The International Journal of Biochemistry & Cell Biology 45 (2013) 1255–1264

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Amadori products promote cellular senescence activating insulin-like growth factor-1 receptor and down-regulating the antioxidant enzyme catalase

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ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 8 March 2013
Accepted 28 March 2013
Available online 8 April 2013

Keywords:
Cellular senescence
Amadori product
Glycated albumin
Catalase
Insulin-like growth factor-1
Ras

ABSTRACT

Activation of the insulin growth factor receptor-1 signaling pathways has been largely related to the aging process. Amadori products are produced in pathological conditions such as diabetes and aging, and are potentially involved in diabetic nephropathy or age-associated decline of renal function. We hypothesize that Amadori products induce senescence in primary human mesangial cells through the activation of IGF-1 receptor and investigate, in the present work, the intracellular mechanism involved after this activation. We treated cultured human mesangial cells with glycated albumin, one of the most abundant Amadori product, and senescence was assessed by determining the senescence associated β-galactosidase activity and the expression of the cell cycle regulators p53 and p21. We demonstrated that prolonged exposition (more than 24 h) to glycated albumin induced senescence and, in parallel, incremented the release of IGF-1 and the activation of the IGF-1 receptor. Inhibition of the IGF-1 activation prevented the GA induced senescence. Activation of IGF-1R, after GA addition, promoted a reduction in the catalase content through the constitutive activation of Ras and erk1/2 proteins which were, in turn, responsible of the observed GA-induced senescence.

In conclusion, we propose that the Amadori product, glycated albumin, promotes premature cell senescence in mesangial cells through the activation of the IGF-1 receptor and the subsequent reduction in the antioxidant enzyme catalase.

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1. Introduction

The aging process is regulated by multiple signaling pathways. Many of them have been described in animal models and are regulated by environmental and physiological stimuli (Kenyon, 2010). Among these, the insulin/IGF-1 signaling pathways affects fly, *Caenorhabditis elegans*, mouse and human lifespan through changes in gene expression that acts cumulatively in the aging process (Lapierre and Hansen, 2012), however less is known about the role of this pathway in premature cellular senescence.

Somatic cells come into replicative senescence after a determined number of cell divisions due to the telomere shortening (Blasco, 2005; Campisi, 1996), but cellular senescence can also be induced prematurely by different stimuli, such as constitutively activated oncogenes (Ferbeyre et al., 2002), DNA damage (Gire et al., 2004), oxidative stress (Chen and Ames, 1994) and suboptimal

Abbreviations: CDK, cyclin-dependent kinase; GA, glycated albumin; PKC, protein kinase C; TGF-beta, transforming growth factor-beta; HMC, human mesangial cells; NGA, non-glycated human albumin; CAT, catalase; C12FDG, 5-dodecanoylaminofluorescein di-beta-D-galactopyranoside; TTBS, Tween Tris buffered saline; GST-Raf1-RBD, Ras binding domain of Raf1; SA-β-gal, senescence associated β-galactosidase; IGF-1R, insulin-like growth factor 1 receptor; HuVEC, human umbilical vein endothelial cells; FGF23, fibroblast growth factor 23; AGEs, advanced glycation end products; MAPK, mitogen-activated protein kinases.

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http://dx.doi.org/10.1016/j.biocel.2013.03.018

cell culture conditions (Balin et al., 2002). Senescent cell withdraws from the cell cycle, but continue to function metabolically active and can remain viable (Michaloglou et al., 2005), developing a particular morphology typified by a flattened, enlarged shape and increased focal adhesion (Goldstein, 1990), and displaying some phenotypic markers such as the senescence associated β-galactosidase activity (SA-β-gal) (Dimri et al., 1995). The major biochemical mediators of senescence in human cells are $p16^{INK4a}$ and p53 tumor suppressor protein, which induces the expression of $p21^{Cip1}$ (Brown et al., 1997; el-Deiry et al., 1993). $p21^{Cip1}$ and $p16^{INK4a}$ promote cell cycle arrest by inhibiting the cyclin-dependent kinase (CDK) required for the cycle progression (Alcorta et al., 1996). However, the signal cascades that activate p53 and $p16^{INK4a}$ pathways remain poorly understood.

Amadori products resulting from the non-enzymatic glycosylation or glycation of macromolecules, especially proteins, play an important role in diseases (Jaleel et al., 2005). Glycated albumin (GA) is a predominant Amadori-modified early glycated protein accumulated in diabetic patients and has been directly implicated as a causal factor in several major complications of diabetes, especially in nephropathy (Chen et al., 2000). When added to the culture media of glomerular mesangial and endothelial cells, glycated albumin stimulates protein kinase C (PKC) activity, increases transforming growth factor-beta (TGF-beta) bioactivity and induces production of extracellular matrix proteins (Chen et al., 2001).

In the present work, we test the hypothesis that Amadori product promotes premature cellular senescence in human mesangial cells, and explore the intracellular mechanisms involved.

2. Materials and methods

2.1. Cell culture and reagents

Human mesangial cells (HMC) were cultured according to previously described procedures (Iglesias-De La Cruz et al., 2001). Briefly, portions of macroscopically normal cortical tissue were obtained from human kidneys immediately after nephrectomy for renal cell carcinoma. Isolated glomeruli were treated with collagenase type IA (Sigma Chemical, St. Louis, MO, USA), plated in plastic culture dishes. They were maintained with RPMI 1640 (LONZA, Basel, Switzerland), supplemented with 10% fetal bovine serum, L-glutamine (1 mM), penicillin (0.66 μg/ml), streptomycin sulfate (60 μg/ml), and buffered with HEPES and bicarbonate, pH 7.4, in a 5% CO_2 atmosphere. Culture media were changed every 2 days. When the cells reached confluence, they were split at a ratio of 1:4 using the same incubation medium. The experiments were performed on passages 3–7 in cells seeded in tissue culture dishes at a density of 4×10^4 cells/cm^2. For the experiments HMC were treated with human glycated albumin (GA, 100 μg/ml), non-glycated human albumin (NGA, 100 μg/ml), catalase (CAT, 320 U/ml) which were purchased from Sigma; PD98059 (50 μM) and IGF-I receptor inhibitor (12 μM) were purchased from Calbiochem (Gibbstown, NJ, USA).

2.2. Klotho stable cell lines generation

Subconfluent HMC cultured in 6-well plate were transfected with 1 μg of a plasmid containing the transmembrane form of mouse klotho cloned into pEF1/*Myc*-His vector (HMCKL) or with the empty pEF1/*Myc*-His vector as controls (HMC Empty). For transfection, Optimem medium (Gibco, Paisley, UK) and lipofectamine (Invitrogen Ltd., Paisley, UK) were used. To select the transfected HMC, cells were treated with 200 μg/ml of G418 (Invitrogen) for 14 days (Kurosu et al., 2006).

2.3. Measurement of mRNA Klotho expression

Total RNA from HMC was isolated using Trizol reagents according to the manufacturer's protocol. The RNA integrity was checked using agarose–formaldehyde gels, and the RNA concentration was measured using a Vis–UV spectrophotometer (Nanodrop). cDNA was synthesized using a High Capacity™ kit (Applied Biosystems Inc., Foster City, CA, USA), Klotho expression was measured by quantitative RT-PCR (qPCR) (ABI Prism 7000), using Taqman™ predeveloped assays and Double delta Ct method. Klotho (Rn00580132_m1), and as endogenous controls, 18S (Eukaryotic 18s rRNA Endogenous Control Reagent, Applied Biosystems) and GAPDH (Rn99999916_m1) were used.

2.4. Flow cytometric determination of senescence associated β-galactosidase activity

To determinate the cellular senescence, β-galactosidase activity was measured by flow cytometry, using the fluorogenic substrate 5-dodecanoylaminofluorescein di-beta-D-galactopyranoside (C_{12}FDG) (Dimri et al., 1995; Kurz et al., 2000). HMC monolayers were treated with 300 μM chloroquine (Sigma) for 2 h to induce lysosomal alkalinization. After that, cells were incubated with 33 μM C_{12}FDG (Invitrogen) for 4 h. At the end of the incubation, HMC were washed twice with ice-cold PBS, harvested by trypsinization, centrifuged and analyzed immediately using a BD FACSCalibur (Becton Dickinon, BD Bioscience, Franklin Lakes, NJ, USA) flow cytometer. The fluorescent signal was measured with a 488 nm argon laser and β-galactosidase activity was estimated using the mean fluorescence intensity of the population. Autofluorescence of HMC was assessed in parallel in cells not exposed to C_{12}FDG. Data were analyzed with WinMDI software (The Scripps Research Institute, La Jolla, CA, USA).

2.5. Protein extraction and immunoblot analysis

Total protein extracts were prepared by cell lysis with 0.3 ml of lysis buffer composed of 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, 100 mM KCl, 1% Triton X-100, 0.5% (v/v) β-mercaptoethanol, 5 mM NaF, 0.2 mM $NaVO_4$, 5 mM $MgCl_2$, and with protease cocktail inhibitor (Roche, Mannheim, Germany). The resulting solution was spun at 13,000 rpm for 5 min at 4 °C. The protein concentration in the supernatants was determinate by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein from each sample were separated in SDS-polyacrylamide gels under reducing conditions and transferred to PVDF membranes (Perkin Elmer, Boston, MA, USA). Membranes were blocked with 5% non-fat dried milk or 5% bovine serum albumin (BSA) in tween Tris buffered saline (TTBS) (20 mM Tris–HCl, 0.9% NaCl, 0.05% Tween 20) 1 h at room temperature. Monoclonal mouse anti-β-tubulin, monoclonal rabbit anti-actin, monoclonal mouse anti-human p53, monoclonal mouse anti-human p16INK4/CDKN2 (Sigma), monoclonal anti-human p21, monoclonal mouse anti-human Ras (BD Biosciences, Erembodegem, Belgium), polyclonal rabbit anti-ERK 1/2, polyclonal rabbit anti-phospho-ERK 1/2 (Thr202/Tyr204), monoclonal anti-IGF-I receptor β rabbit antibody, monoclonal rabbit anti-phospho-IGF-1 receptor β antibody (Tyr1135/1136), anti-AKT rabbit antibody, anti-phospho-AKT (Ser473) rabbit antibody, anti-FOXO1 rabbit antibody and anti phospho-FOXO1 (Ser319) rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) and polyclonal rabbit anti-human catalase (EMD Biosciences, Gibbstown, NJ, USA), were used to incubated membranes overnight at 4 °C. After being washed in TTBS, the membranes were incubated with anti-mouse IgG peroxidase-conjugated (Sigma) or anti-rabbit IgG peroxidase-conjugated (Dako, Glostrup, Denmark) secondary antibodies. The immunoblots were developed with ECL Western

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