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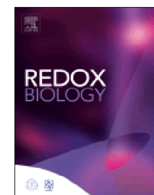

Research Paper

Copper accumulation in senescent cells: Interplay between copper transporters and impaired autophagy

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ABSTRACT

Cellular senescence is characterized by irreversible growth arrest incurred through either replicative exhaustion or by pro-oncogenic cellular stressors (radioactivity, oxidative stress, oncogenic activation). The enrichment of senescent cells in tissues with age has been associated with tissue dyshomeostasis and age-related pathologies including cancers, neurodegenerative disorders (e.g. Alzheimer's, Parkinson's, etc.) and metabolic disorders (e.g. diabetes). We identified copper accumulation as being a universal feature of senescent cells [mouse embryonic fibroblasts (MEF), human prostate epithelial cells and human diploid fibroblasts] *in vitro*. Elevated copper in senescent MEFs was accompanied by elevated levels of high-affinity copper uptake protein 1 (Ctr1), diminished levels of copper-transporting ATPase 1 (Atp7a) (copper export) and enhanced antioxidant defence reflected by elevated levels of glutathione (GSH), superoxide dismutase 1 (SOD1) and glutaredoxin 1 (Grx1). The levels of intracellular copper were further increased in senescent MEFs cultured in copper supplemented medium and in senescent Mottled Brindled (Mo^{br}) MEFs lacking functional Atp7a. Finally, we demonstrated that the restoration/preservation of autophagic-lysosomal degradation in senescent MEFs following rapamycin treatment correlated with attenuation of copper accumulation in these cells despite a further decrease in Atp7a levels. This study for the first time establishes a link between Atp7a and the autophagic-lysosomal pathway, and a requirement for both to effect efficient copper export. Such a connection between cellular autophagy and copper homeostasis is significant, as both have emerged as important facets of age-associated degenerative disease.

1. Introduction

Cellular senescence represents a critical barrier against cellular transformation and prevents the uncontrolled proliferation of cells that are irrevocably damaged [13,63]. Senescent cells actively secrete a variety of proteins, including pro-inflammatory mediators (cytokines and chemokines) to elicit immunological self-clearance [13,73]. Other secreted factors facilitate communication with the local microenvironment and can establish senescence in neighbouring cells in a paracrine manner [50,53]. The beneficial roles of senescent cells, for instance in wound healing, tissue remodelling and against cancer development, are considered conditional on their efficient and timely tissue clearance [13,73]. However, senescent cells accumulate in tissues and organs with age and contribute to age-related pathologies and dysfunction, in part, through promoting chronic inflammation [13,73]. Why senescent cells accumulate with age is unclear, but it is likely to be associated with age-associated immunodeficiency and therefore reduced clearance [17,54,67,74]. Recently developed strategies to clear senescent cells *in vivo* have provided remarkable improvement to the healthspan of mice by attenuating age-related pathologies and tissue dysfunctions [2–4,16,77].

We previously provided an aetiological link between senescent cell

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enrichment and iron dyshomeostasis, both of which occur in tissues with ageing and at sites of age-related pathologies [44]. We demonstrated that senescent cells accumulate substantial amounts of intracellular iron (up to 30-fold) as a consequence of impaired ferritinophagy (ferritin degradation) [44]. Elevated ferritin proved to be a robust biomarker for cellular senescence, for associated iron accumulation and for resistance to iron-induced toxicity, including ferroptosis. [44]. In this study, we further investigated whether other biologically relevant metals are altered in cellular senescence and identified a consistent increase in intracellular copper. Several groups have previously reported that elevated copper (250–500 μM) induces premature senescence in certain cell types [e.g. human diploid fibroblasts and fetal lung fibroblasts] [8,41,47,48,60], which can be attenuated by the copper chelator resveratrol [6,48,69]. Furthermore, replicative senescent human diploid fibroblasts (HDFs) have been shown to accumulate copper (2-fold) [8] and harbour increased mRNA transcript levels for copper-regulated genes, *heat shock protein 70*, *metallothionein 2A* and *prion protein* [60]. We hypothesized that copper accumulation is a feature of cellular senescence and investigated the cellular modifications that potentially contributed to elevated copper levels, such as enhanced antioxidant mechanisms and alterations to key copper homeostasis proteins. Further, we describe the key role of lysosomal dysfunction in senescence-associated copper accumulation.

2. Experimental

2.1. Chemicals and reagents

Rapamycin was purchased from Thermo Fischer Scientific (Cat#FSBBP2963-1). Bafilomycin A1 (Baf A1) (Cat# B1793) was purchased from Sigma Aldrich. All other reagents were purchased from Sigma Aldrich unless otherwise stated.

2.2. Isolation and culturing of primary mouse and human cells

Primary mouse embryonic fibroblasts (MEFs) were collected from sacrificed pregnant mice [C57BL/6 wild-type, Li-Fraumeni syndrome C57BL/6, agouti, mottled brindle (Mo^{br}) mice] at 13 day post-*coitum* and cultured as described previously [44]. Li-Fraumeni syndrome leads to a predisposition to tumor development in individuals that carry inherited mutations in tumor suppressor gene *TP53* [39]. The Li-Fraumeni syndrome C57BL/6 mice, that harbour a *TP53* germline mutation (G515A nucleotide mutation), were a kind gift from Prof. Guillermina Lozano (University of Texas) [39]. This study was approved by the Deakin University Animal Ethics Committee (AEC) (Id#G01–2014). Primary human diploid fibroblasts (HDFs) (S103) obtained from the Murdoch Children's Research Institute, Melbourne, and human primary prostate epithelial cells (PrECs), purchased from Lonza (Cat#CC-2555) were cultured as described previously [44].

2.3. Screening of MEFs carrying copper-transporting ATPase 1 (Atp7a)-Mo^{br} mutation

MEFs were screened for presence of *Atp7a-*Mo^{br} mutation by polymerase chain reaction (PCR) as described previously [42]. Briefly, sense oligonucleotides MMNK22 (5'-GGCAAAACCTCCGAGGCAAAG) specific for *Atp7a-*Mo^{br} mutation or MMNK23 (5'- CAAAACCTCCGAGGCTCT TGC) specific for wild-type *Atp7a* and antisense oligonucleotide MMNK24L (5'-AGGAGGAGATTTTCAGAGTTCAG-3') were used to amplify products of 83 bp and 87 bp, respectively, in separate reaction tubes. PCR conditions were as follows: 96 °C for 4 min, 63 °C for 1 min, 72 °C for 1 min for two cycles followed by 96 °C for 1 min, 63 °C for 1 min, 72 °C for 30 s for 35 cycles. The PCR products were resolved on a 4% agarose (agarose 3:1 High Resolution Blend, AMRESCO) gel and detected by staining with ethidium bromide.

2.4. Senescence induction by ionizing radiation

Senescence was induced in primary mouse and human cell lines (MEFs, HDFs and PRECs) as previously described [44]. Briefly, cells were cultured to ~ 90% confluence in 25 or 75 cm^2 flasks (Cellstar®, Cat#690175 or 658175, respectively) and subjected to 10 Gray (Gy) gamma irradiation using a calibrated Cesium-137 source (Gamma Cell 40, Atomic energy of Canada Limited). Cellular senescence was assessed at appropriate time points (in days) post-irradiation by senescence-associated β-galactosidase (SA-βgal) activity staining, as detailed in the Results.

2.5. Retrovirus production and transduction of primary MEFs

HEK293T cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (20 U/mL) and streptomycin (20 μg/mL), and were used as a packaging cell line for retrovirus production used for transduction of MEFs as described previously [44]. To produce retrovirus containing the SV40 Large T antigen (SV40 LgT), equimolar amounts of pBabe-neo large TcDNA plasmid (Addgene, Cat#1780) along with the packaging plasmid pCl-Eco (Addgene, Cat#12371) were used. Retrovirus containing the oncogene *H-Ras*^{V12} was produced with pWZL-Hygro H-*Ras* V12 plasmid (Addgene, Cat#18749), while control retrovirus was produced with empty pWZL-Hygro plasmid (Addgene, Cat#18750).

2.6. Senescence-associated β-galactosidase assay in cells

SA-βgal staining of cultured cells was performed as previously described [44]. The percentage of cells stained for SA-βgal activity was determined using an inverted microscope (Olympus IX51), by counting cells in four random fields of view at a magnification of 200/400X. Images were taken with Canon 1100D digital camera.

2.7. Western blotting analyses

Cell lysates were prepared and fractionated as described previously [44]. Gel fractionated protein samples were transferred to nitrocellulose membrane using Bolt® transfer system and buffer containing Tris-HCl (25 mM), glycine (192 mM) and 15% methanol (5% for Atp7a). Membranes were blocked for 1 h at room temperature using 5% (w/v) skimmed milk in wash (TBS-T) buffer containing Tris-HCl [10 mM (pH 8.0), NaCl (150 mM) and 0.1% Tween-20]. The following antibody dilutions were used: anti-CCS (Cat#FL274, 1:1000) was purchased from Santa Cruz Biotechnology, USA. Anti-β-actin (Cat#A5441, 1:10000), anti-LC3B (Cat#L7543, 1:1000) and HRP conjugated anti-goat IgG (Cat#A5420, 1:5000) were purchased from Sigma Aldrich. Anti-high-affinity copper uptake protein 1 (Ctr1) (Cat#EPR7936, 1:1000) and anti-Grx1 (Cat#AB16877, 1:1000) were purchased from Abcam. Anti-SOD1 (ADI-SOD-100, 1:2000) was purchased from Enzo Lifesciences, USA. Anti-Atp7a (R17, 1:500), goat polyclonal, was raised in-house and affinity purified. HRP conjugated goat-anti-mouse (Cat#P0447) and goat-anti-rabbit (Cat#P0448) antibodies were purchased from Dako. The membranes were subsequently developed using ECL reagent (Millipore, Cat#WBKLS0500) and bands visualized on Gel Dock™ XR+ system (Bio-Rad). At least three independent experiments were used for all comparisons.

2.8. Determination of total intracellular copper

Total copper concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700, Varian). Tissue culture cells were prepared for ICP-MS analyses as previously described [44]. Unit conversions from raw ppb values were performed as follows: (ng/million cells) = (raw ppb values × dilution factor/cell number).

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