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Chronic NF-κB activation delays RasV12-induced premature senescence of human fibroblasts by suppressing the DNA damage checkpoint response

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ABSTRACT

Normal cells divide for a limited number of generations, after which they enter a state of irreversible growth arrest termed replicative senescence. While replicative senescence is due to telomere erosion, normal human fibroblasts can undergo stress-induced senescence in response to oncogene activation, termed oncogene-induced senescence (OIS). Both, replicative and OIS, initiate a DNA damage checkpoint response (DDR) resulting in the activation of the p53–p21$^{Cip1/Waf1}$ pathway. However, while the nuclear factor-kappaB (NF-κB) signaling pathway has been implicated in DDR, its role in OIS has not been investigated. Here, we show that oncogenic Ha-RasV12 promoted premature senescence of IMR-90 normal human diploid fibroblasts by activating DDR, hence verifying the classical model of OIS. However, enforced expression of a constitutively active IKKβ T-loop mutant protein (IKKβca), significantly delayed OIS of IMR-90 cells by suppressing Ha-RasV12 instigated DDR. Thus, our experiments have uncovered an important selective advantage in chronically activating canonical NF-κB signaling to overcome the anti-proliferative OIS response of normal primary human fibroblasts.

1. Introduction

Normal diploid mammalian cells undergo a finite number of cell divisions in culture, a phenomenon termed cellular senescence (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). Several possible mechanisms have been suggested to explain the manner in which diploid cells senesce and by which immortal cells evade senescence. In addition to telomere length and telomerase activity (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Sedelnikova et al., 2004), the pRb and p53 pathways are also involved in regulating cellular senescence through the CDKN2A locus which encodes the p16^{INK4A} and ARF proteins by alternative splicing in both human and mouse cells (Hahn and Weinberg, 2001; Serrano and Blasco, 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007).

Telomere shortening is not the only inducer of the senescent phenotype. Normal cells possess anti-proliferative mechanisms to counteract the consequences of oncogenic mutations, and these natural cell defenses are often disrupted during tumor development. These anti-proliferative mechanisms are often activated in response to oncogenic stress that delivers excess mitogenic signaling leading to cell growth arrest or premature cell senescence (Hahn and Weinberg, 2001). Oncogenic Ha-RasV12 (RasV12 thereafter) (Franza et al., 1986; Serrano et al., 1997; Lin et al., 1998; Woo and Poon, 2004) or Raf (Zhu et al., 1998) promote uncontrolled mitogenesis but when expressed in primary cells including normal human diploid fibroblasts (HDFs) and rodent cells they provoke a permanent cell cycle arrest with features of senescence, in the absence of telomere shortening (Serrano and Blasco, 2001; Hahn and Weinberg, 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). Induction of human cell senescence by oncogenic RasV12 is accompanied by increased expression of

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$p16^{INK4a}$ and requires the MAPK cascade (Ras-Raf-MEK) (Serrano et al., 1997; Lin et al., 1998). These anti-proliferative responses of primary cells to activated oncogenes also explain why the formation of a transformed cell clone often depends on the properties of additional altered genes, which neutralize the anti-proliferative defense mechanism that was triggered by the primary activated oncogene (Weinberg, 1997; Hahn and Weinberg, 2001). Because it entails an essentially irreversible growth arrest, the p53 and pRb dependent senescence responses induced by either telomere shortening or oncogenic stimuli likely represents a highly conserved surveillance-like, tumor suppressive mechanism (Hahn and Weinberg, 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007).

Induction of senescence due to telomere erosion (d'Adda di Fagagna et al., 2003; Herbig et al., 2004) or oncogenic stress (Bartkova et al., 2006) such as that induced by RasV12 (Di Micco et al., 2006, 2007; Mallette et al., 2007) initiates a DNA double strand break (DSB) checkpoint response (DDR), which involves activation of the kinases ATM/ATR, Chk1 and Chk2 and their downstream effector p53 (Bartek and Lucas, 2007; Hemann and Narita, 2007; Di Micco et al., 2007; Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). Previous studies showed that oncogenic RasV12 induces ARF (Palmero et al., 1998) leading to the induction of p53 phosphorylation at serine 15, a target site of ATM/ATR, and ARF which has been implicated in the modulation of NF-κB function by repressing the transcriptional activity of the anti-apoptotic Rel (p65) NF-κB subunit (Rocha et al., 2003, 2005). Furthermore, in response to DSBs ATM activation induces the inhibitor of NF-κB kinase (IKK) complex (Li et al., 2001) and after the induction of DSBs, NEMO/IKKγ was also shown to associate with ATM to directly orchestrate IKK activation (Wu et al., 2006). This DNA damage-induced NF-κB signaling response has been proposed to be necessary for cell survival during the DDR (Janssens and Tschopp, 2006; Wu and Miyamoto, 2007; Ahmed and Li, 2008; Brzóska and Szumiel, 2009).

The NF-κB transcription factors are pivotal regulators of gene expression programs culminating in stress-like responses. They bind to DNA as hetero- or homodimers that are selectively derived from five possible subunits (RelA/p65, c-Rel, RelB, p50 and p52). All NF-κB family members contain an N-terminal Rel homology domain that mediates DNA binding and dimerization and a nuclear localization domain. The Rel subfamily members RelA/p65, c-Rel and RelB also contain a C-terminal transactivation domain which is absent in the p50 and p52 subunits. In addition the p50 and p52 subunits are processed from precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. The p50/p65 heterodimers are bound to IκBs (inhibitors of NF-κB) thereby sequestering them in the cytoplasm of most cells in the absence of a stress-like response. Kinases that directly activate NF-κB mediate the site-specific phosphorylation of two amino-terminal serines on each IκB (serines 32 and 36 of IκBα), which makes adjacent lysines targets for ubiquitination thereby resulting in 26S proteasome mediated IκB degradation. NF-κB is then free to translocate to the nucleus and bind DNA leading to the activation of a host of target genes. IκB phosphorylation is mediated by a high molecular weight signalsome complex comprising two direct IκB kinases, IKKα and IKKβ, and two molecules of a regulatory, docking/adapter protein, NEMO. IKKα and IKKβ are serine/threonine kinases possessing an amino-terminal catalytic domain and two carboxyl-proximal interaction motifs resembling leucine zipper and helix-loop-helix domains. Activation of IKKβ depends upon signal-induced phosphorylation of serines 177 and 181 in its T-activation loop (Hayden and Ghosh, 2004; Karin and Greten, 2005; Scheidereit, 2006; Perkins, 2006, 2007).

Whereas the expression and activity of NF-κB have been extensively studied following oxidative stress or during inflammation, apoptosis and transformation, the available data on potential roles of NF-κB in ageing-related changes, and in particular during *in vitro* replicative senescence of human fibroblasts, is relatively scant (Gosselin and Abbadie, 2003). Moreover, contrary to the aging-induced up-regulation of NF-κB binding activities in tissues (Gosselin and Abbadie, 2003; Adler et al., 2007; Kriete and Mayo, 2009), data from cultured human cells have also produced apparently conflicting observations (Dimri and Campisi, 1994; Aggarwal et al., 1995; Helenius et al., 1996, 1999; Ikebe et al., 2000) and suggested a cell-intrinsic activation of NF-κB different from the canonical pathway (Kriete et al., 2008; Kriete and Mayo, 2009).

Oncogenic RasV12 can activate multiple effector pathways that give rise to different outputs depending on the cellular context. Thus, RasV12 can induce senescence of primary cells (Serrano et al., 1997), cooperate with other genes to induce neoplastic transformation (Hahn and Weinberg, 2001), and also suppress or induce apoptosis (Cox and Der, 2003). While OIS of primary cells was shown to be due to DDR (Di Micco et al., 2006, 2007; Bartkova et al., 2006; Mallette et al., 2007) and DSBs activate NF-κB (Janssens and Tschopp, 2006; Habraken and Piette, 2006; Wu and Miyamoto, 2007), it remains unclear whether NF-κB activation influences the outcome of OIS.

Here we directly assessed the consequences of chronic NF-κB activation in OIS by the classical model of oncogenic RasV12-induced senescence of IMR-90 HDFs. IMR-90 cells stably expressing oncogenic RasV12 or IKKβca (a constitutively active IKKβ T-loop mutant) or both genes together were generated by stable retroviral transduction. We found that IKKβca delayed oncogenic RasV12-induced premature senescence of IMR-90 by suppressing the DDR triggered by oncogenic stress.

2. Experimental procedures

2.1. Cell culture

Human diploid fibroblasts IMR-90 and amphotropic phoenix cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. To retain equivalence between passage number and population doublings (Pdls), a 1:2 split was counted as 1 Pdl and a 1:4 as 2 Pdls.

2.2. Retroviral vectors and infections

The retroviral vectors used were: pBabe-Hygro, pWZLH-Ha-RasV12 (71), CLXSN-ires-GFP (CLXSN-iG) and CLXSN-IKKβca-iG carrying a constitutively active IKKβ T-loop mutant protein of human IKKβ (denoted IKKβca). A constitutively activated human Flag-IKKβ (IKKβca) mutant in pcDNA3.1 was generated by changing the T-loop activation serines (177 and 181) to glutamic acids with a QuickStart PCR mutagenesis kit (Stratagene) (PE Massa and KB Marcu, unpublished data). The IKKβca cassette was removed from pcDNA3.1 and placed under the control of a moloney 5′ LTR in CLXSN-iG by standard sub-cloning procedures. CLXSN-iG expresses a neomycin resistance gene (Neo) under the control of the SV40 promoter/enhancer (SN cassette) and IKKβca was inserted in between the retroviral LTR and an Ires-GFP sequence upstream of the vector's SN cassette (Zhang et al., 2005).

Young IMR-90 cells (P11) were infected with high-titre retroviruses carrying either oncogenic RasV12 or IKKβca or both genes together or with the corresponding control vectors, generated following transfection of amphotropic phoenix cells. Large polyclonal populations of stable retroviral transduced cells were obtained by selection in hygromycin B (HygroB) or G418 for 2 and 3 weeks, respectively. Neo-resistant IKKβca cells were infected with a RasV12-Hygro virus and submitted to selection in HygroB and G418 for an additional 3 weeks. HygroB- and/or G418-resistant polyclonal cell populations were used in all subsequent analyses.

2.3. Analysis of growth properties

Uninfected and retrovirus-infected IMR-90 were plated at a density of 2×10^4 cells per well in 24-multiwell plates. Cell growth was monitored by counting the cells from two wells every two days using a hemocytometer over a period of 12 days. The experiment was repeated three times and growth curves were constructed.

Exponentially growing IMR-90 cells were subjected to flow cytometric analysis using a cycle test™ plus DNA reagent kit (Becton Dickinson) according to manufacturer's instructions and analyzed on a FACScan Becton Dickinson flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Cell morphologies were observed under a Zeiss Model Axiovert S100, Germany and photographed.