The International Journal of Biochemistry & Cell Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Autophagy deficient melanocytes display a senescence associated secretory phenotype that includes oxidized lipid mediators

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ARTICLE INFO

Article history:
Received 30 June 2016
Received in revised form 5 October 2016
Accepted 6 October 2016
Available online xxx

Keywords:
Autophagy
Senescence
Oxidized lipids
SASP
DAMP

ABSTRACT

Autophagy is a recycling program which allows cells to adapt to metabolic needs and to stress. Defects in autophagy can affect metabolism, aging, proteostasis and inflammation. Autophagy pathway genes, including autophagy related 7 (Atg7), have been associated with the regulation of skin pigmentation, and autophagy defects disturb the biogenesis and transport of melanosomes in melanocytes as well as transfer and processing of melanin into keratinocytes. We have previously shown that mice whose melanocytes or keratinocytes lack Atg7 (and thus autophagy) as a result of specific gene knockout still retained functioning melanosome synthesis and transfer, and displayed only moderate reduction of pigmentation. In cell culture the Atg7 deficient melanocytes were prone to premature senescence and dysregulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling. To elucidate the biochemical basis of this phenotype, we performed a study on global gene expression, protein secretion and phospholipid composition in Atg7 deficient versus Atg7 expressing melanocytes. In cell culture Atg7 deficient melanocytes showed a pro-inflammatory gene expression signature and secreted higher levels of C-X-C motif chemokine ligand -1,-2,-10 and −12 (Cxcl1, Cxcl2, Cxcl10, Cxcl12), which are implicated in the pathogenesis of pigmentary disorders and expressed higher amounts of matrix metalloproteinases -3 and -13 (Mmp3, Mmp13). The analysis of membrane phospholipid composition identified an increase in the arachidonic- to linoleic acid ratio in the autophagy deficient cells, as well as an increase in oxidized phospholipid species that act as danger associated molecular patterns (DAMPs). The secretion of inflammation related factors suggests that autophagy deficient melanocytes display a senescence associated secretory phenotype (SASP), and we propose oxidized lipid mediators as novel components of this SASP.

1. Introduction

Macroautophagy (hereafter autophagy) is the bulk degradation of cellular cargo in double membrane vesicles called autophagosomes which fuse with lysosomes in which the cargo is subsequently hydrolyzed. Cells can adapt to low carbon source availability by activating autophagy to recycle damaged or momentarily nonessential biomolecules and releasing their metabolites for survival, the mammalian target of rapamycin complex 1 (mTORC1) being the central switch for nutrient sensing. Autophagy also has a fundamental role as a cellular waste disposal mechanism. It allows degradation of oxidized and crosslinked macromolecules that can accumulate under cellular redox stress and withstand proteasomal degradation (Filomeni et al., 2015). Reactive oxygen species (ROS) such as the superoxide anion (O_2^-), hydrogen peroxide, as

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http://dx.doi.org/10.1016/j.biocel.2016.10.006
1357-2725/

well as reactive nitrogen species (RNS) have been recognized as inducers of autophagy (Wen et al., 2013; Chen et al., 2009; Scherz-Shouval et al., 2007). Autophagy is also regulated by Nrf2 which induces expression of the autophagic cargo adapter Sequestosome 1 (p62) upon redox stress, and high levels of p62 again activate Nrf2 nuclear translocation (Komatsu et al., 2010; Taguchi et al., 2012; Jain et al., 2010). Together, these cytoprotective pathways limit excessive cellular ROS formation and resulting damage.

Melanocytes (MC), the pigment producing cells of the skin, need finely tuned redox chemistry, as they are permanently exposed to ROS from ultraviolet light, and their major task of synthesizing melanin can yield highly redox reactive side products, including quinones (Urabe et al., 1994). Melanocyte (-stem cell) dysfunction has consequences on skin pigmentation and hair greying. The major pigment disorder, vitiligo is also correlated to ROS imbalance in the affected tissues and melanocyte senescence (Yamaguchi and Hearing, 2014; Bellei et al., 2013). In vitiligo cells of the innate and adaptive immune system are targeted to destroy melanocytes by secretion of chemokines and presentation of “eat me” signals and DAMPS (Xie et al., 2016).

Consequently, the role of autophagy in pigmentation regulation, pigment disorders, skin cancers and skin aging is being intensively investigated in melanocytes and keratinocytes (Ho and Ganesan, 2011). Functional genomics has identified autophagy genes as regulators of melanogenesis (Ganesan et al., 2008). Both melanosome formation (Ho et al., 2011) and processing of melanosomes in human keratinocytes (Murase et al., 2013) was affected by targeting autophagy genes in human culture systems. We generated mice deficient in the essential autophagy gene Atg7 (and functional macroautophagy) in melanocytes, keratinocytes or both and found only mildly reduced pigmentation, and functional synthesis and transfer of melanosomes (Zhang et al., 2015). We observed however that cultured Atg7 deficient melanocytes showed a premature senescent phenotype in vitro and also dysregulated activation of Nrf2. Further, these cells accumulated oxidized phospholipids and high molecular weight, crosslinked p62 protein.

Here we extended our investigations of the molecular basis of the early senescent phenotype by examining how autophagy deficiency would affect the transcriptome, the protein secretion and the accumulation and secretion of bioactive phospholipid oxidation products (OxPL) in cultured melanocytes.

2. Materials and methods

2.1. Cell culture

Atg7-floxed mice (WT), Tyr:Cre mice and Atg7 f/f Tyr:Cre mice (KO) have been described previously (Delmas et al., 2003; Komatsu et al., 2005; Zhang et al., 2015). Genotyping and sex determination by PCR were carried out as described (Clapcote and Roder, 2005; Colombo et al., 2007). Institutional approval for animal material was granted under decree of the Federal Ministry of Science, Research and Economy (GZ 66.009/0255-II/3b/2013 ff). Melanocytes were isolated from 1- to 5-day-old pups as described before in (Zhang et al., 2015). Cells from female animals were suspended in melanocyte growth medium (MGM; Lonza, Basel, Switzerland) and kept in culture for two passages. After 22 days RNA, protein lysate, supernatants and phospholipids were isolated from WT and KO melanocytes.

2.2. Microarrays and bioinformatic analysis

Total RNA from two series of triplicate cultures was extracted with TriFast Reagent (VWR Peqlab, Radnor, PA, USA) according to the manufacturer’s instructions. After purification with RNeasy MinElute Cleanup Kit (Qiagen, Redwood City, USA), RNA was pooled to yield two analytical samples for each genotype. One hundred nanograms of total RNA were used for gene expression analysis with mouse gene level 2.0 ST arrays from Affymetrix (Sta. Clara, CA, USA). Hybridization and scanning were performed according to manufacturer’s protocol (http://www.affymetrix.com) and robust multi-array average (RMA) signal extraction and normalization were performed using custom chip description file. Data were submitted to the gene expression omnibus (GEO) repository (GSE83856). Genes (probe sets) with a mean RMA > 50 in at least one condition and >1.3 fold regulation in both biological series were used for further analysis. Heatmaps were generated with Net Walker 1.0 (https://netwalkersuite.org), using centered log2 values. We conducted Gene Ontology (GO) enrichment analyses for the biological process_5 (BP_5) using the DAVID web server (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) for the regulated genes. Pathway- and upstream regulator analyses were performed through the use of QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN, www.qiagen.com/ingenuity). This software was used to calculate which upstream regulatory events were likely to cause the observed gene expression changes between the analyzed groups and which signaling pathways are likely to be activated, both based on evidence from the literature. We restricted analysis to the molecule type “Genes, RNAs and Proteins”.

2.3. Protein arrays

To screen the production and secretion of mouse chemokines, WT and KO cultures supernatants were collected 24 h after medium change. The supernatants were subjected to a membrane-based sandwich immunoassay (Proteome Profiler Mouse Cytokine Array, Panel A, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.4. ELISA

To quantify the production and secretion of Cxcl10 and Mmp 3, supernatants were collected 24 h after medium change and analyzed with enzyme-linked immunosorbent assay (ELISA, Mouse Cxcl10 Quantikine ELISA Kit and Mouse Total Mmp-3 Quantikine ELISA kit, respectively (both R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Optical density at 450 nm was measured using a microplate reader (Fluostar Optima, BMG Labtech, and Offenburg, Germany).

2.5. qRT-PCR

RNA was isolated using the RNeasy 96 system (Invitrogen/Life Technologies, Grand Island, NY) or with TriFast Reagent (VWR, Radnor, PA), according to manufacturer’s instructions. 400 ng of total RNA were reverse-transcribed with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, and CA) and quantitative PCR (qRT-PCR) was performed on a LightCycler 480 using LightCycler 480 SYBR Green I Master chemistry (Roche, Basel, Switzerland). Primers were as follows: matrix metallopeptidase 13 (Mmp13 f: 5′-tttattgttgctgcccatga −3″, r: 5′-ctctggtgttttgggatgct-3″); C-X-C motif chemokine 10 (Cxcl10 f: 5′-aatcatccctgcgagcctat-3″, r: 5′-gaggctctctgctgtccatc −3′). Expression of target genes was normalized to the expression of beta-2 microglobulin (B2m f: 5″ attcacccccactgagactg r: tgctatttctttctgcgtgc −3″). Relative quantification was performed according to (Pfaffl, 2001). The significance of differences in relative expression between two biological triplicate groups was determined using Student”s *t*-test ($p < 0.05$).

Please cite this article in press as: Ni, C., et al., Autophagy deficient melanocytes display a senescence associated secretory phenotype that includes oxidized lipid mediators. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.10.006