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Role of galactose in cellular senescence

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ABSTRACT

Cellular senescence has been proposed to play critical roles in tumor suppression and organismal aging, but the molecular mechanism of senescence remains incompletely understood. Here we report that a putative lysosomal carbohydrate efflux transporter, Spinster, induces cellular senescence in human primary fibroblasts. Administration of D-galactose synergistically enhanced Spinster-induced senescence and this synergism required the transporter activity of Spinster. Intracellular D-galactose is metabolized to galactose-1-phosphate by galactokinase. Galactokinase-deficient fibroblasts, which accumulate intracellular D-galactose, displayed increased baseline senescence. Senescence of galactokinase-deficient fibroblasts was further enhanced by D-galactose administration and was diminished by restoration of wild-type galactokinase expression. Silencing galactokinase in normal fibroblasts also induced senescence. These results suggest a role for intracellular galactose in the induction of cellular senescence.

1. Introduction

Cellular senescence was originally described as irreversible proliferation arrest that limits the proliferation of human primary cells in culture (Hayflick and Moorhead, 1961), which is now known to be caused by progressive shortening of telomeres. Various stresses such as DNA damage or oncogene expression can also induce similar persistent proliferation arrest which is called stress-induced senescence (Campisi and D'adda di Fagagna, 2007; Kuilman et al., 2010). Cells with characteristics of senescence accumulate with age in multiple tissues and such cells are also found at sites of age-related pathologies, raising the possibility that senescent cells contribute to aging phenotypes and age-related diseases. Indeed, selective elimination of p16-positive senescent cells in a mouse model of accelerated aging retarded or diminished the aging phenotypes (Baker et al., 2011). Furthermore, the pioneering work by Campisi and others (Coppe et al., 2008) as well as our comprehensive quantitative proteomic analyses (Elzi et al., 2012b) established that senescent cells display profoundly altered protein secretion, which can affect the architecture and function of the surrounding tissues, likely contributing to organismal aging. Therefore, if we understand the molecular mechanism of senescence, we could eliminate senescent cells and might be able to retard or diminish aging and age-related diseases in humans. However, although the phenotypes of senescent cells are well known (such as persistent proliferation arrest, flat and enlarged morphology, senescence-associated β gal [SA-β gal] activity, senescence-associated heterochromatic foci, and senescence-associated secretory phenotype), the molecular basis for these senescence phenotypes and hence how senescence occurs remain largely unknown.

Chronic low-dose D-galactose administration results in accelerated organismal aging in mice, rats, and *Drosophila* (Cui et al., 2004; Ho et al., 2003). Animals chronically treated with D-galactose display shortened lifespan, cognitive dysfunction, neurodegeneration, and impaired immune responses, which resembles natural aging. While D-galactose-induced aging models have been used for the study of the aging process and screening of drugs, how galactose effects organismal aging remains unclear.

Here we report several lines of evidence implicating intracellular galactose in cellular senescence.

2. Methods

2.1. Reagents

LysoTracker Red was purchased from Life Technologies. Hoechst 33342 was from Cell Signaling Technology. Human Spinster (SPNS1) and galactokinase cDNAs were purchased from GE Healthcare Dharmacon and cloned to pCDF1-Puro lentiviral vector (System Biosciences). Lentiviral vectors expressing human galactokinase shRNAs were purchased from GE Healthcare Dharmacon. Puromycin and glucose were from EMD Millipore. Hygromycin was from Invitrogen/Life Technologies. Galactose was from Thermo Fisher

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Scientific. N-acetylcysteine was from Acros Organics. The target sequences for shRNAs are as follows: human galactokinase shRNA-1, GGGCCAACTATGTCAAGGGAG; human galactokinase shRNA-2, CCAG ACTCGGGCACAATAGCT; human galactokinase shRNA-3, CACCAACTCT AATGTCCGCCA; human galactokinase shRNA-4, GTACAACTGGAAGAGC TAGAG; human p53 shRNA, GACTCCAGTGGTAATCTACT; human Spinster shRNA, TACATTGCAGGCTCCAAAGTGA; luciferase shRNA, GCACTCTGATTGACAAATACGATTT; scrambled shRNA, CCTAAGGTTAAG TCGCCCTCGCT.

2.2. Cell culture

IMR-90 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. 293T cells were cultured in DMEM supplemented with 10% calf serum. Galactokinase-deficient GM00334 fibroblasts were purchased from Coriell Institute and cultured in Eagle's MEM supplemented with 15% fetal calf serum. Lentiviruses were prepared by transfection in 293T cells following System Biosciences' protocol. The cells infected with lentiviruses were selected with 2 μg/ml puromycin or 0.15 mg/ml hygromycin for 48 h.

Live cells were stained with LysoTracker Red and Hoechst 33,342 for 10 min at 37 °C as described by the manufacturer's protocol. Senescence-associated beta-galactosidase assays (Dimri et al., 1995) and detection of senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003) were conducted as described (Elzi et al., 2012a,b). A minimum of 100 cells were counted.

2.3. Immunoblotting

Thirty micrograms of whole cell lysate was separated by SDS-PAGE and analyzed by immunoblotting as described (Elzi et al., 2012a,b). The following antibodies were used: mouse monoclonal anti-nucleolin (C23, Santa Cruz Biotechnology); rabbit polyclonal anti-p16 (C20, Santa Cruz Biotechnology); mouse monoclonal anti-p21 (SX118, BD Pharmingen); rabbit polyclonal anti-p53 (FL-393, Santa Cruz Biotechnology); rabbit monoclonal anti-phospho Rb (S780, D59B7, Cell Signaling Technology); rabbit polyclonal anti-phospho Rb (S807/811, #9308, Cell Signaling Technology); mouse monoclonal anti-Rb (G3-245, BD Pharmingen); rabbit polyclonal anti-Spinster (ProSci); and mouse monoclonal anti-tubulin (DM1A, Sigma-Aldrich).

2.4. RT-PCR

Total cellular RNA was prepared using TRIzol reagent (Invitrogen/Life Technologies) and RT-PCR was performed as described (Elzi et al., 2012a,b). The following PCR primers were used: RNA polymerase II (Pol II) 5′ primer, GGATGACCTGACTCACAAACTG, 3′ primer, CGCCCA GACTTCTGCATGG; Spinster 5′ primer, TCATCTTTGGACTCATCACCTGC, 3′ primer, GCCCAGTTCATGGACAGGA.

3. Results and discussion

A genetic screening for inducers of SA-β gal activity in zebrafish identified the defect in a putative lysosomal carbohydrate efflux transporter, Spinster, as a mediator of senescence and aging phenotypes (Kishi et al., 2008). Senescence as well as autophagic impairment induced by Spinster defect in zebrafish was further shown to be differentially modulated by p53 and Beclin 1 (Sasaki et al., 2014). On the other hand, exogenous Spinster expression in immortalized human cell lines resulted in autophagic cell death (Yanagisawa et al., 2003). To test the effect of manipulation of Spinster on cellular senescence of human primary cells, we first exogenously expressed human Spinster (SPNS1) in human IMR-90 primary fibroblasts. We found that expression of Spinster in IMR-90 fibroblasts results in proliferation arrest, increased lysosomes (Fig. 1A), increased SA-β gal activity (Figs. 1B and 2A), increased senescence-associated heterochromatic foci (Fig. 1C), flat and enlarged morphology (Fig. 1D), induction of cyclin-dependent kinase inhibitor p21 and dephosphorylation of Rb (Fig. 1E). These results indicate that exogenous expression of Spinster induces cellular senescence in human primary fibroblasts. Silencing p53 in IMR-90 cells abrogated p21 induction by Spinster (Fig. 1F), suggesting that p21 induction by Spinster is p53-dependent. Senescence induction by Spinster was abolished by an anti-oxidant, N-acetylcysteine, implicating oxidative stress in Spinster-induced senescence (Fig. 1G). Conversely, silencing endogenous Spinster in IMR-90 cells (Fig. 1H) also resulted in increased SA-β gal activity (Fig. 1H). These findings might suggest that both up- and down-regulation of Spinster results in cellular senescence in human primary fibroblasts.

Spinster belongs to a family of lysosomal efflux transporters, lysosomal membrane proteins required for the export of lysosomal degradation products such as amino acids and monosaccharides. Loss of the *Drosophila* Spinster homolog, benchwarmer, results in lysosomal carbohydrate storage which can be visualized by the periodic acid Schiff (PAS) staining (Dermaut et al., 2005). Similarly, knockdown of Spinster in normal rat kidney cells results in accumulation of carbohydrate in lysosomes as determined by PAS staining (Rong et al., 2011). These findings suggest that Spinster is a lysosomal monosaccharide efflux transporter. We became interested in the role of monosaccharides in Spinster-induced senescence and tested the effect of addition of two common monosaccharides, glucose and galactose, to the culture medium. As shown in Fig. 2A, galactose enhanced Spinster-induced senescence in IMR-90 cells whereas glucose had no effect on senescence induction. Interestingly, Spinster E164K mutant, which is a loss-of-function mutant due to the substitution of a highly conserved glutamate residue in the transmembrane domain (Dermaut et al., 2005; Rong et al., 2011), abolished galactose-induced senescence in IMR-90 cells (Fig. 2B and C), suggesting that Spinster mediates senescence by exporting galactose from lysosome to cytoplasm.

Cytoplasmic galactose is metabolized to galactose-1-phosphate by galactokinase (Fig. 2D) and mutation of galactokinase results in accumulation of intracellular galactose and a disease, type 2 galactosemia (Lai et al., 2009). We found that type 2 galactosemia patient fibroblasts (GM00334, harboring homozygous Val32Met inactivating mutation in galactokinase (Stambolian et al., 1995)) are slow growing and display increased baseline senescence (Fig. 2E), which was significantly enhanced by the addition of galactose to culture medium (Fig. 2E, +gal) and was diminished by the restoration of wild-type galactokinase expression [Fig. 2E, GALK(+)]. Furthermore, silencing galactokinase in IMR-90 fibroblasts (Fig. 2F) resulted in cellular senescence (Fig. 2G). These results are consistent with the notion that intracellular galactose promotes the induction of cellular senescence.

How intracellular galactose contributes to senescence is not yet clear. One possibility is that excess intracellular galactose forces cells to rely more heavily on mitochondrial respiration for energy production (Marroquin et al., 2007; Rossignol et al., 2004), leading to overproduction of reactive oxygen species and induction of senescence. It was suggested that oxidative stress also plays a role in accelerated organismal aging induced by chronic galactose administration in *Drosophila*, mice, and rats (Cui et al., 2004; Cui et al., 2006; Du et al., 2015). Alternatively, downregulation of glutamine synthetase, which was observed upon galactose-induced aging of astrocytes (Shen et al., 2014), might also play a role in galactose-induced senescence of non-neuronal cells. Although galactose oxidase reaction has been commonly used to quantify D-galactose in a variety of biological samples (Frings and Pardue, 1964), we found that this method is not sufficiently sensitive to quantify intracellular galactose in fibroblasts. In future studies, the HPLC–ESI-MS approach could be used to quantify intracellular galactose in cells induced to senesce by different stimuli such as replicative telomere shortening, oncogenic Ras, DNA damage, and oxidative stress, which may provide additional insight into the role of intracellular galactose in mediating cellular senescence.