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A novel quantitative morphometry approach to assess regeneration in dystrophic skeletal muscle

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Abstract

Duchenne muscular dystrophy is an inherited degenerative muscle disease with progressive weakness of skeletal and cardiac muscle. Disturbed calcium homeostasis and signalling pathways result in degeneration/regeneration cycles with fibrotic remodelling of muscle tissue, sustained by chronic inflammation. In addition to altered microarchitecture, regeneration in dystrophic muscle fibres is often only classified by centrally located nuclei but correlation of the regeneration process to nuclear volumes, myosin amounts, architecture and functional quality are missing, in particular in old muscles where the regenerative capacity is exhausted. Such information could yield novel regeneration-to-function biomarkers. Here we used second harmonic generation and multi photon fluorescence microscopy in intact single muscle fibres from wild-type, dystrophic mdx and transgenic mdx mice expressing an Δex 17–48 mini-dystrophin to determine the percentage of centronucleated fibres and nucleus-to-myosin volume ratio as a function of age. Based on this ratio we define a 'biomotoric efficiency' as an optical measure for fibre maturation, which is close to unity in adult wild-type and mini-dystrophin fibres, but smaller in very young and old mdx mice as a result of ongoing cell maturation (young) and regeneration (aged). With these parameters it is possible to provide a quantitative measure about muscle fibre regeneration.

Keywords: Muscular dystrophy; Nucleus; Myosin; Regeneration; Efficiency; Myonuclear domain; Second harmonic generation

1. Introduction

Duchenne muscular dystrophy (DMD) is the most commonly inherited X-linked muscle disease (1:3500 male births [1]), which results in progressive muscle degeneration and muscle weakness. The complete absence of dystrophin is due to several mutations in the dystrophin gene which encodes for the 427 kDa isoform of this protein [2], Missing dystrophin results in altered intracellular calcium homeostasis [3–7] and activity of ion channels [8] in the mdx mouse, an animal model for DMD. The mdx mouse presents with a more slowly progressing phenotype compared to DMD in humans and with only a 20% reduced lifespan compared to wild-type (wt) mice [9]. The cause of increased calcium permeability of sarcolemma is still under debate [10].

The onset of the disease usually appears at 4–8 weeks in the mdx mouse by an altered morphology due to massive necrosis followed by regeneration/degeneration cycles [11]. With ongoing pathology, age dependent abnormalities in cell structure and function accumulate [11–14]. Specifically, such abnormalities are probably caused by age-dependent changes in the microarchitecture that structurally determine dysfunction and weakness on a

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cellular level [15,16]. The chronic remodelling in muscle tissue is additionally associated with progressive fibrosis [17] and global muscle weakness [12] on a macroscopic level. On a microscopic level, branched or grossly malformed muscle fibres [13] and centronucleated fibres [11,18] prevail and become more and more prominent with age. These cellular and subcellular remodelling patterns seem to be a direct consequence of the disrupted and/or massive regenerative process, as very recently shown using a dystrophin-positive massive regeneration mouse model that also produced this abnormal cell architectures [19].

It is well known, that the central myonuclei in muscle fibres indicate ongoing regeneration, but mdx muscle loses its ability to regenerate over time [20]. Nuclei in healthy muscle fibres are usually located in the periphery. The determination of the percentage of muscle fibres with central nuclei, therefore, provides information about the degree of muscle regeneration. The changes in the microarchitecture in mdx muscle fibres indicate that the regeneration is incomplete, i.e. degenerative processes are more common than regenerative processes or the latter are of impaired order and quality. But this also means that the number of fibres with signs of regeneration must first increase with age and then remain at the same level when the maximum regeneration capacity is reached. Additionally, the number of nuclei in dystrophic muscle is increased [21], which means that the myonuclear domain, i.e. the myonuclei number per cytoplasm volume, is reduced [21]. Thus, the myonuclear domain is a way to correlate general functional changes with structural changes in muscle fibres [22,23]. Whether this ratio is altered in progressive muscle disease, like muscular dystrophy or chronic inflammatory myopathies, is currently unknown.

To approach this question, we investigated the age-dependent changes in distribution and volume of myonuclei using advanced microscopy techniques based on nonlinear optical effects on hundreds of intact living single fibres from wt mice, dystrophic mdx mice and transgenic, Δex 17–48 mini-dystrophin (MinD) expressing, mdx mice [24]. The visualisation of myosin in muscle fibres is performed by the so-called second harmonic generation (SHG) microscopy without using any external dyes [25,26]. At the same time, the cell nuclei are stained with a fluorescent dye and visualised by multi photon excited fluorescence (MPE) microscopy. In this way, the cellular volumes of myosin and myonuclei can be determined. Based on these parameters the *nucleus-myosin-volume ratio* (NMV), a measure similar to the myonuclear domain, can be calculated. Assuming that the volume of myosin with unaltered acto-myosin interaction [16] is a measure for motoric capacity and the volume of myonuclei is a measure for gene regulatory capacity of muscle fibres (underlying protein synthesis balance), we define the *biomotoric efficiency* (BE) of muscle fibres based on these morphometric parameters. Because of the incomplete regeneration that requires increasing regulatory processes in mdx muscle fibres [27] we hypothesised that BE decreases with disease progression in mdx muscle. The increase of regulatory processes is also supported by the increased ubiquitin levels in muscles from mdx mice according to earlier histological investigations from Smith et al. [28].

2. Materials and methods

2.1. Single-fibre preparation and staining

The sacrifice of animals and all experiments were authorised by the local Animal Ethics Committee (T-27/09, University Heidelberg). The single fibres preparation from interossei toe muscles of wild-type (C57), mdx and mini-dystrophin (Δex 17–48 deletion [26], molecular weight of 228 kDa) expressing mice were obtained by mild enzymatic dissociation. Mice were killed by an overdose of CO_2 atmosphere and the hindlimb paws were cut off at the ankle to dissect interossei muscles using a stereo microscope (Olympus SZ 60, Olympus Hamburg, Germany). The isolated muscles were bathed in physiological saline (140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM KCl, 5 mM glucose) containing 0.5–1 mg/ml (depending on the enzyme activity) collagenase (Typ IA, Sigma–Aldrich, Taufkirchen, Germany) for 30 min at 30 °C. After three washes the muscles were incubated in a physiological saline containing 0.01% (v/v) ethidium bromide (EtBr) for 15 min at room temperature. The single fibres were separated using a fire-polished Pasteur pipette.

2.2. SHG- and MPE-imaging

Single fibres were transferred to a recording chamber and imaged using a TCS SP2 Leica confocal microscope (DM IRBE, Leica, Wetzlar, Germany) with settings described in [25]. A mode-locked Ti:Sa-Laser (2 ps, Newport Spectra-Physics, Darmstadt, Germany) at 880 nm wavelength simultaneously generated the SHG signal from myosin and the multi photon fluorescence signal of ethidium bromide stained nuclei. To do so, two or more photons are required for excitation of the fluorophore, yet the use of photons with lower energy (i.e. higher wavelengths) allows a high penetration depth (optical window) with a high resolution (small cross section) in living tissue. Single fibres were completely z-scanned using a 0.285 μm step size (voxel-size: 0.116 μm × 0.116 μm × 0.285 μm).

2.3. Image processing

The two-channel (EtBr and SHG) tiff-stacks were displayed with Fiji based on ImageJ (National Institutes of Health, Bethesda, MD), the segmentation operation by thresholding extracts the nucleus volume (NV) and myosin volume (MV) by counting the segmented voxels