Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fractal dimension analysis reveals skeletal muscle disorganization in *mdx* mice

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ARTICLE INFO

Article history:
Received 22 May 2018
Accepted 28 May 2018
Available online xxx

Keywords:
Duchenne muscular dystrophy
Co-culture
Fractal dimension analysis
Histopathology
Osteoglycin

ABSTRACT

Duchenne Muscular Dystrophy (DMD) is characterized by muscle extracellular matrix disorganization due to the increased collagen deposition leading to fibrosis that significantly exacerbates disease progression. Fractal dimension analysis is a method that quantifies tissue/cellular disorganization and characterizes complex structures. The first objective of the present study was use fractal analysis to evaluate extracellular matrix disorganization in *mdx* mice soleus muscle. Next, we mimic a hyper-proliferation of fibrogenic cells by co-culturing NIH3T3 fibroblasts and C2C12 myoblasts to test whether fibroblasts induce disorganization in myoblast arrangement. Here, we show *mdx* presented high skeletal muscle disorganization as revealed by fractal analysis. Similarly, this method revealed that myoblasts co-cultured with fibroblast also presented cellular arrangement disorganization. We also reanalyzed skeletal muscle microarrays transcriptomic data from *mdx* and DMD patients that revealed transcripts related to extracellular matrix organization. This analysis also identified Osteoglycin, which was validated as a potential regulator of ECM organization in mdx dystrophic muscles. Our results demonstrate that fractal dimension is useful tool for the analysis of skeletal muscle disorganization in DMD and also reveal a fibroblast-myoblast cross-talk that contributes to "*in vitro*" myoblast disarrangement.

1. Introduction

Skeletal muscle primary diseases, such as muscular dystrophies, are characterized by muscle dysfunction due to muscle degeneration accompanied by chronic inflammation and fibrosis [1]. Fibrosis is a pathological feature of muscle biopsies from patients with Duchenne Muscular Dystrophy (DMD) that contributes to the lethal DMD phenotype [2]. Disrupted extracellular matrix (ECM) in dystrophic muscles is the result of increased collagen deposition and cross-linking [3] leading to fibrosis that significantly exacerbate disease progression [4]. This aberrant deposition of ECM in muscular dystrophies is also associated with hyper-proliferation and over-activation of fibrogenic cells that modifies tissue architecture [1]. However, to our knowledge, there is no study quantifying skeletal muscle disorganization in DMD, which can be used to better characterization of muscle structural changes.

Fractal dimension is a differentiated and innovative methodology that allows the quantification of tissue and cells disorganization with important clinical applications for histopathological studies in neurology, ophthalmology, and hepatology [5,6]. Fractal dimension has also been used to evaluate myocytes preservation in isolated rat hearts, to determine myocardial cellular rejection after transplantation, and most recently, to quantify right ventricular structural changes in a rat model of pulmonary arterial hypertension [7–9]. In skeletal muscles, fractal dimension analyses were performed in histological slides for fiber type characterization, and in electromyography studies to evaluate muscle fatigue [10–12].

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https://doi.org/10.1016/j.bbrc.2018.05.189
0006-291X/

Considering the different cellular and molecular mechanisms underlying skeletal muscle tissue architecture modifications associated with dystrophin deficiency that have been described in patients and animals models [2], we hypothesized that ECM disorganization in *mdx* mice soleus muscle can be accessed by fractal dimension analysis. We also mimic a hyper-proliferation of fibrogenic cells by co-culturing NIH3T3 fibroblasts and C2C12 myoblasts, and applied fractal dimension analysis to test whether "*in vitro*" fibroblasts induce disorganization in myoblast arrangement. Our data demonstrate that fractal dimension is useful tool for the analysis of skeletal muscle disorganization in DMD and reveals a fibroblast-myoblast cross-talk that contributes to "*in vitro*" myoblast disarrangement. We also reanalyzed microarrays transcriptome data of skeletal muscle from *mdx* and DMD patients that revealed transcripts related to biological process of matrix organization. This analysis identified Osteoglycin (Ogn) as potential regulator of ECM organization in dystrophic muscles.

2. Materials and methods

2.1. Animals

Male C57BL/10-DMD^{mdx}/PasUnib (*mdx*) and C57BL/10-ScCr/PasUnib (Ctrl) mice obtained from the mouse breeding colony of the Sao Paulo State University (UNESP) were housed under controlled temperature conditions with a 12/12-h light/dark cycle and free access to food and water. Soleus muscles were dissected from three aged adults (13 months-old) *mdx* and three C57BL/10 (control) mice for histology analysis. All experiments were performed in accordance with the guidelines for the use of animals set forth by our institution. The study was approved by the local Ethics Committees (CEUA #461).

2.2. Histology

Cryostat transverse-sections of the soleus muscle (7 μm thick) were examined in *mdx* and Ctrl animals. Collected samples were fixed in Bouin or methanol for hematoxylin and eosin (HE) and Picrosirius red stained (PSR), respectively, according to standard laboratory histological protocol. Soleus sections stained with PSR were used to quantify ECM; analysis was performed using *ImageJ* following software instructions for collagen quantification. Tissue fractal dimension was assessed in both soleus sections stained with PSR or HE of three random sections from each animal, using a light microscope (Olympus, Japan) and 10X and 20X objective. Myoblasts images were analyzed in triplicate with four images per well by using phase-constrast inverted microscopy with coupled digital camera AxioCam ICc5 (Carl Zeiss, Germany).

2.3. Co-culture of myoblasts and fibroblasts

C2C12 mouse myoblasts (MB) and NIH3T3 mouse fibroblasts (FB) were first cultured separately in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) with 1% Penicillin–Streptomycin (Thermo Fisher Scientific, USA) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) at 37 °C and 5% CO2 for growth and expansion. After growth and harvest, the myoblasts were plated into six-well plates at a density of 1×10^5 cells/well in 2 ml of DMEM, and fibroblasts or myoblasts were seeded at same concentration into ThinCert™ Cell Culture Inserts (Greiner Bio-One) containing membrane with 0.4 μm pore size. After 50% of confluence of both cells, inserts were placed into six-well plates containing myoblasts. Experimental group were composed by fibroblasts on the inserts (FB/MB), and control group were composed by myoblasts on the inserts (MB/MB). Co-cultures were maintained for 24 h.

2.4. Fractal dimension analysis

To analyze the fractal dimension of soleus muscle and C2C12 myoblasts, the photographed slides and cell culture images were binarized by ImageJ, and the fractal dimension was estimated by using box-counting. This tool, in the image processing program, is required to quantify pixels distribution in the space, without considering image texture. All the steps in fractal dimension analysis in histological slides and cell culture images was based on Pacagnelli et al. (2016) [9]. The fractal dimension value is always expressed from 0 to 2, where values close to 2 represent higher tissue or cell disorganization.

2.5. Differential gene expression and ontology analysis

Microarray data from patients with DMD and *mdx* mice are available in Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo). The array data of GSE1007 included 20 samples of quadriceps from patients with DMD and normal skeletal muscle [13], and GSE466 are composed by 10 gastrocnemius samples of 16-weaks-old *mdx* and controls [14]. Those data were analyzed with GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/), which were used to determine the differentially expressed genes in patients or animals with muscular dystrophy [15]. Considering adj. $P < 0.05$ and |Fold Change (FC)|>2 as cut-off criterion for differential expressed genes. Gene enrichment analysis were performed by Gene Ontology Consortium database (http://geneontology.org/) [16] using the up-regulated transcripts in DMD and *mdx*, separately. Ontology terms with $P < 0.05$ were considered enriched.

2.6. Western blotting analysis

Soleus muscles from *mdx* and Ctrl were lysed in assay lysis buffer containing freshly added protease and phosphatase inhibitors (1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 100 mM sodium pyrophosphate, 100 mM NaF, 10 mM sodium orthovanadium, 10 mM EDTA, 2 mM PMSF, and 10 μg/ml aprotinin). The samples were centrifuged at 12.581 × *g* for 20 min at 4 °C. Protein concentration in soleus muscle was determined by Bradford protein assay kit according to the standard protocol [17] (Bio-Rad Laboratories, USA). Subsequently, Laemmeli buffer (Sigma, USA) were added to each soleus muscle samples, and maintained at 100 °C for 10 min. After samples preparation, the proteins at 30 ng of concentration were placed on 10% polyacrylamide gels in SDS-PAGE electrophoresis. After electrophoresis, the separated proteins were subjected to nitrocellulose membranes (Bio-Rad, USA) for electrotransference. A 5% of nonfat dry milk solubilized in TBS buffer containing 0.5% Tween 20 (TBST) were used to block the electrotransferred membranes for 2 h at room temperature, and then incubated overnight at 4–8 °C with specific Ogn primary antibody (Santa Cruz Biotechnology, USA; 1:1000 dilution). Mouse secondary antibody was maintained for 2 h at room temperature to promote binding of the primary antibody with peroxidase conjugated secondary antibody. Proteins were detected by using enhanced chemiluminescence (Amersham Biosciences, USA) in ImageQuant™ LAS 4000 (GE Healthcare, USA) autoradiography equipment. Quantification analyses of blots were performed by ImageJ software. Ogn levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7. Statistical analysis

The data are expressed as the mean ± standard deviation (SD).

Please cite this article in press as: S.S. Cury, et al., Fractal dimension analysis reveals skeletal muscle disorganization in *mdx* mice, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.189