Mechanisms of Ageing and Development 164 (2017) 82–90

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Original Article

Skin melanocytes and fibroblasts show different changes in choline metabolism during cellular senescence

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ARTICLE INFO

Keywords:
Cellular senescence
^{1}H NMR spectroscopy
Glycerophosphocholine
Choline metabolism
HEPES

ABSTRACT

Unmodified cells undergo only a limited number of cell divisions until they enter a state termed cellular senescence. Other triggers like cytotoxic compounds can also induce cell senescence. Since cell senescence represents a major mechanism of tumor suppression this cellular state has attracted increasing attention. Different markers like senescence-associated β-galactosidase (SAβGal), senescence-associated heterochromatic foci (SAHF) or certain metabolic changes have been identified to be characteristic for senescent cells; however, data is often limited to fibroblasts − the cardinal model system for cellular senescence. In order to investigate whether metabolic changes during senescence are cell type independent, skin fibroblasts and skin melanocytes have been examined. Expression of the senescence marker p16 could be detected in skin fibroblasts but not in melanocytes of this specific donor, rendering the senescent phenotype not fully ascertained for the melanocytes. Metabolic profiles of senescent cells and controls have been determined using NMR spectroscopy. Changes in metabolism are different for fibroblasts and melanocytes. Senescent melanocytes showed lower levels of phosphocholine whereas for fibroblasts in accordance with literature, levels of glycerophosphocholine were increased during senescence. Although no general metabolic marker for cellular senescence exists, the same metabolic pathway seems to be affected for both cell types.

1. Introduction

The phenomenon of cellular senescence was described by Hayflick and Moorhead first, when they recognized that fibroblasts can undergo only a limited number of cell divisions (Hayflick, 1965; Hayflick and Moorhead, 1961). The loss of telomere length is causal for cellular senescence (Bodnar et al., 1998; Harley et al., 1990). However, not only extensive replication results in a senescent state. It has also been described in literature that certain stressors can cause cell senescence like overexpression of oncogenes or DNA damage (Hewitt et al., 2012; Suram et al., 2012).

Along with the increasing interest in cellular senescence due to its potential role as a tumor suppressor mechanism (Campisi and d'Adda di Fagagna, 2007) different senescence markers have been identified. For instance morphological changes of the cells (Matsumura et al., 1979), senescent-associated β-galactosidase activity (SAβGal) (Dimri et al., 1995) and senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003). Very early in senescence research increased diameters of senescent cells have been observed (Greenberg et al., 1977; Macieira-Coelho and Ponten, 1969) and subsequent studies showed also an increase in cell size (Mitsui and Schneider, 1976; Schneider and Fowlkes, 1976). This gain in cell volume is also accompanied by a larger variation in cellular size (Mitsui and Schneider, 1976; Schneider and Fowlkes, 1976) therefore, cell size seems probably not an adequate marker for senescence.

Changes in lipid metabolism play also an important role in cellular senescence (Ford, 2010). A very recent study of Quijano and co-workers identified pronounced changes in the lipid metabolism during oncogene-induced senescence in fibroblasts by using mass spectrometry (Quijano et al., 2012). Our group identified by employing NMR spectroscopy increased levels of glycerophosphocholine (GPC) as a metabolic marker for senescence in WI-38 fibroblasts − independent of the trigger causing the senescent state (Gey and Seeger, 2013).

Since fibroblasts are the cardinal model system for investigating cellular aging, data on senescence is somehow biased towards this cell type. Melanocytes have been proposed as an alternative model system

Abbreviations: cPDL, cumulative population doubling level; DMSO, dimethyl sulfoxide; GPC, L-α-glycerophosphocholine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PC, phosphocholine; PC1/2/3, principal component 1 or 2 or 3; PCA, principal component analysis; PMA, phorbol 12-myristate 13-acetate; SAβGal, senescence-associated β-galactosidase; SAHF, senescence-associated heterochromatic foci; TSP-d_4, 3-(Trimethylsilyl)proponic acid

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http://dx.doi.org/10.1016/j.mad.2017.05.001
Received 22 December 2016; Received in revised form 25 April 2017; Accepted 2 May 2017
Available online 02 May 2017
0047-6374/

for characterization of senescence in non-fibroblastic cells (Bandyopadhyay et al., 2001). Contrary to other senescence markers like SAβGal (Dimri et al., 1995), morphological changes (Haferkamp et al., 2009), and SAHF (Haferkamp et al., 2009) no data on metabolic changes are reported for melanocytes so far. To fill this knowledge gap, we addressed the question whether changes in glycerophosphocholine present a metabolic marker for senescence independent of the cell type.

Human skin cells (fibroblasts and melanocytes) were cultured until they stopped replication. Metabolic profiles of proliferating cells with a low population doubling level, replicative senescent cells (or cells showing a senescence-like growth arrest) and cells with DNA damage-induced senescence have been determined. We were able to show differences between young and senescent cells, but there is no general metabolic marker that is characteristic for the senescent state. Senescent human skin fibroblasts show the same changes i.e. elevated levels of GPC as senescent lung fibroblast in our previous study (Gey and Seeger, 2013). In contrast, melanocytes showed no change in GPC but a decrease in phosphocholine (PC) levels was detected indicating that the same metabolic pathway − choline metabolism − is influenced. Therefore we conclude that choline metabolism is a commonly changed metabolic pathway during senescence independent of the cell type.

2. Materials and methods

2.1. Cell lines and culture

Replicative and DNA damaged-induced senescence were investigated using human unmodified fibroblasts and melanocytes. The cell lines − skin fibroblasts (GM21808) and skin melanocytes (GM21810) isolated from foreskin of one donor with Caucasian origin − have been obtained from the Coriell Institute for Medical Research/Cell Repositories (Camden, US) with a low population doubling level. At the time of sampling the donor was one day old. Cells were cultivated in a 5% carbon dioxide and 21% oxygen incubator at 37 °C.

2.1.1. Replicative senescence

Skin fibroblasts of a cPDL 1.6 (GM21808) have been cultured in a 1:1 mixture of Ham's F12 Medium/Dulbecco Modified Eagles Medium supplemented with 2 mM L-glutamine and 15% fetal bovine serum (not heat inactivated). Skin melanocytes of cPDL 1 (GM21810) have been cultured in serum free melanocyte growth medium with the corresponding supplement added (M254, GIBCO). M254 contains phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) as a supplement for stimulating growth. Cells at a cPDL of about 10 were considered to be presenescent/young cells and are referred to as control. Cells were considered to be senescent when the growth curve showed only a minimal increase in the cPDL and cells were positive for senescence markers. Western blot analysis showed no detectable expression of p16 and p21 in melanocytes and thus the senescent state cannot unequivocally ascertained by alterations of these markers (see below). Therefore, it referred to as a senescence-like growth arrest instead of replicative senescence for melanocytes that have undergone extensive replication. To ensure identical cultivation conditions as for DNA damaged-induced senescence DMSO was added to the media of the control group and replicative senescent cells with a final concentration of 0.05% (v/v) (vide infra).

2.1.2. DNA damage-induced senescence

The fibroblast and the melanocyte cell line have been also used to investigate cellular senescence induced by DNA damage. This was achieved as described previously (Gey and Seeger, 2013; Jeanblanc et al., 2012) by adding etoposide to a final concentration of 20 µg/ml (Sigma E1383) for 5 days to cells at a cPDL of about 10. Etoposide was prepared as stock solution in DMSO leading to a final concentration of DMSO of 0.05% (v/v) in the cell culture media. DMSO was added at the same concentration to the media of the control group and replicative senescent cells.

2.2. Staining for senescence markers

For all groups the senescence marker SAβGal according to the protocol of Dimri et al. (1995) and SAHF have been determined.

Detection of senescence markers was done as described in (Gey and Seeger, 2013). In brief: cells grown on six well plates were fixated with paraformaldehyde, washed with PBS (supplemented with 1 mM $MgCl_2$) and incubated over night with the staining solution (1 mg/ml X-Gal; 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 150 mM NaCl; 2 mM $MgCl_2$ in citric acid/sodium phosphate buffer pH 6) at 37 °C. The percentage of SAβGal positive cells was calculated by counting at least 50 cells from two or more different images (Lee et al., 2006).

For DAPI staining the cells were grown on cover slips and stained with DAPI upon mounting. To assess morphology, images have been taken from un-fixated, living cells over the period of cultivation.

2.3. Western blot analysis of p16 and p21 expression

Western blot analysis was used to analyze expression levels of senescence markers p16 and p21 in whole cell lysates. Experiments were performed as described previously (Pientka et al., 2012). In brief: extracts were obtained by cell lysis with 10 mM Tris/HCl (pH 6.8), 6.7 M urea, 10 M Glycerin, 1% SDS and 5 mM DTT. 100 µg protein as determined by a Bradford assay using BSA as standard have been subjected to SDS-PAGE analysis with a 15% gel. Transfer onto nitrocellulose membranes (Amersham Hybond-ECL, GE Healthcare) was done by semidry blotting and successful transfer was verified by staining with Ponceau S (Sigma). After blocking the membranes (4 °C for two hours with 5% nonfat milk powder in PBS) they were incubated with the primary antibodies (anti-CDKN2A/p16INK4a antibody [EPR1473], anti-p21 antibody [EPR362]; abcam) with a 1:1000 dilution over night at 4 °C followed by an incubation with a goat anti-rabbit polyclonal antibody conjugated with horseradish peroxidase (Dako, Germany) for one hour at room temperature. Detection of chemiluminescence was done by incubation of the membrane with ECL detection reagents (Amersham ECL Western Blotting Detection Reagents, GE Healthcare) for 1 min, followed by exposure to X-ray films (Amersham Hyperfilm MP, GE Healthcare). Equal protein loading was verified by a murine anti-β-actin monoclonal antibody (8H10D10, Cell signaling) followed by an anti-mouse antibody (Dako, Hamburg) with incubation times of 2 h and 1 h, respectively at room temperature.

2.4. Sample preparation and NMR-measurements

Cells harvested from three confluent Petri dishes (10 cm diameter) have been pooled for NMR analysis. A detailed description of the sample preparation is provided in (Gey and Seeger, 2017). After a wash step with PBS cells were harvested by scraping and the pellet was stored at −80 °C until further sample processing. In case of melanocytes the cells were washed three times and a sample of the supernatant of the last wash step was collected. Typically 3.5–5.5 × 10^6 cells per NMR sample were obtained in case of fibroblasts and 4–7 × 10^6 cells per NMR sample in case of melanocytes. Typically, six NMR samples per group have been analyzed.

Water soluble metabolites have been isolated by a methanol/chloroform extraction as described by Lee and colleagues (Lee et al., 2009). All steps have been carried out at 4 °C or on ice unless otherwise stated. To each pellet 900 µl of a 2:1 (v/v) mixture of chloroform/methanol was added. After mixing and cell lysis via sonification (highest setting at Sonifier Bandelin HD 200 with MS72 tip for 3s) chloroform saturated water (900 µl) was added. Phase separation was achieved by centrifugation (20 min, 16,000*g*). The solvent from the aqueous phase was removed using a centrifugal vacuum concentrator