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Short Communication
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Muscle Remodelling on the Basis of Extracellular Matrix Seeded with Cells

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Short Communication

Components of Extracellular Matrix (ECM) incl. basal laminas, connective tissue fibers, multiadhesive glycoproteins, proteoglycans play a key role in maintaining the structural integrity of tissues, organs and organisms. In addition to a supportive function, ECM allows cell adhesion and regulates cell behaviour through binding cell membrane receptors (e.g. integrins), accumulating bioactive growth factors and altering local tissue stiffness [1,2]. Assembly of ECM components in mammalian organs is highly ordered and currently available artificial matrices and constructs do not correspond to complexity of biological systems. Decellularization procedure allows to obtain a biostroma while retaining organ microstructure and original composition of structural and bioactive molecules that support cell attachment and organisation. Although acellular scaffolds are immunologically inert and maintain organ shape and size, after transplantation into the body they support regeneration only to a certain extent because of the lack of viable cells. On the other hand, grafts coated with living cells improved their integration into the recipient tissues. Recently several groups established a proof of concept for utilization of decellularized bioscaffold reseeded with cells in reengineering of complex bioartificial organs like the heart [3], lung [4] or liver [5]. Such organs are transplantable and functional as demonstrated by Uygun et al. 2010 in liver grafts or by [6] *in veins*.

Skeletal muscles lost in severe injuries may be replaced by autografts in reconstruction surgery; unfortunately, the availability of a tissue for autografts is severily limited. This problem may be overcome by construction of artificial muscles prepared after colonization of decellularized muscles with suitable cells. In the proposed project we plan to exploit our experience gained over years in the stem cell research. We anticipate the recellularized muscle grafts share several common features with isotransplants of entire muscles (e.g. of extensor digitorum muscle), which show an excellent muscle reconstitution after transplantation in recipients [7]. In addition to degeneration of myofibres, other structures of muscle organ incl. exteroreceptors are affected by denervation and ischaemia resulting from muscle avascularisation. Nevertheless, basal laminas and satellite cells are left intact in the is transplanted tissue and they initiate my genesis accompanied with vascularisation, innervation and renewal of muscle sensory reception incl. Formation of muscle spindles as documented by our manuscripts [7].

Earlier studies exploited tube-like components of an acellular muscle as a scaffold promoting axonal regrowth after spinal cord hemidissection, to bridge a gap in the sciatic nerve defects or to fill the missing muscle tissue in defects created in experimental lesions [1,7,8]. Several reports described protocols for gaining acellular muscle grafts (e.g., [9-11]. Reseeding acellular muscle grafts with myoblasts gave rise to engineered muscle constructs with histomorphologic characteristics that resembled native muscle, which under in vitro conditions were capable of generating contractile force [3,9]. However, evaluation of grafts reoccupied with myogenic and non-myogenic cells after implantation in animal models has not yet been performed. Suitable cells for acellular muscle graft recellularization involve myogenic cells as well as non-myogenic cells that participate in reconstitution of a tendon, muscle stroma and graft revascularization. The principal cells responsible for muscle regeneration are satellite cells located at the surface of muscle fibres just under the basal lamina. A satellite cell is frequently considered to be a progenitor cell due to its unipotency. The engraftment capacity of isolated satellite cells into injured skeletal muscle is poor [10] and cells from other sources, e.g. mesoangioblasts [11-14]. Engraft into musle fibres with a greater efficacy. However, population of satellite cells is highly heterogenous; some are derived from dermatomyotomes, the others from prechordal mesoderm and few possible other sources. A minor subpopulation of satellite cells may include myogenic stem cells [14]. Isolated a rare subset of muscle Side Population (SP) cells characterized by co-expression of ABCG2, Syndecan4 and

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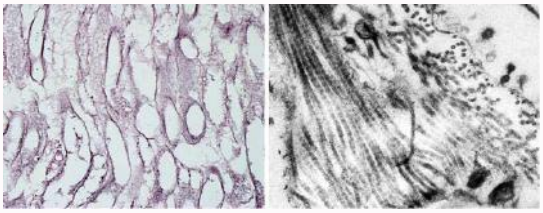

Figure 1: A scaffold of decellularized mouse abdominal muscle we prepared by immersion in 1% SDS and 0.02% EDTA for 32 hrs. Gomori impregnation confirms preservation of ECM muscle microstructure of tiny reticular fibres and absence of sarcoplasm and cell nuclei from myofibers and endomysium (left). Although a transmission electron microscopy reveals submicroscopic defects caused by osmotic shock and detergents such as partial disintegration of basal laminas and interstitial edema it also confirms a good preservation of collagen fibrils with their characteristic periodic striation and their organization in bundles (right).

Pax7 which occupied satellite cell niche and exhibited more robust stem cell potential than previously characterized muscle progenitor cells; after transplantation these were capable of repopulating 75% of mature myofibers.

Despite muscle-derived cells, another promising candidate for muscle graft reseeding can be found in the bone marrow-derived cells. Not only the bone marrow cells contribute to reconstitution of the stroma in many organs of irradiated animals (our unpublished results) and tissue revascularization [15,16], they also contribute to my genesis [17].

Project Objectives

The main aim of the project is to reconstruct the skeletal muscle organ using ECM scaffold. Since acellular biological matrix can be used only as a passive element (e.g. bridge) that is colonized from outside by adjacent recipient cells (that lack any navigation to enter the graft) we plan to colonize the graft with donor-derived cells immediately prior graft implantation. We hypothesize the combination of myogenic and non-myogenic cells can be optimal for stimulating ingrowth of recipient cells responsible for graft vascularization, innervation and infiltration with inflammatory cells (that initiate the tissue repair). The resulting interaction between recellularized scaffold and recipient cells will support remodelling of the scaffolds microenvironment through partial disruption of basal laminas, local change in ECM composition and reduced stiffness which are critical prerequisites for successful muscle regeneration.

The Main Aim will be Reached Through who Following Principal Activities

Preparation of acellular ECM from the muscle

Muscle injury

In vitro analysis

In vivo analysis

Research Plan

Preparation of acellular ECM from the intact skeletal muscle tissue

Rationale: The acellular bioscaffold preserves internal microscopic structure of the muscle that will provide navigation clues to facilitate entrance and 3D organization of cells participating in the scaffold reoccupation.

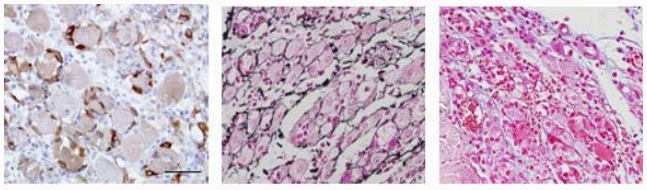

Figure 2: Muscle regeneration on day 4 after CTX injection. Nestin^{+} myoblasts derived from activated sattelite cells identified by imunoperoxidase immunohistochemistry (left), reticular fibres delineating myotubes idenitifed by Gomori impregnation (middle), ECM sulphated mucopolysaccharides by alcian blue and cell nuclei by Nuclear Fast Red (right).

Method

Tissue scaffolding will be prepared from decellularization of the anterior tibial m. and external digitorum longus m. of C57BL/6J and GFP (3 month-old) mice using different protocols. GFP mice are the strain C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb mice (Riken) – cooperating laboratories (Dept. Histology and Embryology and Dept. Biochemistry) are included in the register of GMO users (see http://www.mzp.cz/www/env-gmo.nsf/e7244034d91e3db0c1256e7e003a0828/2c41eb4ec5cda35dc1256fe9004176d4?OpenDocument). Scaffolds are prepared by combination of initial osmotic shock followed by immersion with ionic or zwitterionic detergents that are then replaced with prolonged washes with PBS with the aim to remove cell cytoplasmic components and preserve ECM proteins and glycosaminoglycans (in our previous experiments better results for muscle acellularization were reached with immersion technique than with whole-body perfusion decellularization.

A methodical part of the study plans to look the way for optimal storage of decellularized matrix with the use of physical and chemical approaches (cryopreservation, solutions for keeping the graft in aseptic conditions in the fridge or at room temperature, mild fixation with paraformaldehyde/glutaraldehyde). In addition to processing the entire muscle for decellularization, we also plan to decellularize small muscle units (from tissue cubes 1-2 mm in length to smaller fascicles). Processing small muscle pieces consisting of few myofibers should offer several advantages: we expect, their decellularization occurs faster with lower deteriorative effects on ECM and small units become more easily colonized by the cells. Because the basic skeletal muscle architecture is formed by multiple parallelly arranged myofibers, the parallelly arranged subunits could be used to fill in the large gaps in the traumatized muscle. Re-assembly of the small muscle units will be studied in vitro, the precise orientation and arrangement of units with adhered paramagnetic particles will be achieved with a magnetic field and with avidin to bridge biotinylated unit tips. Efficacy of the decellularization process will be evaluated with light, histochemical (Sirius red, Gomori impregnation, alcian blue, dimethylmethylene blue, biotinalyed hyaluronic acid binding protein to detect collagen and reticular fibres, proteoglycans and hyaluronic acid) and fluorescent microscopic methods; quantification is performed with Image analysis (Image Pro). Transmission electron microscopy is used to confirm removal of cellular components incl. chromatin and preservation of ECM structure; Western blotting is used to assess the presence of biogenic ECM molecules incl. decorin, biglycan, fibronectin, laminin and heparin sulphate proteoglycans in the grafts and to confirm a removal of such cellular proteins like actin. An advantage of preparing scaffolds from transgenic mice with an enhanced GFP expressed under the control of a chicken beta-actin promoter is a rapid assessment of decellularization efficacy because a

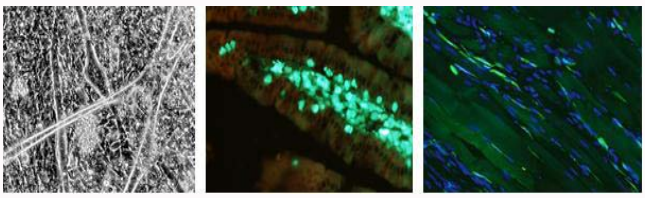

Figure 3: Formation of multinucleated myotubes from embryonic myogenic cells is facilitated by a decrease in serum concentration and cultivation on a soft hydrogel (left). Fluorescent identification of GFP⁺ bone marrow cells transplanted to irradiated mice; after 70 days the cells participate in stroma repair (middle). GFP⁺ bone marrow cells transplanted to animals 28 days after CTX muscle injury infiltrate the muscle stroma (right).

removal of cellular components correlates well with a decline and loss in GFP fluorescence.

Muscle Injury Analysis

Rationale: Precise knowledge of events associated with adult myogenesis incl. their timing (inflammatory reactions, cell activation, proliferation, differentiation) and characterization of changes during muscle ECM remodelling (matrix metalloproteinases /MMPs, ECM glycoproteins deposition etc.) is of critical importance for creating promyogenic niche that stimulates muscle regeneration.

Method

We plan to exploite the model of adult muscle injury induced by 100µl 10µM cardiotoxin (CTX) injection into the anterior tibial muscle of 2-3 moth-old C57Bl6/J or GFP mice. Our previous findings documented that following a removal of necrotic myofibres on day 3, the myogenesis occurs after activation of intact satellite cells left in situ in the close vicinity to necrotic myofibres and includes both extrafusal and intrafusal myofibres [17]. In addition to characterization of myogenic cells, an attention will be paid to expression of MMPs (particularly those involved in satellite cell activation – MMP9, degradation of basal membrane components - MMP7 [18], MMP10, degradation of entactin – MMP7, 8, 13) and expression of ECM glycoproteins (fibronectin, laminin, entactin). The site of degeneration/regeneration will be analyzed histologically in defined intervals of time (on day 3, 4, 5, 7, 10 and 21 post injury) using histochemistry, electron microscopy as well as immunohistochemical detection of myogenic markers (Myf5, MyoD, myogenin, striated muscle myosin heavy chains /SM1 and SM2/, intermediate filaments incl. nestin, vimentin, desmin) to get a detailed knowledge of temporal processes associated with muscle regeneration, markers of muscle cell proliferation, activation (integrins β1 [mediate myogenesis, i.e. heterodimers with α1, α3, α4, α5, α6, α7 α5, αv]; β3 [αvβ3 in myoblasts and activated satellite cells - [19]; β4 [interstitial cell progenitors - [20] and ECM glycoproteins.

Injured muscles will also serve as controls to myogenesis in decellularized and recellularized grafts and for isolation of activated myogenic cells (see below). Regenerating muscles on day 4-5 from CTX injury which undergo substantial ECM remodelling with promyogenic niche (caused e.g. by perisatellite deposition of cellular fibronectin [21] and other bioactive molecules) will be decellularized and the resulting scaffolds used for recellularization experiments.

In Vitro Analysis

Rationale: Muscle regeneration requires the coordinated interaction of multiple cell types. Myogenic cells alone are not able to reconstruct complex structures in the muscle organ. Therefore we plan to seed the acellular scaffolds with distinct cell types: either with myogenic cells alone or non-myogenic cells alone or combination of both types. The most promising cell populations will be defined from analysis cell behaviour on acellular scaffold surfaces grown *in vitro*.

Method

In vitro tests are based on cultivation of cells on 2D acellular scaffold cryosections attached to the bottom of cultivation dishes and in 3D scaffolds cultured for 1-2 weeks. When using small decellularized muscle units, the constructs will be seeded with cells after the re-assembling; following cell immigration and differentiation, morphology of such constructs will be compared to large recelularized scaffolds. Scaffolds will be examined in their natural stage after their preparation or after modification with MMP7 (to inititate remodelling of basement membranes) or treatment with fibronectin and Wnt7 (to stimulate satellite cells). As a control, acellular scaffolds from non-muscle organs, peripheral nerve or liver will be used.

Myogenic cells used for *in vitro* tests include C2C12 myoblasts (an immortal line of mouse skeletal myoblasts originally derived from satellite cells) and muscle-derived stem cells. The latter population is obtained from intact or injured muscle using a modified preplate technique described by [21]. as the cells received from muscle dissociation with the slowest adhesion to the flasks (after 5-7 serial transfers). The stem cell population (Sca-1⁺, CD34⁺) expand *in vitro* in DMEM supplemented with 20% FBS within 10 days and is maintained in undifferentiated conditions at low cell density culture. This cells population shows increased capacity to engraft into myofibres and satellite cell niche [22]. Additional immunomagnetic separation for β4 integrin or ABCG2/Syndecan4 will be used to yield a subpopulation of myogenic cells with robust engraftment capacity [23-25].

Non-myogenic cells will be obtained from the bone marrow mesenchymal cells or muscle connective tissue fibrobalsts. We expect non-myogenic cells to participate in formation of muscle microenvironment, coverings and tendon etc. From the bone marrow, all differentiated blood elements are removed with lineage cell depletion kit; the resulting suspension enriched for progenitor and stem cells will be used for graft recellularization. Alternatively positive immunoselection will be performed to collect cells expressing the required markers (CD117/c-kit, Sca-1, CD133). Muscle connective tissue cells are obtained from a preplate technique described above as the cells received from muscle dissociation with increased adhesion to the flasks (after 2-4 serial transfers). Cells that reveal myogenic potential in differentiation culture are eliminated with CTX.

Isolated cells are characterized in our labs with measurement of cell kinetics, viability and immunophenotypization (immunofluorescence, flow cytometry using the flow cytometer CyAN-ADP (DakoCytomation) and with the expression of cell-specific markers (e.g. myogenic regulatory factors MyoD, Myf5, myogenin). Identification of primary cillia (using anti-acetylated α tubulin or glutamylated tubulin immunofluorescence) will be examined in cells because these sensory structures are associated with multiple signalling pathways; *Wnt* and *Shh* signalling in myogenic cells (e.g. [26] offer possibility for modulation of stem/progenitor cell differentiation toward required myogenic phenotypes.

The acellular grafts are recellularized by multiple injections of

10^3 to 10^4 donor cells (depending on the graft size) in different sites of the graft under the visual control with a stereomicroscope and cultured *in vitro*. The cells with best potential to infiltrate the scaffold and participate in myogenesis are to be utilized for recellularization. 3D scaffolds seeded with cells are analyzed for biocompatibility, adhesion, cell surival, proliferation, migration and differentiation depending on number, and type of seeded cells and time period using histological examination and compared to regeneration induced by CTX. Presence and length of primary cillia will be correlated with the cell capacity to colonize acellular grafts.

In Vivo Analysis

Rationale

A complete tissue remodelling requires participation of recipient and its inflammatory cells, vascularisation, innervation etc. For that reason acellular and recellularized bioscaffolds will be implanted into the recipient muscle; rate and efficacy of muscle remodelling will be evaluated histologically. We expect recellularized scaffolds to show higher rate of remodelling because cells inside the scaffold become hypoxic after transplantation and trigger signalling through proangiogenic factors to attract blood capillary ingrowth. Graft vascularization is crucial not only for substituting cells with oxygen and nutrients to support cell survival and growth but it allows entry and spreading of other mesenchymal elements via perivascular spaces and involvement of new circulating stem/progenitor cells.

Method: Small scaffolds will be placed in a cavity created within the anterior tibial muscle of anaesthetised animales and covered with sutured fascia. For recellularization, GFP^+ myogenic and/or non-myogenic cells will be used to allow identification of cells after transplantation into histocompatible GFP^- C57Bl6/J mice. Implantation of decellularized and recellularized muscles grafts is performed in the same way as isogenous transplantations described by us earlier [27]. Proximal tendon of the graft is ligated, pulled distoproximally through the exposed host extensor digitorum longus muscle then sutured at the proximal pole of the host muscle and the distal tendon of the graft is connected to the distal pole of the host muscle. After transplantation, the cut tendon of the host tibialis anterior muscle is sutured to the site of its insertion and then the skin is sutured as well. After 24 hrs mice are treated with 33 mg bromodeoxyuridine i.p. injection and after 72 hrs with iododeoxyridine to allow sequential staining of different subpopulations of activated cells. Experimental animals will be divided into four groups (scaffolds acellular, recellularized with myogenic cells only, non-myogenic cells only and both populations) at a minimum of six animals each (for each survival interval).

Muscles are explanted under a dissecting stereomicroscope (after 4, 7, 21 days) and examined under the UV lamp. Sections will be cut on a cryostat and examined under a fluorescent microscope. Precise morphological examination is performed in paraffin-embedded sections (GFP^+ cells are detected with anti-GFP immunohistochemistry). For identification of phenotype of transplanted cells, co-expression of cell-specific antigens (myogenic markers), bromodeoxyuridine and laminin, the immunofluorescent staining will be performed. Image analysis (Image Pro) is used to evaluate the extent of cell engraftment and for estimation of muscle regeneration by calculating number of myotubes with centrally located nuclei. Graft innervation is assessed from S100 or neurofilament immunohistochemistry; graft vascularization from PECAM or nestin immunostaining or after Indian ink perfusion of bloood vessels. Transmission electron microscopy: Characterization of graft scaffolding, initial steps of graft repair, examination of nerve fibres (e.g. their myelination) and blood vessel wall maturation (e.g. presence of pericytes) is done with TEM in Durcupan-epoxy resins after osmification [27]. Quantitative PCR is used as an independent method to verify the presence of GFP cells in DNA of the examined tissue containing exogenous cells using modification of the procedure described by us [28]. Functional tests of mice injured, transplanted or implanted with bioscaffolds will include Rotarod tests and Foot print analysis. Data analysis will be performed by appropriate statistical methods using software Statistica v. 9.0 (StatSoft) and GrapPad Prism v. 5.01 (GraphPad).

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