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TNF-α upregulates macroautophagic processing of APP/β-amyloid in a human rhabdomyosarcoma cell line

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ABSTRACT

Sporadic inclusion body myositis is a chronic progressive, inflammatory disorder of the skeletal muscle. No effective treatment is available for this debilitating condition and the complex disease pathology is far from being understood. The major hallmark of the pathomechanisms is the co-occurrence of inflammatory as well as degenerative cascades including aggregates consisting of β-amyloid within skeletal muscle fibers. Macroautophagy, a homeostatic process that shuttles cytoplasmic constituents into endosomal and lysosomal compartments, has recently been shown to be upregulated via the proinflammatory cytokine TNF-α in human skeletal muscle cells. In a human cell line from rhabdomyosarcoma as a model to study muscle cells, we here show that TNF-α-mediated upregulation of macroautophagy modulates APP and β-amyloid load and can be blocked by inhibition of macroautophagy. Thus, macroautophagy may be a crucial mediator between inflammation and β-amyloid-associated degeneration in skeletal muscle.

1. Introduction

Autophagy is a set of highly conserved catabolic pathways during which cell organelles and long-lived proteins are degraded in lysosomes in order to recycle nutrients for cell survival when facing starvation [1]. During macroautophagy cytosolic material is sequestered within a double-membraned organelle called the autophagosome, which subsequently fuses with lysosomes and late endosomes to form the autolysosome in which the cargo faces degradation via acidic lysosomal hydrolases [2]. Aside from its involvement in homeostasis, cell death [3] and immunity [4], autophagy appears to play a crucial role in the elimination of abnormal intracellular protein aggregates of numerous neurodegenerative disorders [5,6]. Apart from other stimuli, macroautophagy can be regulated via cytokines [7]. Amongst them, tumor necrosis factor (TNF)-α has proven to be involved in the induction of macroautophagic activity in several cell types [8,9].

Sporadic inclusion body myositis (sIBM) is the most common inflammatory myopathy in patients above the age of 50 and is characterized by the concurrent presence of both immune mediated as well as degeneration driven pathomechanisms [10–12]. Aside from $CD8^+$ T cell mediated cytotoxicity and upregulation of proinflammatory cytokines like TNF-α and interleukin (IL)-1β, muscle fibers from sIBM patients display distinctive accumulations of aberrant molecules including aggregates of β-amyloid as well as overexpression of the amyloid precursor protein (APP) [13].

It has been previously reported that muscle fibers of sIBM patients feature increased frequencies of autophagosomes in comparison with nonmyopathic muscle and that intracellular APP as well as β-amyloid showed a significant level of co-occurrence with the autophagosomal marker LC3 [14]. We could recently show that the proinflammatory cytokine TNF-α induced macroautophagic activity and regulates MHC expression in human skeletal muscle cells [15].

In this study we investigated whether TNF-α mediated upregulation of macroautophagy was associated with increased APP expression and intracellular β-amyloid load in a human cell line from rhabdomyosarcoma as a model to study muscle cells.

2. Materials and methods

2.1. Cell culture

Human rhabdomyosarcoma CCL136 cells (*ATCC*; *American Type Culture Collection*, Manassas, USA), a standard in vitro model to study

Abbreviations: sIBM, sporadic inclusion body myositis; APP, amyloid precursor protein; 3MA, 3-methyladenine; CQ, chloroquine; FKBP12, FK-binding protein 12; mTORC1, mammalian target of rapamycin complex1; BACE1, β-site APP-cleaving enzyme 1; AD, Alzheimer's dementia.

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muscle cells [13–15], were cultured in Dulbecco's modified eagle medium with 10% fetal calf serum, 1 mM glutamine, 110 μg/ml sodium pyruvate, and 2 μg/ml gentamycin (all cell culture reagents from *Invitrogen*, Carlsbad, USA). Chamber slides and culture wells in duplicates were exposed to the cytokine TNF-α (5–10 ng/ml, *R&D Systems*, Minneapolis, USA) and the reagents chloroquine (50 μM, *Sigma*, St. Louis, USA) and rapamycin (1 μg/ml, *Sigma*, St. Louis, USA) in serum free X-vivo medium (*Cambrex Bio Science*, Wiesbaden, Germany).

2.2. Thioflavin-S-fluorescence

For fluorescent thioflavin-S-staining, cultured CCL136 muscle cells were seeded in 8-chamber slides (*Nunc*, Rochester, USA). Cells were fixed in 4% paraformaldehyde (*Electron Microscopy Sciences*, Hatfield, USA) in PBS for 10 min at room temperature, followed by further fixation in methanol at −20 °C for 10 min. Staining of amyloid aggregation was achieved by thioflavin-S (*Sigma*, St. Louis, USA) at 1% in distilled H_2O for 5 min at room temperature. Nuclei were counterstained by 4,6-diamidino-2-phenylindole (0.5 μg/ml, *Invitrogen/Molecular Probes*, Carlsbad, USA) for 1 min; slides were mounted in Fluoromount G (*Electron Microscopy Sciences*, Hatfield, USA). Digital photography was performed on an Axiophot microscope (*Zeiss*, Göttingen, Germany). Appropriate filters for green (488 nm) and blue (350 nm) fluorescence, a cooled charge-coupled device digital camera (Retiga 1300; *Qimaging*, *Burnaby*, Canada) and the Image-Pro software (*Media Cybernetics*, Inc., Bethesda, USA) were used. For quantitative assessment a grayscale analysis was performed using the Scion image software (*Scioncorp.*, Frederick, USA) and the value was expressed as arbitrary units.

2.3. Western blot

CCL136 cells were lysed in lysis buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, 1% NP40, pH 7.9) containing protease inhibitors (*Roche*, Mannheim, Germany). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (*Schleicher & Schuell*, Dassel, Germany). After blocking with 5% skimmed milk in TBS for 1 h, membranes were incubated over night at 4 °C with the primary antibodies anti-APP/β-amyloid (mouse monoclonal 6E10, diluted 1/1000; *Signet*, Dedham, USA) and anti-β-actin (mouse monoclonal, diluted 1/10,000; *Sigma*, St. Louis, USA). Horseradish peroxidase-conjugated goat anti-mouse antibodies (*Jackson Immuno Research*, Suffolk, UK) were used as secondary reagents. Blots were developed with the enhanced chemiluminescence technique (*ChemiGlow West*; *Alpha Innotech*, San Leandro, USA) following the supplier's protocol.

2.4. Inhibition of macroautophagy

3-Methyladenine (3MA) (*Sigma*, St. Louis, USA) and Atg12 siRNA (*Qiagen Inc.*, Valencia, USA) together with respective control siRNA (*Applied Biosystems*, Foster City, USA) were used to inhibit autophagic activity. For 3MA treatment CCL136 myoblasts were plated in 8-chamber slides in serum free X-vivo medium supplemented with 10 mM 3MA with or without TNF-α. All experiments were terminated after 48 h and cells were analyzed using fluorescence microscopy.

For macroautophagy inhibition by RNA interference, Atg12 specific siRNA or negative siRNA controls were used. Cells were seeded in 8-chamber wells in serum free X-vivo medium and transfected with Atg12 siRNA and negative control siRNA respectively via Nanofectin (*PAA*, Pasching, Austria) following the manufacturer's protocol.

2.5. β-Amyloid$_{1–40}$ ELISA

All samples were analyzed at least in triplicates using the β-amyloid (1–40) assay (*IBL*, Minneapolis, USA). This kit is designed to measure full-length β-amyloid peptides with an intact N terminus. It uses the mouse anti-β-amyloid antibody clone 1A10 as a capture antibody and an HRP-conjugated polyclonal rabbit anti-human β-amyloid as a detection antibody. Samples of 20–40 μg total protein were sonicated and diluted in EIA buffer to a volume of 100 μl and treated according to the manufacturer's instructions. The colorimetric reaction was measured at 450 nm with a 1420 Multilabel Counter Victor 2 (*PerkinElmer*, Rodgau, Germany).

3. Results

3.1. TNF-α induced macroautophagy regulates APP- and β-amyloid load in human skeletal muscle cells

To analyze if TNF-α mediated upregulation of macroautophagy leads to modulation of APP-processing in human skeletal muscle cells CCL136 from rhabdomyosarcoma were incubated with TNF-α compared to rapamycin, which induces autophagosome formation via binding the cytosolic *FK-binding protein 12* (FKBP12) and subsequent inhibition and binding of the mammalian target of rapamycin complex1 (mTORC1). Cells were left untreated or treated with the lysosomal acidification inhibitor chloroquine (CQ). CQ raises the lysosomal pH, which leads to inhibition of lysosomal protein degradation; thus, the accumulation of autophagic vesicles under CQ treatment indicates autophagic flux. For these experiments concentrations of TNF-α and rapamycin were used which have previously been proven to upregulate macroautophagy in this cell line [15]. Upon 48 h incubation with TNF-α or rapamycin muscle cells showed an increase in the APP-signal compared to untreated controls, particularly with the lysosomal acidification inhibitor CQ (Fig. 1A), which reached statistical significance upon rapamycin. The observed increase in the APP-signal upon TNF-α treatment was concentration dependent (data not shown). To determine whether TNF-α-mediated stimulation of CCL136 cells would also lead to accumulation of the APP cleaving-product β-amyloid, the muscle cells were incubated for 48 h with TNF-α. Upon exposure to TNF-α or rapamycin, a significant signal increase of a size of 7 kD was detected by western blot, which likely represents dimers of β-amyloid (Fig. 1B).

3.2. Thioflavin-S-fluorescence upon inhibition of macroautophagy in human skeletal muscle cells

To further identify protein aggregation of β-amyloid, a fluorescent thioflavin-S staining was carried out to visualize β-sheet structures [13]. An increase in thioflavin-S-fluorescence could be detected upon a 48 h incubation of muscle cells with TNF-α (Fig. 2). To analyze if the TNF-α-mediated increase in the thioflavin-S-signal was dependent on macroautophagy, we analyzed fluorescence upon specific blockade. Knockdown of Atg12, a gene essential for autophagosome formation, significantly decreased the thioflavine-S signal and completely abolished the TNF-α-induced upregulation of autophagic activity (Fig. 2). Comparable findings were observed with 3MA as a pharmacological inhibitor of macroautophagy (data not shown).

An ELISA-assay was used to complement the analysis by immunohistochemistry: the concentration of β-amyloid$_{1–40}$ in supernatants from muscle cells was elevated upon TNF-α and downmodulated after pharmacological inhibition of macroautophagy using 3MA (Fig. 3). Although no statistical significance was reached, the trend of these data was in line with the results obtained by Western blot and immunocytochemistry.

4. Discussion

In this study we show that incubation of rhabdomyosarcoma-derived human skeletal muscle cells with TNF-α led to an overexpression of APP and accumulation of β-amyloid. Autophagy appears to have a protective role against diverse pathologies owing to its cellular clearance function and removal of damaged or aggregate-prone proteins