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Homologous muscle acellular matrix seeded with autologous myoblasts as a tissue-engineering approach to abdominal wall-defect repair

Maria Teresa Conconi, Paolo De Coppi, Silvia Bellini, Gabriella Zara, Morena Sabatti, Maurizio Marzaro, Giovanni Franco Zanon, Pier Giorgio Gamba, Pier Paolo Parnigotto, Gastone Giovanni Nussdorfer*

Section of Anatomy, Department of Human Anatomy and Physiology, University of Padova, Via Gabelli 65, I-35121 Padova, Italy

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Abstract

Myoblasts were obtained by culturing in vitro, single muscle fibers, isolated by enzymatic digestion from rat *flexor digitorum brevis*, and their phenotype was confirmed by myogenic differentiation factor, myogenic factor-5, myogenin and desmin. Cultured myoblasts were harvested and seeded on patches of homologous acellular matrix, obtained by detergent-enzymatic treatment of abdominal muscle fragments. Myoblast-seeded patches were inserted between *obliqui abdominis* muscles on the right side of 1-month-old rats, while non-seeded patches were implanted on the left side. Thirty days after surgery, non-seeded patches were completely replaced by fibrous tissue, while the structure of myoblast-seeded patches was well preserved until the 2nd month. Seeded patches displayed abundant blood vessels and myoblasts, and electromyography evidenced in them single motor-unit potentials, sometimes grouped into arithmic discharges. Ninty days after implantation, the thickness of myoblast-seeded patches and their electric activity decreased, suggesting a loss of contractile muscle fibers. In conclusion, the present results indicate that autologous myoblast-homologous acellular muscle matrix constructs are a promising tool for body-wall defect repair, and studies are under way to identify strategies able to improve and maintain the structural and functional integrity of implants for longer periods.

Keywords: Bioprosthesis; Abdominal wall defects; Satellite cells; Acellular matrix

1. Introduction

Compelling evidence indicates that satellite cells are committed myogenic progenitors that mediate both postnatal growth and regeneration after injury of muscles [1–3]. Satellite cells reside beneath the basal lamina of adult skeletal muscles, accounting for 2–5% of sublaminal nuclei, and are normally mitotically quiescent. Upon activation satellite cells, now called myogenic precursor cells, undergo multiple rounds of division before their terminal differentiation [4,5].

One of the strategies for muscle tissue engineering involves the harvesting of satellite cells, their expansion in vitro, and their subsequent autologous implantation in vivo into the sites requiring repair or replacement. One of the main obstacles in the formation of new muscle tissue is the lack of an adequate support for expanded satellite cells. To overcome this obstacle, many research groups are trying to develop adequate synthetic and biological delivery systems for implanted cells.

Synthetic materials, such as Dacron [6] and polytetrafluoroethylene [7], have been used to repair congenital muscle defects, e.g. onfalocele and gastroschisis [8]. However, all these materials do not allow cell ingrowth and do not follow the host development. Evidence has been provided that biological

*Corresponding author. Tel.: +390498272317; fax: +390498272319.
E-mail address: gastone.nusdorfer@unipd.it (G.G. Nussdorfer).

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materials can support in vivo and in vitro cell adhesion and proliferation: acellular matrices are remodeled in living tissues and can function as bladder, urethra, and small bowel substitutes [9–11]. However, previous results showed that homologous muscle acellular matrix is not per se able to repair defects of abdominal wall in rabbits and is rapidly transformed into fibrous tissue [12]. The presence of autologous cells was found to improve the biocompatibility of the matrices used to repair artificially created defects of dorsal muscle wall in the rat, but although the structure was well preserved until the 4th week, newly formed myocytes were not visible inside the implants [13].

In light of these considerations, it seemed worthwhile to ascertain whether better results can be achieved by the use of cell-matrix constructs obtained in vitro by seeding myoblasts on the scaffolds.

2. Materials and methods

2.1. Animals and reagents

Male Sprague–Dawley rats were purchased from Charles-River (Como, Italy), and the experiment protocol was approved by the local Ethics Committee for Animal Studies and the Italian Health Ministry, and followed the Italian Law for Animal Care (D.L.G.S. 116/92).

Horse serum (HS), chick embryo extract (CEE), fetal calf serum (FCS) and TRIzol reagent were provided by Invitrogen Italia (Milan, Italy). Type-I collagenase was obtained from Worthington Biochemicals (Lakewood, NJ), Matrigel® from Becton-Dickinson (Bedford, MA), monoclonal anti-myogenic differentiation factor (MyoD) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), glutaraldehyde from Merck (Darmstadt, Germany), Zoletil from Laboratories Virbac (Carros, France), and Rompun from Bayer (Leverkusen, Germany). Dulbecco's modified Eagles medium (DMEM), antibiotic and antimycotic solution (AF), bovine serum albumin (BSA), phosphate buffered saline (PBS), and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Muscle acellular matrices

Abdominal muscles were excised from rats, rinsed four times in PBS (containing 1% AF), and then treated as previously described [13]. Briefly, the samples were processed twice as follows: distilled water for 72 h at 4 °C, 4% sodium deoxycholate for 4 h, and 2000 kU DNase-I in 1 M NaCl for 3 h. Matrices were stored in PBS at 4 °C until use. The absence of cells was checked histologically (hematoxylin-eosin staining).

2.3. Myoblast isolation and culture

Myoblasts were isolated from single muscle fibers, as previously detailed [14]. Rats were anesthetized with Zoletil (35 mg/kg) and Rompun (2 mg/kg), and the *flexor digitorum brevis* was removed by microdissection. The muscle was washed in DMEM, and incubated for 2 h at 37 °C in 35-mm dishes, containing 0.2% type-I collagenase. Then tissue was transferred in to plates with medium composed of DMEM added with 10% HS, 1% AF and 0.5% CEE ultrafiltrate. Under a transilluminating microscope, single muscle fibers were isolated by repeated pipetting. Fibers were then plated in Matrigel (1 mg/ml)-precoated dishes, and incubated at 37 °C in an atmosphere of 95% air-5%CO_2. Myoblasts migrating from myofibers were cultured in proliferation medium (DMEM, containing 20% FCS, 10% HS, 1% AF and 1% CEE). The myogenicity of the single fiber-derived cells was confirmed by inducing their differentiation with a low-serum medium (DMEM, containing 2% HS, 1% AF and 1% CEE).

2.4. Immunocytochemistry

To confirm the purity of the cultured myoblasts, an immunocytochemical analysis was performed using the muscle-specific anti-MyoD antibody. Cells ($5 \times 10^3/cm^2$) obtained from primary cultures were seeded in chamber slides (Nunc, Wiesbaden, Germany) and grown for 3 days. The cultures were fixed with 2% paraformaldehyde, permeabilized using 0.2% Triton X-100 in PBS, washed three times with PBS, and treated with 10% normal goat serum in PBS, as blocking reagent. The cells were then incubated with the primary antibody (1:100 dilution in 1% BSA-PBS), and then labeled with avidin–biotin amplified immunoperoxidase method, using the Large Volume Dako LSAB Peroxidase Kit (Dako, Glostrup, Denmark). Counterstaining was performed by hematoxylin. The samples were mounted and examined under a light Laborlux-S Leitz microscope. Negative controls were carried out by similarly treating cultures and omitting the primary antibody.

2.5. Reverse transcription (RT)-polymerase chain reaction (PCR)

Total mRNA was extracted from 5×10^6 cells, using TRIzol reagent, and reverse transcribed using MultiScribe RT (Applied Biosystems, Monza, Italy) and Random Hexamers as primers for the first strand cDNA synthesis. The amplification of the resulting cDNA was carried out using specific primers. Briefly, in a thermal cycler (I-Cycler; Bio-Rad, Milan, Italy), after an initial denaturation step at 95 °C for 10 min, we used a denaturation step at 95 °C for 30 s, an annealing step at 60 °C for 45 s, and an extension step at 72 °C for 45 s