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Loss of HuR leads to senescence-like cytokine induction in rodent fibroblasts by activating NF-κB

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ABSTRACT

Background: HuR (human antigen R) is a ubiquitously expressed member of the Hu/ELAV family of proteins that is involved in diverse biological processes. HuR has also been shown to play an important role in cell cycle arrest during replicative senescence in both human and mouse cells. Senescent cells not only halt their proliferation, but also activate the secretion of proinflammatory cytokines. A persistent DNA damage response is essential for the senescence-associated secretory phenotype (SASP), and increasing evidence has suggested that the SASP is associated with malignancy.

Methods: Senescence-associated phenotypes were analyzed in MEFs and other cell line in which HuR expression is inhibited by sh-RNA-mediated knockdown.

Results: RNAi-mediated HuR inhibition resulted in an increase in SASP-related cytokines. The induction of SASP factors did not depend on ARF–p53 pathway-mediated cell cycle arrest, but required NF-κB activity. In the absence of HuR, cells were defective in the DNA-damage response, and single strand DNA breaks accumulated, which may have caused the activation of NF-κB and subsequent cytokine induction.

Conclusions: In the absence of HuR, cells exhibit multiple senescence-associated phenotypes. Our findings suggest that HuR regulates not only the replicative lifespan, but also the expression of SASP-related cytokines in mouse fibroblasts.

General significance: RNA-binding protein HuR protects cells from undergoing senescence. Senescence-associated phenotypes are accelerated in HuR-deficient cells.

1. Introduction

Cellular senescence halts the proliferation of cells at risk of malignant transformation and acts as a potent tumor-suppressive mechanism in mammals [1,2]. Senescence occurs following a period of cell proliferation, or is acutely induced in response to various cellular insults such as oxidative stress, oncogene activation, or DNA damaging agents [3]. Cellular senescence depends on the activation of two major tumor suppressor pathways, the $p19^{ARF}$ ($p14^{ARF}$ in human)–p53 and $p16^{Ink4a}$–pRB pathways, which play critical roles in inducing and maintaining cell cycle arrest during senescence [4,5]. The inactivation of these pathways bypasses senescence, allowing damaged cells to proliferate.

Besides cell cycle arrest, senescent cells exhibit several characteristics including a flattened morphology, the formation of senescence-associated heterochromatin foci, and an increase in senescence-associated β-galactosidase activity [6]. Furthermore, recent studies have confirmed that senescent cells secrete numerous inflammatory cytokines, which is called the senescence-associated secretory phenotype (SASP) [7]. Interleukin 6 (IL-6) is the most prominent cytokine among the SASP cytokines, and is known to associate with DNA damage or oncogene-induced senescence in several types of mouse and human cells [8]. The consequence of the SASP varies depending on the biological context. For example, some SASP factors can reinforce cell growth arrest and activate immune systems to clear senescent cells from tissues [9–12]. On the other hand, the SASP also has deleterious effects such as the promotion of malignant phenotypes, angiogenesis, and epithelial–mesenchymal transition [8,13–15]. The SASP may also contribute to age-related diseases because senescent cells accumulate in tissues with age [16]. The mechanism of SASP induction is not fully understood. However, NF-κB, which is activated by a persistent DNA damage response (DDR) or p38 signaling pathway, as well as C/EBPβ appear to play pivotal roles in the induction of SASP-related cytokines [17].

HuR is a ubiquitously expressed member of the ELAV/Hu family and is involved in diverse biological processes [18,19]. HuR is an RNA-

Abbreviations: SASP, senescence-associated secretory phenotype; HuR, human antigen R; IL-6, interleukin 6; MEF, mouse embryonic fibroblast; sh-RNA, short hairpin RNA; DDR, DNA damage response; IR, ionizing radiation; UV, ultraviolet

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binding protein that controls the stability, translation, splicing, and intracellular trafficking of its target mRNA [20,21]. HuR was previously shown to be down-regulated during the senescence of human diploid fibroblasts and human tissues with age [22]. $p16^{Ink4a}$ plays pivotal roles in the induction of cellular senescence in human cells, and HuR destabilizes *Ink4a* mRNA in human cells [23]. In contrast, $p16^{Ink4a}$ is dispensable while the ARF–p53 pathway is essential for senescence in rodent cells [24]. We recently demonstrated that HuR suppressed $p19^{ARF}$ expression in mouse embryonic fibroblasts (MEFs), thereby maintaining the replicative lifespan of these cells [25]. The loss of HuR results in premature cellular senescence with concomitant activation of the ARF–p53 pathway, and deletion of either the *ARF* or *p53* gene enables MEFs to grow in the absence of HuR expression. Thus, HuR plays essential roles in the regulation of cellular senescence in both humans and mice.

We herein showed that HuR regulates not only the replicative lifespan, but also the expression of SASP-related cytokines in MEFs. RNAi-mediated silencing of HuR resulted in the induction of several SASP factors including IL-6. Unlike senescence-associated cell cycle arrest, the *ARF* and *p53* genes were dispensable for the induction of cytokines. NF-κB was activated in HuR knockdown cells, and pharmacological inhibition of NF-κB abrogated the cytokine induction in these cells. Under these conditions, we observed the accumulation of single strand DNA breaks, which may have caused the activation of NF-κB activation and subsequent cytokine expression. Thus, this study confirmed the novel role of HuR in the regulation of senescence-associated phenotypes.

2. Materials and methods

2.1. Cells and culture conditions

NIH-3T3 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin–streptomycin. Mouse embryonic fibroblasts (MEFs) were cultured in medium supplemented with 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg/ml gentamicin instead of penicillin and streptomycin. To analyze mRNA stability, cells were treated with 5 μg/ml actinomycin D. To perform the clonogenic survival assay, 10^4 cells were seeded on 10-cm-diameter culture dishes and subjected to irradiation (IR or UV). After 12 days, cultured cells were stained with crystal violet, and the number of colonies per dish was counted. To inhibit NF-κB activity, cells were treated with 5 μM BAY 11-7082 (Wako Pure Chemicals Insustries, Osaka, Japan) for 24 h. To inhibit p38 activity, cells were treated with 5 μM SB203580 (Merck Millipore, Billerica, MA) for 24 h.

2.2. Virus production and infection

293T cells were transfected with sh-SCR or sh-HuR retroviral expression plasmids together with a helper plasmid [26,27]. Viruses were harvested 24 to 60 h after transfection, pooled, and stored on ice. Exponentially growing cells in 10-cm-diameter culture dishes were infected with 3 ml of fresh virus-containing supernatant in complete medium containing 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Infected cells were selected in the presence of 5 μg/ml puromycin (Sigma-Aldrich).

2.3. Annexin V staining

Annexin V staining was performed using Tali® Apoptosis Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instruction. 50,000 cells were stained for each sample.

2.4. Realtime PCR analysis

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. cDNA was generated using a PrimeScript RT reagent kit with gDNA Eraser (TAKARA BIO, Otsu, Japan), and subjected to PCR using the following primers: for 18S, 5′-AGTCCCTGCCCTTTGTACACA-3′ (sense) and 5′-GATCCGAGGGCCTCACTAAAC-3′ (antisense); for *IL-6*, 5′-CAAGAAAGAC AAAGCCAGAGTC-3′ (sense) and 5′-GAAATTGGGGTAGGAAGGAC-3′ (antisense); for *IL-1beta*, 5′-GAAATGCCACCTTTTGACAGTG-3′ (sense) and 5′-CTGGATGCTCTCATCAGGACA-3′ (antisense); for *Ccl-2*, 5′-TAAA AACCTGGATCGGAACCAAA-3′ (sense) and 5′-GCATTAGCTTCAGATTTA CGGGT-3′ (antisense); for *VEGF*, 5′-CCGAAACCATGAACTTTCTG-3′ (sense) and 5′-AGATGTACTCTATCTCGTCG-3′ (antisense); for *Cxcl1*, 5′-ACTGCACCCAAACCGAAGTC-3′ (sense) and 5′-TGGGGACACCTTTTAG CATCTT-3′ (antisense); for *Cyclin D1*, 5′-GCGTACCCTGACACCAATCTC-3′ (sense) and 5′-CTCCTCTTCGCACTTCTGCTC-3′ (antisense); for *GAPDH*, 5′-AATGGTGAAGGTCGGTGTG-3′ (sense) and 5′-GAAGATGG TGATGGGCTTCC-3′ (antisense). Real-time PCR analysis was performed on a Chromo4 realtime PCR system (Bio-Rad, Hercules, CA). Values were normalized to 18S rRNA or *GAPDH* in each sample.

2.5. Immunoblotting

Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (10 mM Na-phosphate pH 7.2, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, and 1% NP-40) containing protease inhibitors (Roche, Indianapolis, IN). Nuclear and cytoplasmic lysates were prepared as previously described [26]. Lysates were separated on denaturing polyacrylamide gels containing 0.1% SDS and transferred to PVDF membranes (Millipore, Billerica, MA). Proteins were detected using antibodies to HuR (3A2; Santa Cruz Biotechnology, Santa Cruz, CA), Lamin A/C (H-110; Santa Cruz Biotechnology), α-Tubulin (B-5-1-2; Sigma-Aldrich), phospho-H2AX (γH2AX) (Ser139; Millipore), p38 (Cell Signaling Technology, Danvers, MA), and phospho-p38 (Thr180/182; Cell Signaling Technology). Antibody binding sites were detected using HRP-conjugated antibodies to mouse or rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

2.6. Analysis of mRNA and ribosome association

In vivo ribosome association was analyzed as previously described [25]. Briefly, cytoplasmic extracts were prepared from MEFs expressing GFP or GFP-L10, and immunoprecipitated using GFP antibody-coated magnetic beads (Medical & Biological Laboratories, Nagoya, Japan). RNA was extracted from immune complex, and subjected to realtime PCR analysis.

2.7. Immunofluorescence

Cells seeded on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized in 0.5% Triton X-100/PBS for 15 min at room temperature, and stained with antibodies to NF-κB (C-20; Santa Cruz Biotechnology) or γH2AX. Washed coverslips were incubated with Alexa488-conjugated antibodies to rabbit IgG (Jackson ImmunoResearch) and mounted using Vectashield and 4′, 6′-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Cells were grown to confluent in a 24-well dish and cultured in 1 ml/well fresh cell culture medium for 4 days. The amount of the IL-6 protein in each conditioned medium was determined using the mouse IL-6 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.