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Research paper

xCT deficiency induces autophagy via endoplasmic reticulum stress activated p38-mitogen-activated protein kinase and mTOR in sut melanocytes

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ABSTRACT

xCT, the functional subunit of the system x_c^- encoded by the Slc7a11 gene, plays an important role in maintaining intracellular glutathione (GSH) levels. In previous study, we have indicated that xCT deficiency induces OS and that OS triggers apoptosis through JNK pathway, however, this induction of apoptotic features did not fully explain the cell death induced by xCT deficiency. In the current study, we demonstrated that sut melanocytes of xCT deficiency showed activation of both ER stress and autophagy. And that the activation of autophagy by xCT deficiency was mediated by ER stress induced activation of p38 MAPK and NF-κB pathways and subsequently inhibited functions of Akt/mTOR/p70S6K survival pathways, ultimately led to autophagic cell death of sut melanocytes. Our novel results provided important insights into understanding the mechanism associated with xCT deficiency.

1. Introduction

xCT is the functional subunit of the system x_c^- encoded by the Slc7a11 gene, expression of xCT at the cell membrane is essential for the uptake of cystine required for intracellular GSH synthesis. Therefore, xCT plays an important role in maintaining the intracellular redox balance (Shiro and Noriko, 1986; Patel et al., 2004).

The subtle gray (sut) mutant mouse, a putative mouse model of Hermansky Pudlak syndrome (Li et al., 2004), carries a large deletion in the Slc7a11 gene, creating a new splice site and replacement of exon 12 with exon 12′. Due to a new stop codon in exon 12′, a mutant xCT with a modified carboxyl terminus is encoded. sut mouse also has moderate deficiencies of electron microscopically observable platelet dense granules, qualifying it as a model for a mild form of Hermansky-Pudlak syndrome (HPS), a genetically heterogeneous inherited disease characterized by abnormalities in biosynthesis and/or trafficking of lysosome-related organelles including melanosomes, platelet dense granules and lysosomes (Chintala et al., 2005).

The fibroblasts and melanocytes from sut mutant mice grow very poorly under normal culture conditions. In previous study, we have indicated that misfolded xCT induces OS and that OS triggers apoptosis through the JNK pathway and the latter induces the activation of the caspase (caspase-9-caspase-3). However, this induction of apoptotic features did not fully explain the cell death induced by xCT deficiency (Qiao et al., 2008).

The endoplasmic reticulum (ER) is a cellular membrane compartment for secretion and membrane protein synthesis in eukaryotic cells, most nascent proteins are synthesized and able to fold to their native conformation in the ER. Unfolded or misfolded proteins, however, trigger ER stress (also known as unfolded protein response) and are targeted to be degraded through a highly conserved, ER-associated quality control degradation system. Three ER transmembrane sensor molecules, PERK, ATF6 and IRE1, are involved in this process. In resting cells, the functions of the three ER stress sensors are inhibited by association with GRP78. Changes in physiological condition, however, will cause GRP78 dissociation from the sensors and trigger ER stress (Xu et al., 2005).

Autophagy is a cellular process that mediates the recycling of cytoplasmic macromolecules and structures through the formation of membrane double bounded vacuoles, called autophagosomes, that engulf and degrade large portions of cells (Martinet et al., 2009; Mizushima et al., 2008). Autophagy has also been associated with

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the induction of non-apoptotic cell death (Kim et al., 2008). The accumulation of misfolded protein aggregates in the ER that cannot be degraded by the proteosome results in the upregulation of the UPR(unfolded protein response) and the expression of autophagy-related genes (Kouroku et al., 2007; Ogata et al., 2006). Although both the UPR and autophagy can function independently, recent studies have shown that these processes may be linked and share a common function, exerting either cytoprotective (under basal or metabolic stress conditions) or cytocidal effects (after acute cellular damage) (Kondo et al., 2005; Moenner et al., 2007).

In order to fully elucidate the mechanism of cell death induced by xCT deficiency, in the current study, we also demonstrate that xCT deficiency could induce ER stress and autophagy, and that the activation of autophagy by xCT deficiency was mediated by ER stress induced activation of p38 MAPK and NF-κB pathways and subsequently inhibit functions of Akt/mTOR/p70S6K survival pathways, ultimately led to autophagic cell death of sut melanocytes.

2. Material and methods

2.1. Cell culture

The melanocyte line from xCT −/−mice (sut) and the mela a cell line from wild-type mice were gifted by Wei li and cultured as described (Li et al., 2004). To maintain the cells, 100 μM BME (Amresco, Solon, OH, USA) was added in the medium at 37 °C in a 5% CO2 incubator.

2.2. Electron microscopy

Cells were fixed using a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h. After fixation, the samples were post fixed in 1% OsO4 in the same buffer for 2 h and were dehydrated with anethanol gradient. Then, the cells were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (HITACHI −600, Japan).

2.3. Western blotting

Cells were washed with PBS, harvested and lysed in a buffer (0.5 M Tris-HCl, 0.4% SDS, 20% glycerol, 1.5% Bromphenol Blue, 1.0% beta-mercaptoethanol) by boiling for 5 min. Equal amounts of protein were subjected to 10–15% denaturing SDS electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were probed with antibodies as indicated below and detected using ECL Advance or Plus (Millipore). The blots were probed with the following antibodies, mouse anti-ATF6 (1:1000, Santa Cruz), anti-rabbit HRP-Conjugated secondary antibody (1:5000), anti-mouse HRP-conjugated secondary antibody (CST 1:7000), and monoclonal anti-beta-actin (1:8000) (Sigma), rabbit monoclonal to S6K (phospho T389) (ab32359), rabbit polyclonal to pan-AKT (phospho T308) (ab38449), LC3(1:3000, Abcam), p38 MAPK (1:1000, Cell Signalling Technology), PERK (1:1000, Cell Signalling Technology), NF-κB (1:1000, Cell Signalling Technology), mouse monoclonal anti-Beclin1 (1:200, Santa Cruz) (sc-48341).

2.4. Real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the sut cell lines and mela a cell lines using TRIzol® reagent (Invitrogen). Reverse transcription reactions containing 2 μg of total RNA were performed using RNA PCR Kit (TaKaRa Dalian Biotechnology Co., Ltd., Dalian, China). The individual cDNA species were amplified in a reaction mixture containing 1 unit of TaqDNA polymerase performed using TransStart® Top Green qPCR SuperMix (TransGen Biotech) and Bio-Rad S1000 Thermal Cycler (Bio-Rad, America) real time PCR system. Genes were quantified using GAPDH. All the sequences of the primers are mouse sequences. The primers that were

Atg5 Forward 5′-AGCCAATGTTGGAAACACCTCTGC-3′
Reverse 5′-TCCTTCAATCTGTTGGCTGTGGGA-3′
Atg7 Forward 5′-ATGCCAGGACACCCTGTGAACTTC-3′
Reverse 5′-ACATCATTGCAGAAGTAGCAGCCA-3′
Atg12 Forward 5′-CCCAGACCAAGAAGTTGGAA-3′
Reverse 5′-CAGCACCGAAATGTCTCTGA-3′
GAPDH Forward 5′-AACTTTGGCATTGTGGAAGG-3′
Reverse 5′-ACACATTGGGGGTAGGAACA-3′
Xbp1 Forward 5′-GGAGTGGAGTAAGGCTGGTG-3′
Reverse 5′-CCAGAATGCCCAAAAGGATA −3′

3. Results

3.1. xCT deficiency induces ER stress in sut melanocytes

As OS could induce ER stress and ER stress may cause autophagy. In our previous study, we found that xCT deficiency induces OS, implicating that xCT deficiency induce OS and substantially trigger ER stress. To determine whether the death of melanocytes from sut mutant mice induced by xCT deficiency was mediated by ER stress/UPR signaling, first we examined the expression of key molecules in the three main signal transduction cascades activated by ER stress. We monitored PERK, ATF6 and IRE1 activities overtime. To measure ATF6 activation, we followed the liberation of its cleaved cytosolic fragment, ATF6f that recapitulated ATF6 processing upon induction of ER stress. We saw that when BME was washed away with fresh medium without BME, rapid production of ATF6f (Fig. 1B) and significant increase in P-eIF2a protein (Qiao et al., 2008) in sut melanocytes, but we did not detect the cleaved XBP1 in sut melanocytes without BME(Fig. 1A), indicating that PERK and ATF6 pathway was activated while XBP1 was not activated in sut melanocytes without BME.

3.2. xCT deficiency induce autophagy and autophagic cell death in sut melanocytes

As induction of apoptotic features did not fully explain the cell death induced by xCT deficiency, to determine whether autophagy exists in sut melanocytes,we assessed the autophagy induction by electron microscopy, we found that xCT deficiency induced the appearance of lipid droplets and dense bodies, the latter scattered throughout the cytoplasm with morphological characteristics of autophagosomes. The abundance of these dense bodies was time-dependent (Fig. 2A). While, these structures were not observed in sut melanocytes with BME. The cytoplasm of sut melanocytes with BME was densely packed with abundant mitochondria, peroxisome and other subcellular organelle (Fig. 2A).

To further confirm that autophagy was induced in sut melanocytes, we assessed the expression of the autophagy related gene ATG5, ATG7, ATG12, autophagy markers LC3BI, LC3BII and Beclin1 by RT-PCR and western bloting. We found that when BME was washed away with fresh medium without BME, the expression levels of ATG5 ATG7 and ATG 12 increased in sut melanocytes (Fig. 2B), the LC3-II and Beclin1 proteins were also up-regulated in sut melanocytes compared to sut melanocytes with BME(Fig. 3), suggesting that autophagy was induced in sut melanocytes without BME. Using the inhibitor of autophagy 3-MA, partial cytoprotection was observed after 48 h when BME was washed away with fresh medium without BME (Fig. 4), indicating that autophagy was involved in cell death induced by xCT deficiency in sut melanocytes without BME.

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