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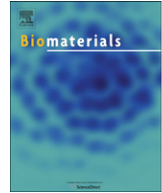

The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle

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ABSTRACT

In the pursuit of a transplantable construct for the replacement of large skeletal muscle defects arising from traumatic or pathological conditions, several attempts have been made to obtain a highly oriented, vascularized and functional skeletal muscle. Acellular scaffolds derived from organ decellularization are promising, widely used biomaterials for tissue engineering. However, the acellular skeletal muscle extra cellular matrix (ECM) has been poorly characterized in terms of production, storage and host–donor interactions. We have produced acellular scaffolds at the whole organ scale from various skeletal muscles explanted from mice. The acellular scaffolds conserve chemical and architectural features of the tissue of origin, including the vascular bed. Scaffolds can be sterilely stored for weeks at +4 °C or +37 °C in tissue culture grade conditions. When transplanted in wt mice, the grafts are stable for several weeks, whilst being colonized by inflammatory and stem cells. We demonstrate that the acellular scaffold *per se* represents a pro-myogenic environment supporting *de novo* formation of muscle fibers, likely derived from host cells with myogenic potential. Myogenesis within the implant is enhanced by immunosuppressive treatment. Our work highlights the fundamental role of this niche in tissue engineering application and unveils the clinical potential of allografts based on decellularized tissue for regenerative medicine.

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1. Introduction

A hallmark of biomaterials, an evolving concept recently reviewed by Williams [1], is the *in vivo* interaction with the host's biological components. The various strategies used to obtain biomaterials for tissue engineering (TE) applications include allografts produced by the recellularization of previously decellularized tissues. Such allografts are widely used, especially for *in vivo* replacement of tissues and organs whose spatial organization and biochemical composition are complex. The decellularization of an explanted tissue can be achieved through various approaches, all of which eliminate the cellular compartment and leave a spatially and chemically preserved ECM [2]. These approaches have been successfully used to produce transplantable vessels, skin and cardiac tissues [3–5]. The ECM is, by definition, nature's ideal biological scaffold material. Indeed, it is specifically synthesized by the resident cells of each tissue and is obviously biocompatible since host cells produce their own matrix. The ECM also provides a supportive medium for blood or lymphatic vessels and for nerves. As reviewed in detail by Badylak, the ECM possesses all of the characteristics of the ideal tissue-engineered scaffold or biomaterial [6]. However, since complex three-dimensional organization of the structural and functional molecules that make up the ECM has not yet been fully characterized, synthesis of this material cannot be fully reproduced in the laboratory. ECM can be obtained from allogenic or syngenic donors, which may pose the histocompatibility and resorption problems that are typical of allografts. ECM scaffold materials that are resistant to degradation appear to elicit a pro-inflammatory macrophage (M1)-like response, whereas the anti-inflammatory (M2) macrophage phenotype prevails in native ECM scaffold materials, which are consequently readily degraded [7].

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Recent advances in skeletal muscle TE have opened new perspectives for the replacement of this tissue in common clinical applications, such as traumatic injury, extended tissue ablation or denervation [8]. Key issues in skeletal muscle TE are the composition and architecture of the ECM of this tissue, which is characterized by a highly ordered and hierarchical organization of muscle fibers. Several attempts have been made to address this issue by seeding fibrillar matrices with myoblasts or myogenic stem cells [8]. Skeletal muscle constructs have been obtained by using the ECM deriving from decellularized tissue. In particular, acellular muscles have been used by Borschel et al. as a substratum for C2C12 myoblast cultures, thus producing constructs capable of longitudinal contractile force upon electrical stimulation [9]. The same authors have obtained vascularized constructs by culturing C2C12 cells in a fibrinogen hydrogel contained within cylindrical silicone chambers and transplanting them around the femoral vessels in isogeneic adult recipient rats [9]. Patches of homologous muscle acellular matrix seeded with autologous myoblasts have been used to repair abdominal wall defects in rodents [10]. Minced muscle replaced in its bed has been shown to effectively regenerate fibers, though such fibers are spatially disorganized probably owing to the loss of ECM spatial orientation [11]. ECM and growth factors deeply affect various aspects of cell behavior, including survival, proliferation and differentiation, and are therefore key issues in TE applications [12]. In both pathological conditions, such as Duchenne's muscular dystrophy, and healthy conditions, such as after strenuous exercise, skeletal muscle tissue is maintained and repaired through regeneration [13]. As regeneration is strongly influenced by ECM and growth factors, ECM extracts have been used to coat culture dishes to induce muscle differentiation *in vitro* [14]. The role of growth factors in muscle regeneration has also been investigated in depth both *in vitro* and *in vivo* [15–17]. In order to promote host–donor tissue integration and vascularization, engineered factor VIII-releasing synthetic fibers have been used as a scaffold for myoblast cell culture before transplantation of the constructs into murine recipients [18].

While the generation of a whole functional, bioengineered rodent heart has demonstrated that it is possible to produce highly complex whole organs *in vitro* [5], a functional, anatomically defined skeletal muscle transplantable *in vivo* has not yet been produced. Few studies have evaluated and characterized the host immune response to non-autologous ECM scaffold materials. To this purpose, we generated an acellular scaffold from skeletal muscle and transplanted it into syngeneic hosts. This approach allowed us to extensively characterize histocompatibility, bioactivity and integration of acellular scaffolds in a murine model.

2. Materials and methods

2.1. Animals

Adult sex-matched BALB/C mice were used throughout this study as both donors and hosts. For specific experiments, adult nude athymic mice (strain NU/NU Crl:NU-Foxn1nu, Charles River, Milano, Italy) were used. Mice were treated according to the guidelines of the Institutional Animal Care and Use Committee. Donor animals were sacrificed before skeletal muscle removal, while host animals were anesthetized before muscle dissection and replacement with the acellular scaffold. The transplantation procedure is described in detail below. Immunosuppressive treatment consisted of 1 μl/g body weight of a 100 mg/ml Cyclosporin A (CSA) solution in peanut oil injected i.p., as previously described [19]. Vehicle was used for controls.

2.2. Decellularization of skeletal muscle

Freshly dissected *Tibialis anterior* (TA) and *Extensor digitorum longus* (EDL) were immediately incubated in sterile 1% SDS in distilled water respectively for 24 and 48 h, at RT under slow rotation. At least 10 ml of SDS solution was used for each pair of muscles. At the end of the decellularization procedure, the muscles were thoroughly washed by means of 3 incubations lasting 30 min each in sterile PBS. Decellularized scaffolds were used on the same day as they were produced or were stored for specific experiments.

2.3. Experimental design and surgical procedure

TA acellular scaffolds were used to replace TA of inbred, age- and sex-matched wt mice, unless otherwise indicated. The grafts were subsequently dissected from the host one, two, three and four weeks following transplantation. The production of an adequate number of acellular scaffolds was planned according to the surgical procedures. Following anesthesia with Avertin A (tribromoethanol and 2-methilbutanol from Sigma–Aldrich, St Louis, MO, USA), the skin over the left TA was sterilized and the hair removed. An incision was created in the skin layer and the dermal flap opened to expose the TA epimisium, which was in turn carefully lifted on one side to allow dissection of the underlying TA. The distal tendon and a small fragment of the muscle inserted proximally on the tibia were left in place as substrata for suturing the grafted material. The acellular scaffold was properly oriented and sutured with silk thread (USP 5-0 TR-17 black silk 45 cm). The scaffold was then covered by the epimisium, and the latter sutured with silk thread. Lastly, the skin flap was used to cover the wound and closed with 3–4 stitches of silk thread (USP 3-0 TT-26 black silk 45 cm). The filmed surgical procedure is included in Supplementary material.

2.4. Histological and histochemical analysis

The TA was dissected, embedded in tissue freezing medium (Leica, Wetzlar, Germany) and frozen in liquid nitrogen-cooled isopentane. Cryosections (8 μm, unless otherwise specified) were obtained from the mid-belly of the graft using a Leica cryostat. For histological analysis, the sections were stained with hematoxylin and eosin using standard methods and Masson's trichrome staining kits (Sigma).

Esterase staining was adapted from Davis [20] as previously reported [21]: cryosections of each muscle were incubated for 5 min in a staining solution containing: 3 mg alpha-naphthyl acetate, 0.375 ml acetone, 6.25 ml 0.2 M sodium phosphate and 0.4 ml of a solution containing equal volumes of 2% pararosaniline (Sigma) and 2% sodium nitrite. Photomicrographs were obtained using an Axioscop 2 plus system equipped with an Axiocam HRc (Zeiss, Oberkochen, Germany) at 1300×1030 pixel resolution.

2.5. Immunofluorescence analysis

Transverse cryosections were fixed in 4% paraformaldehyde for 10 min at room temperature. After incubation with 1% BSA (Sigma) for 30 min, the samples were incubated with a 1:100 dilution in 1% BSA of polyclonal anti-laminin or anti-fibronectin Ab (Sigma), followed by incubation with 0.5 ug/ml Hoechst 33342 (Sigma). A 1:500 dilution in BSA of anti-mouse-Alexa 488 Ab was used to detect endogenous IgG on cryosections of the grafted material, while a 1:500 dilution in BSA of anti-rabbit-Alexa 488 or anti-rabbit-Alexa 568 Ab was used to detect the polyclonal anti-laminin or anti-fibronectin Abs above. An aliquot of 25,000 cells from flow cytometry experiments was cytocentrifuged and used for the immunofluorescence analysis. To detect PW1 (recently highlighted as a stem cell marker that specifically identifies stem cells with myogenic potential [22,23]) expression, we used a 1:2000 dilution in 1% BSA of a polyclonal anti-PW1 custom-made Ab, followed by incubation with a 1:500 dilution in 1% BSA of anti-rabbit-Alexa 568 (Molecular Probes, Eugene, OR, USA). Pre-immune serum was used for the negative control. Hoechst was used as above to counterstain cell nuclei. Photomicrographs were obtained by means of an Axioskop 2 plus system (Zeiss) or a Leica Leitz DMRB microscope fitted with a DFC300FX camera for confocal analysis (Leica).

2.6. Dot blot analysis

Three types of samples were analyzed with this approach: freshly prepared acellular scaffolds, acellular scaffolds stored for two weeks at +4 °C in PBS, and acellular scaffolds stored for two weeks at +37 °C (in a 5% CO_2 atmosphere) in DMEM. For each type of sample, four scaffolds were pooled and homogenized in 320 μl of Laemli sample buffer. For each sample, 1–4 μl were then spotted on a nitrocellulose membrane. The aspecific binding sites were blocked with 5% milk in TBST for 30 min. The membrane was rinsed for 5 min with TBST and incubated with Rabbit anti-laminin antibody (Sigma) diluted 1:1000 in 5% BSA/TBST for 30 min at RT. The membrane was then rinsed 3 times, for 5 min each, in TBST and incubated it with HRP goat anti-rabbit antibody (Biorad) diluted 1:10000 in 1% milk in TBST for 30 min at RT. Following extensive rinsing in TBST, ECL was developed with Super signal west Pico chemiluminescent substrate (Pierce).

2.7. Flow cytometric analysis

The graft, the contralateral TA and a TA from a healthy mouse that did not undergo surgery were separately dissected, minced and digested in a solution containing 50 U/ml type II collagenase (Sigma), 35 U/ml type IV hyaluronidase (Sigma) and 1 mg/ml collagenase/dispase in phosphate buffered solution (PBS) for

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