissue Engineering (ATE) therefore emerged like a promising technique for development of biological substitutes which promote not only volume regeneration but also function restoration of soft tissues with minimal rejection.

Tissue regeneration by ATE is achieved not only by controlling cell sources and biochemical environment but also through suitable scaffolds, which are required for support, migration and proliferation of anchorage-dependent adipocytes and preadipocytes [2]. Scaffolds for ATE may be either synthetic or natural, regarding the biomaterial chosen for their construction. Up to date, synthetic scaffolds based on e.g. polylactic acid, hydrogels and poly(lactic-co-glycolic acid) have been used successfully in ATE [6–8]. Nevertheless, they have some disadvantages such as lack of mechanical integrity and stiffness mismatch compared to native tissue, lack of biodegradability or generation of acidic microenvironments upon degradation with eventual inflammatory reactions and implant rejection [9–11]. Therefore, biocompatibility and biomimeticity hold by natural scaffolds become a main advantage over aforementioned synthetic structures. Several natural biomaterials like e.g. fibrin, gelatin, hyaluronan and matrigel have been studied for such purpose [12–14]. However, collagen remains as the most widely used scaffold natural material for ATE given its ability to support adipogenesis from several cell sources [15–18]. An alternative collagen-based biomaterial with extremely low antigenicity is produced by telopeptide

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removal from natural collagen molecule and it is known as atelocollagen which has been used mainly for e.g. cartilage restoration, bone and connective tissue engineering [19–21]. Nevertheless, preliminary works of this group showed that atelocollagen scaffolds also enhanced bone and adipose tissue formation [22,23]. Therefore, in this study we investigate and systematically evaluate *in vivo* adipogenic induction ability of fibrous-structured atelocollagen (FSA) scaffolds and honeycomb atelocollagen (HCA) scaffolds as a first approach concerning adipose tissue restoration.

2. Materials and methods

2.1. Animals

Twelve 4-week-old male severe combined immunodeficient (SCID) mice were used in this study in accordance with the Guidelines for Animal Experiments at Graduate School of Medicine and Dentistry Okayama University, Japanese Government Animal Protection and Management Law (No. 105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

2.2. Intramuscular implantation and explantation

FSA and HCA scaffolds (Fig. 1) of $3 \times 3 \times 2$ mm^3 were used for this work (Koken, Japan). SCID mice were subjected to intramuscular anesthesia with Ketamine (Fuji Chemical Industry, Japan) and Dormitol (Meiji Seika, Japan). The skin of the legs was shaved and disinfected with 70% alcohol and iodine. Subsequently, the scaffolds were implanted into intramuscular pockets made by blunt dissection between *tibialis anterior* and *soleus* muscles. The animals were sacrificed with an overdose of ether at 1, 2, and 4 weeks after implantation. For hematoxylin–eosin (H&E) staining, specimens and surrounding tissues were removed, fixed by 4% paraformaldehyde. Then, the samples were embedded in paraffin, sectioned at 4 μm in thickness and finally stained.

2.3. Adipose tissue staining by Oil Red O

To assess adipogenic differentiation, lipid deposits were visualized through Oil Red O staining. Briefly, frozen sections were prepared by embedding implants in Tissue Tek (Sakura Finetek, USA) followed by specimen freezing in liquid nitrogen bath and trimming. Then, they were dried with warm air and embedded in 60% propanol for 2 min. Subsequently, sections were stained with 0.3% Oil Red O solution at 37 °C for 7 min, provided by Kayayama Chemical (Japan). The dye was washed out with 60% propanol for 2 min. Thereafter, sections were rinsed in distilled water, stained with Mayer's hematoxylin and mounted with Aqueous Mounting Medium.

2.4. Transmission Electron Microscopy (TEM)

TEM of implanted samples was performed by negative staining. Specimens and surrounding tissues were pre-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde solution, post fixed with 1% osmium tetroxide dehydrated with a series of alcohols and infiltrated with resin. The resin sample block was trimmed, thin-sectioned thickness of 70 nm, and collected on formyar-coated copper grids. Before examining under the TEM, these grids were stained by uranyl acetate and lead citrate, followed by blotting with a filter paper and air drying. Samples were examined with Philips CM10 at 200 kV.

2.5. Immunohistochemical staining of CD34 and Pax7

The sections were immunostained with monoclonal antibodies against CD34 (abcam, UK) and Pax7 (Santa Cruz Biotechnology, USA) using Vectastain ABC Rat Kit method (Adivin-Biotin-peroxidase Complex, USA). The main steps were as follows: (1) inactivation of endogenous peroxidase with hydrogen peroxide in methanol for 30 min; (2) the activation of antigenicity was done with microwave treatment before blocking nonspecific protein binding with rabbit normal serum and horse normal serum, respectively, for 10 min at room temperature; (3) incubation with the primary antibody at 4 °C overnight. The optimal dilutions of each primary were (CD34) 1:100 and (Pax7) 1:200; (4) incubation with anti-rat IgG (1:200) and anti-mouse IgG (1:200), respectively, for 30 min; (5) incubation with ABC at a dilution of 1:50 for 30 min; and (6) treatment with DAB color development and counterstaining with Mayer's hematoxylin.

3. Results

3.1. Histological examination

In Vivo response to FSA and HCA scaffolds at weeks 1, 2 and 4 was initially studied through H&E staining. As it is observed in Fig. 2, low or no inflammatory reaction was noticed at FSA scaffold periphery and bulk. At week 1, spindle cells attached to FSA scaffold fibers were stained whereas at week 2 numerous rounded cells were colored. Some of them contained multiple cytoplasmic lipid droplets surrounding the nucleus, as it was confirmed by Oil Red O assay (Fig. 3). Strikingly, H&E assay revealed at week 4 the absence of such cells as well as the presence of white-like adipose tissue, composed by white-like adipocytes with single large cytoplasmic lipid droplets and flattened stained nucleus located on the cell periphery. Similar responses were observed at all FSA specimens.

Oppositely, no tissue regeneration at HCA specimens in addition to an acute inflammatory response at weeks 2 and 4 was observed (Fig. 4). Giant bodies stained at week 4 might indicate scaffold

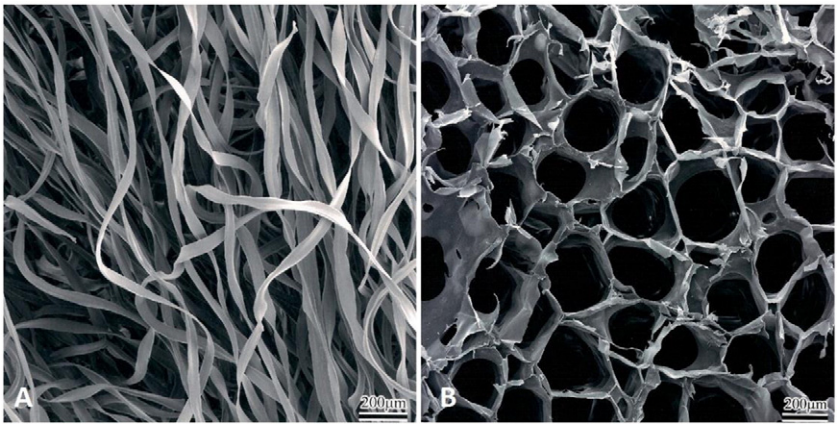

Fig. 1. SEM micrograph showing the (A) fibrillar microstructure of FSA scaffolds; and (B) honeycomb-like structure of HCA scaffolds.