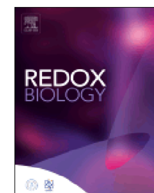
Redox Biology 14 (2018) 100–115

Contents lists available at ScienceDirect

Redox Biology

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



Research Paper

Iron accumulation in senescent cells is coupled with impaired ferritinophagy and inhibition of ferroptosis

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ARTICLE INFO

Keywords:
Senescence
Iron
Ferritinophagy
Ferroptosis
Ferritin
Autophagy
Ageing

ABSTRACT

Cellular senescence is characterised by the irreversible arrest of proliferation, a pro-inflammatory secretory phenotype and evasion of programmed cell death mechanisms. We report that senescence alters cellular iron acquisition and storage and also impedes iron-mediated cell death pathways. Senescent cells, regardless of stimuli (irradiation, replicative or oncogenic), accumulate vast amounts of intracellular iron (up to 30-fold) with concomitant changes in the levels of iron homeostasis proteins. For instance, ferritin (iron storage) levels provided a robust biomarker of cellular senescence, for associated iron accumulation and for resistance to iron-induced toxicity. Cellular senescence preceded iron accumulation and was not perturbed by sustained iron chelation (deferiprone). Iron accumulation in senescent cells was driven by impaired ferritinophagy, a lysosomal process that promotes ferritin degradation and ferroptosis. Lysosomal dysfunction in senescent cells was confirmed through several markers, including the build-up of microtubule-associated protein light chain 3 (LC3-II) in autophagosomes. Impaired ferritin degradation explains the iron accumulation phenotype of senescent cells, whereby iron is effectively trapped in ferritin creating a perceived cellular deficiency. Accordingly, senescent cells were highly resistant to ferroptosis. Promoting ferritin degradation by using the autophagy activator rapamycin averted the iron accumulation phenotype of senescent cells, preventing the increase of TfR1, ferritin and intracellular iron, but failed to re-sensitize these cells to ferroptosis. Finally, the enrichment of senescent cells in mouse ageing hepatic tissue was found to accompany iron accumulation, an elevation in ferritin and mirrored our observations using cultured senescent cells.

1. Introduction

Cellular senescence refers to cells that have undergone irreversible growth arrest through replicative exhaustion, or in response to a variety of pro-oncogenic cellular stresses (e.g. oncogenes, oxidants, and radiation) [1,2]. Irrespective of the stimulus, senescence safeguards against the unrestricted growth of damaged cells and promotes cellular clearance through eliciting the immune system. However, senescent cells accumulate with age and contribute to chronic diseases and age-related dysfunctions, in part, through the pro-inflammatory factors (e.g. cytokines & chemokines) they secrete (senescence-associated secretory phenotype; SASP) [1,2]. Senescent cells and SASP have been linked with chronic inflammation that is often observed during ageing in tissues in the absence of obvious infection [2]. The clearance of senescent cells in mice, using a novel transgene (*INK-ATTAC*) that allows for selective apoptosis of p16-positive senescent cells in vivo, improved healthspan by attenuating age-related pathologies both prophylactically and as treatment [3,4]. Removal of p16-positive senescent cells in ageing mice delayed tumourigenesis and attenuated age-related deterioration of several organs, including kidney, heart and adipose tissue, without adverse side effects [3]. Furthermore, a median lifespan extension was observed, indicating that p16-positive senescent cells negatively impact longevity [3].

Senolytic (anti-senescence) treatment is also sought-after for cancer therapy, as current chemotherapeutics (e.g. DNA damaging agents) can cause cancer cells to become senescent [5–8]. Senescent cells are linked

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http://dx.doi.org/10.1016/j.redox.2017.08.015
Received 10 August 2017; Received in revised form 21 August 2017; Accepted 22 August 2017
Available online 01 September 2017
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with cancer drug resistance and recurrence [9,10]. A novel senolytic agent (FOXO4-p53 interfering peptide) has shown promise in mice, promoting apoptosis of doxorubicin-induced senescent cells, neutralizing doxorubicin-induced toxicity and improving overall health-span [6]. Together, these studies demonstrate tangible health benefits for targeting senescent cells and thereby identify senescent cells as being integral to many age-related pathologies and dysfunctions.

Several reports have described dysfunctional iron homeostasis with ageing, either systemically [11,12], or in specific organ systems affected by age-related pathologies [13–15]. Age-dependent accumulation of iron in various tissues has been reported to occur separately to the enrichment of senescent cells [11,12,16–19]. Diseases associated with the accumulation of senescent cells, such as neurodegenerative disorders (e.g. Alzheimer's and Parkinson's) [20–22], osteoarthritis [23,24] and idiopathic pulmonary fibrosis [25], also exhibit iron dyshomeostasis where often iron burden correlates with disease severity [14,15,26–34]. Iron accumulation occurs in replicative senescent fibroblasts in vitro [35] and the iron storage protein ferritin is enriched in ageing tissues [36,37]. Since excess iron can be toxic through redox activity, iron burden has been hypothesized to cause cellular damage or to promote ferroptosis. However, the relationship between senescent cells and iron dyshomeostasis in ageing, or in age-related pathologies, is unclear. We therefore investigated whether altered iron homeostasis is a characteristic of senescent cells using mammalian cell culture models and aged wild-type (wt) C57BL/6 mice. The aim was to determine if the senescence phenotype is intrinsically linked with changes to cellular iron homeostasis and related cell death pathways.

2. Results

2.1. Senescent cells accumulate large amounts of intracellular iron

We initially studied a well-established model of cellular senescence in mouse embryonic fibroblasts (MEFs) induced by sublethal gamma irradiation [38,39]. The DNA damage incurred sequentially activates the p53-p21 and p16-RB effector pathways, representing distinct phases from early to full senescence [2,40]. When cultured for ≥ 10 days, more than 80% of irradiated MEFs (MEF IR) stained positive for senescence associated beta-galactosidase (SA-βgal) activity (Fig. 1**A(i)**,Fig. S1A). Senescent MEFs remain viable (Fig. S1B) and can be metabolically active in culture for many months [2]. To permit sufficient time for a net change in intracellular iron to occur, we cultured irradiated MEFs (MEF IR) for 21 days, by which time they expressed increased p53 and p16 protein expression consistent with senescence incurred through DNA damage (Fig. 1**A(ii)**). Transcripts for senescent markers, p16, p21 and IL-6 were also increased (Fig. S1C). Intracellular iron was measured by inductively-coupled plasma mass spectrometry (ICP-MS) and at 21 days post-irradiation senescent MEFs (MEF IR) accumulated a large amount of iron (~ 20-fold) when compared to primary non-irradiated MEFs (MEF PRI) (Fig. 1**A(iii)**). The iron accumulation was reliant on p53-mediated senescence induction and was not indirectly caused by irradiation (Fig. S1E). One consequence of cellular senescence is the reported morphological change in cell size (flat-cell phenotype) [41], which is an important factor to consider when quantifying intracellular metals. Consistent with previous reports [41], the diameter of adhered senescent MEFs (at 21 days post-irradiation) was approximately twice that of primary non-irradiated MEFs (MEF PRI) (Fig. S1F). However, diameters of suspended cells measured following dissociation of adherent cells were closely aligned (Fig. S1G). Cellular protein content is considered a surrogate marker for overall cell size, increasing roughly linearly with cell mass and volume [42]. Total cellular protein was slightly elevated (~ 1.3-fold) in senescent MEFs (MEF IR) (Fig. S1G), but could not account for the magnitude of increased intracellular iron (~ 20-fold) (Fig. 1**A(iii)**).

To ascertain whether intracellular iron accumulation occurs when senescence is induced through other stimuli, not just through irradiation, we measured iron in MEFs that underwent replicative senescence (REP), or oncogene (*HRas*^{V12}) induced senescence (OIS). In our hands, replicative senescence in primary MEFs occurred at passage 7 (P7), as established through staining for SA-βgal activity (10 days post-seeding) (Fig. 1**B(i)**) and was accompanied with termination of their replicative potential [population doubling limit (PDL) < 0.2] (Fig. S1H). When MEFs at passage 7 were sustained in culture for 21 days they had increased p53 and p16 expression (Fig. 1**B(ii)**). Concurrently, these replicative senescent MEFs (MEF REP), analogous to irradiation-induced senescent MEFs (MEF IR) (Fig. 1**A**), accumulated a vast amount of intracellular iron (~ 20-fold) (Fig. 1**B(iii)**). Variation in cell diameter or total cellular protein content between primary (MEF PRI) and replicative senescent MEFs (MEF REP) did not account for the degree of increased intracellular iron (Fig. S1F, G). In contrast, the well-characterised NIH(3T3) MEF line, that spontaneously bypassed senescence (43), had intracellular iron levels more comparable to that of primary MEFs (MEF PRI) despite multiple passages (Fig. 1**B(iii)**). We aided isogenic MEFs (our embryo lineage) to spontaneously bypass senescence by using the 3T3 culturing method [ISO(3T3)] [43] and these cells also had minimal change in their cellular iron content (Fig. 1**B (iv)**). Furthermore, primary MEFs immortalised with retrovirus containing SV40 large T antigen (MEF-LT) (at passage 5) (Fig. 1**B(v)**), evaded replicative senescence and continually proliferated, but maintained intracellular iron levels comparable to those of primary MEFs (MEF PRI) (Fig. 1**B(vi)**). Therefore, iron accumulation is associated with replicative senescence in MEFs and is not a feature of MEFs that have either spontaneously bypassed senescence or were immortalised.

Oncogene-induced senescence (OIS) was produced in MEFs by using retroviruses containing *HRas*^{V12} (Fig. 1**C**). HRas^{V12} directly causes senescence by activating the MAPK pathway in murine fibroblasts, arresting cells at the G1 cell cycle stage and is accompanied by an accumulation of p53 and p16 [44]. Oncogene-induced senescence has also been linked to the reactivation of programmed developmental senescence involving p21 and p15 and thus has molecular distinctions from replicative and irradiation-induced senescence that emanate from DNA damage response (DDR) mechanisms [45]. Senescent MEFs (MEF OIS) were determined by SA-βgal activity 8 days after retroviral transduction with *HRas*^{V12} and represented approximately 50% of the cell population (Fig. 1**C(i)**). Despite the limited percentage of senescent cells the accumulation of intracellular iron (~ 4.5-fold) was still evident when compared to MEFs transduced with control retroviruses (Fig. 1**C(ii)**). Immortalised primary MEFs (MEF-LT) transduced with retroviruses containing *HRas*^{V12} showed no signs of cellular senescence and accordingly no iron accumulation (Fig. 1**C(ii)**).

Cellular senescence can be induced by different molecular mechanisms depending upon the cell type and species of origin [2]. We therefore further demonstrated that human primary diploid fibroblast (HDFs) and prostate epithelial cells (PrECs), analogous to MEFs, also accumulated intracellular iron following senescence induction through either irradiation (IR) (Fig. 2**A**) or replicative exhaustion (REP) (Fig. 2**B**). Taken together, these results demonstrate that intracellular iron accumulates in senescent cells irrespective of stimuli, or cell origin (mouse vs. human; fibroblast vs. epithelial) and is therefore possibly a universal feature.

2.2. Altered iron homeostatic mechanisms drive senescent cells to acquire profound levels of intracellular iron

The remarkable increase in intracellular iron in senescent cells would conceivably necessitate numerous adaptive changes by the cell. Iron represents a double-edged sword, as its redox property that is utilised by many biochemical reactions also renders it potentially toxic. Iron can catalyse the production of reactive oxygen species (ROS) and free radicals, including the highly reactive hydroxyl radical [46]. We therefore investigated the levels of key cellular iron homeostasis proteins in senescent MEFs (21 days post-irradiation) (Fig. 3). Western blot

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