A Genetic Screen Identifies Topoisomerase 1 as a Regulator of Senescence

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Abstract

Normal cell growth can be permanently blocked when cells enter a state known as senescence. This phenomenon can be triggered by various stresses, such as replicative exhaustion, oncogenic stimulation, or oxidative stress. Senescence prevents transmission of aberrant signals to daughter cells and thus prevents irreversible damage that could favor cancer development. To identify new genetic events controlling senescence, we have performed a loss-of-function genetic screen on normal human cells. We report that knockdown of topoisomerase 1 (Top1) results in an increased replicative potential associated with a decrease in senescence markers and a diminished DNA damage response. In addition, Top1 depletion also favors a bypass of oncogene-induced senescence. Conversely, Top1 constitutive expression induces growth arrest, the appearance of a senescence marker, and an activation of the DNA damage response. Altogether, these results reveal an unanticipated function of Top1 in regulating senescence. [Cancer Res 2009;69(10);1101–6]

Introduction

Cancer cells derive from normal cells that accumulate genetic and epigenetic alterations. During this process and among other steps, normal cells acquire immortality and enhanced stress resistance (1). Cellular senescence was originally described as a replicative potential limit of normal cells. It has been redefined recently as a cellular stress response program occurring in normal cells and occasionally in cancer cells, depending on the context. It is now widely accepted that the genetic events triggering a bypass of cellular senescence favor tumorogenesis by extending the replicative potential, rendering cells more resistant to cellular stresses, or both (2).

The p53 pathway is a major regulator of senescence and tumorigenesis. During tumorigenesis, multiple cooperating genetic events can result in inactivation of the p53 tumor suppressor pathway (2). One major activator of the p53 pathway is induction of DNA damage. Interestingly, the DNA damage response pathway is activated during senescence and its loss allows senescence escape and is an early step required for tumor development (3–5).

To identify new loss-of-function genetic events involved in the control of cellular senescence, we have performed a genetic screen on normal human diploid fibroblasts (HDF), using an shRNA library. We have thus identified topoisomerase 1 (Top1) as a regulator of senescence, revealing a new function for an old target of cancer treatment.

Materials and Methods

Cell culture and vectors. WI38 and IMR-90 cells (American Type Culture Collection) and packaging GP 293 cells (Clontech) were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and gentamycin (Invitrogen). The pRS retroviral library was prepared from the pRS library (6). The shRNA sequence inserted into the pRS to generate pRS/Top1.2 was 5′-GGTCCATAATTCACCACTCA-3′. The 2nd shRNA used was purchased and used as recommended (V2HS_171464; Open Biosystems). The pMC-GFP-Top1 vector was kindly provided by Dr. M.O. Christensen (Institute of Clinical Chemistry and Laboratory Diagnostics, Dusseldorf, Germany; ref. 7).

Transfection, infection, and genetic screening. GP 293 cells were transfected using PEI reagents (Euromedex). Three days after transfection, viral supernatant mixed with fresh media (1/2) and polybrene (8 μg/mL) was used to infected target cells. In the genetic screening experiments, WI38 cells close to senescence were infected with the pRS control retroviral vector or with the pRS library (6) and infected cells were selected by puromycin treatment at 500 ng/mL. The selection pressure was maintained at 200 ng/mL. WI38 cells were split every week for 3 wk, and the gDNA of proliferating cells was prepared. Nested PCRs were performed with the following primer pairs: PCR1 reverse 5′-GAGACGTTGC-TACTTCCATTGTC-3′ and forward 5′-CCCTTGAACCTCCTCGTTCGACC-3′, PCR2 reverse 5′-TGTTAGGGGACAGGGGAGGAG-3′ and forward 5′-ACCTC-TCTGGTGCGACC-3′. A PCR (20 cycles) was performed with primer pair PCR1, and 1% of this PCR reaction product was then subjected to a 20-cycle PCR with primer pair PCR2. The PCR product was cloned with the help of the TOPO TA cloning kit (Invitrogen) and subjected to sequence analysis.

Colony formation assays. In the WI38 or IMR-90 colony formation assays, 90,000 cells were seeded and left to grow for between 1 and 2 wk. Then the cells were fixed with 4% formaldehyde and stained with crystal violet. In the case of U2OS cells, 200,000 cells were seeded into 10-cm dishes 1 d after transfection and selection was done with 300 ng/mL puromycin for 2 d. The cells were fixed and stained 10 d after seeding. Experiments were at least performed independently twice.

Senescence analysis. When cells acquired typical morphology of senescent cells, analysis of senescence markers was performed. Senescence-associated β-galactosidase activity analysis was performed as described by Dinri and colleagues. (8). For senescence-associated heterochromatin foci analysis (9), cells were fixed by formaldehyde 4%, wash with PBS and stained with Hoechst (Sigma). DNA staining was examined under a Zeiss fluorescence microscope.

Immunofluorescence, immunoblotting, staining, and antibodies. For immunofluorescence, cells were fixed with 4% paraformaldehyde in 1× PBS and stained with Hoechst (Sigma). DNA staining was examined under a Zeiss fluorescence microscope.
PBS, permeabilized with 0.2% Triton X-100 and blocked in PBS/serum 10%. Briefly, cells were incubated with a primary antibody followed by a secondary antibody coupled to Rhodamin (Amersham). Nuclei were stained with Hoechst 33258 at 1 mg/mL for 3 min.

For immunoblotting, cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used were anti-Top1 (556597; BD Biosciences), anti–phospho-p53 (9284; Cell Signaling), anti–phospho-ATM (4526; Cell Signaling), anti-p53 (sc-126; Santa Cruz Biotechnology), anti–phosphoSer10-H3 (ab14955; Abcam), anti-p21 (sc-397; Santa Cruz Biotechnology), and antiactin (A5316; Sigma-Aldrich). The corresponding peroxidase-labeled secondary antibody was detected with Western Lighting Chemiluminescence Reagent Plus (NEL103; Perkin-Elmer).

Comet assay. For each condition, 10,000 cells were embedded in 80 μL of 0.5% low-melting-point agarose at 37°C and the suspension was immediately pipetted onto a TREVIGEN, Inc., cometslide. Agarose was allowed to solidify at 4°C for at least 30 min. The slides were immersed in prechilled lysis solution [2.5M NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, and 1% Triton (pH 10)] and left at 4°C for 90 min in the dark. The cometslides were placed in a horizontal electrophoresis unit, and equilibrated in electrophoresis buffer for 30 min at 4°C in the dark. For assessing DNA strand breaks, the migration step was performed at pH of >13 in alkaline solution (300 mmol/L NaOH, 1 mmol/L EDTA) for 20 min at 1 V/cm, after which the migration slides were neutralized with 0.4 mol/L Tris (pH 7.5). The slides were stained with propidium iodide (2.5 μg/mL). Comet assays were performed twice.

Statistics. Microsoft Excel software was used to perform a student test. P values are directly indicated in the figures.

Results

A decreased Top1 level prevents senescence. In recent years, senescence escape has emerged as a key step in tumor

Figure 1. Top1 down-regulation extends the cellular life span. A, summary of the genetic screen. B, WI38 cells were infected with retroviral vectors encoding an shRNA directed against Top1 or a control vector. After selection, cell extracts were prepared and resolved. The Top1 protein level was revealed using a Top1 antibody and an actin antibody was used as a loading control (ctrl). Alternatively after selection, WI38 and IMR-90 were seeded at low density. After 2 wk, the cells were crystal violet stained. C, WI38 cells were infected with the indicated retroviral vector and puromycin selected. When cell growth was starting to slow down, senescence-associated heterochromatin foci (SAHF) marker was analyzed. D, analysis of senescence-associated β-galactosidase marker (SA-β-Gal).

Figure 2. Top1–down-regulated cells bypass oncogene-induced senescence. WI38 cells were coinfected with a MEK/ER vector and with a control or a shTop1 vector. Cells were selected and seeded at the same density. After 2 d, 4OHTamoxifen was added once at 100 nmol/L. Senescence-associated β-galactosidase analysis was performed after 3 d (A) and growth analysis after 3 wk (B).
Topoisomerase 1 Regulates Senescence Outcome

We next wondered whether Top1 knockdown could favor bypass from oncogene-induced senescence. WI38 cells stably expressing MAP/ERK kinase (MEK)/ER with a control or an shTop1 were seeded at the same density. Three days after 4OHTamoxifen treatment, 50% of control cells were senescence-associated β-galactosidase–positive, whereas no increase was observed in shTop1-expressing cells (Fig. 2A). Three weeks after treatment, cells were stained by crystal violet. Top1-depleted cells treated with 4OHTamoxifen formed colonies, whereas control cells did not (Fig. 2B). Taken together, these results reveal that down-regulation of Top1 expression induces a bypass of senescence.

Top1 constitutive expression blocks cell growth. Because Top1 affects senescence, we next sought to determine whether Top1 expression might influence the growth of normal and cancer cells. For this, we transiently expressed a GFPTop1 fusion protein in U2OS cancer cells and checked its overexpression by immunoblot (Fig. 3A, top band). Proliferation of U2OS or WI38 cells constitutively expressing GFPTop1 was much slower than that of control-transfected U2OS cells (Fig. 3B). This was confirmed by the inability of U2OS cells expressing GFPTop1, after brief selection, to form colonies after 10 days of culture when seeded at low density (Fig. 3C). Interestingly, DNA labeling revealed that Top1 expression in U2OS cells as well as in WI38 cells induced appearance of senescence-associated heterochromatin foci (Fig. 3D). Thus, Top1 constitutive expression induces a growth arrest and the appearance of a senescent marker in WI38 and U2OS cells.

Top1 modulates the DNA damage–p53 response. Because the DNA damage pathway is involved in regulation of senescence (3–5), we next investigated whether the DNA damage pathway might...
be altered by Top1. We first observed the effect of stable Top1 down-regulation on the DNA damage pathway. To this end, we first performed a comet assay that consists in visualizing DNA strand breaks by migrating denatured DNA of individual cells (Fig. 4A). The quantification of these results showed a difference, ~6-fold, between the numbers of cells with DNA breaks in the control senescent HDFs versus the Top1-depleted HDFs (Fig. 4B). This was further confirmed by performing immunoblot analysis. Indeed, Top1-depleted cells displayed lower levels of phospho-ATM, phospho-p53, p53, and its target p21 at protein levels and RNA level for p21 (Fig. 4C and D).

We next used appropriate antibodies (anti–phospho-ATM, anti–phospho-p53, and anti-p53) to examine the expression of key players of the pathway in cells expressing GFP-Top1 or GFP. Interestingly, U2OS cells expressing the GFP-encoding vector displayed weak to no antibody labeling (Fig. 5A, top), whereas expression of the GFP-Top1 protein resulted in accumulation of all three DNA damage markers (Fig. 5A, bottom). In WI38 cells, a majority of GFPTop1-transfected cells were positive for phospho-ATM labeling, whereas ~20% of GFP-transfected cells were positive (Fig. 5B). Furthermore, measurement of p53 activity in the presence of an increasing amount of Top1 with a constant amount of a p53 activity reporter revealed dose-dependent activation of p53 by Top1 (Fig. 5C). To further confirm the involvement of the p53 pathway, we examined the effect of p53 inhibition by a dominant negative form of p53 (p53DN) over the proliferation arrest induced by GFPTop1. Interestingly, p53 pathway inhibition reverted efficiently the proliferation arrest induced by GFPTop1 (Fig. 5D). Altogether, these results support that Top1 acts through the DNA damage-p53 pathway.

**Discussion**

In summary, we here describe a loss-of-function genetic screen, performed on HDFs that enabled us to identify Top1 as a regulator of senescence. We show that Top1 down-regulation is delay senescence of normal cells, and that its overexpression arrests proliferation of both normal and cancer cells.

Top1 is known to attack the DNA and forms a covalent Top1-DNA intermediate required for DNA relaxation during cellular processes such as replication and transcription. Compounds such as camptothecin are used in clinic to provoke death of dividing cells by stabilizing the Top1-DNA intermediate and inducing DNA damage (15). Interestingly, camptothecin also induces senescence in HDFs (16). Analysis on p16 reveals no change in its expression in the presence of an increasing amount of Top1 with a constant amount of a p53 activity reporter revealed dose-dependent activation of p53 by Top1 (Fig. 5C). To further confirm the involvement of the p53 pathway, we examined the effect of p53 inhibition by a dominant negative form of p53 (p53DN) over the proliferation arrest induced by GFPTop1. Interestingly, p53 pathway inhibition reverted efficiently the proliferation arrest induced by GFPTop1 (Fig. 5D). Altogether, these results support that Top1 acts through the DNA damage-p53 pathway.

We checked whether Top1 expression increases during senescence. Even, if Top1 expression increased during oncogene-induced senescence (Supplementary Fig. S3), we did not detect any increase during replicative senescence (Supplementary Fig. S3). However, we cannot discard that Top1 might be regulated, at least during replicative senescence, at different levels than its expression such as by phosphorylation (17) and/or by activity of corepressor or coactivator (18) or/and by acting in synergy with other pathways.

We did not detect change in telomere length between shTop1-expressing cells and control cells, suggesting that Top1 is acting downstream of telomere length. This is supported by the fact that Top1 knockdown favors the oncogene-induced senescence escape, a senescence independent of telomere length. Interestingly, one of the factors contributing to replicative or oncogene-induced senescence is the accumulation of DNA lesions at telomere site or at replicative foci, respectively (3–5). As endogenous DNA lesions can recruit Top1 at the damaged sites (19), Top1 manipulation affects the response to DNA damage.

Top1 down-regulation enables HDFs to keep growing whereas control cells enter senescence, Top1 might thus be expected to exert an antioncogenic function. Yet Top1 expression is generally...
increased in cancer samples (Oncomine database; ref. 20), excepted, eventually, in Top1-inhibitors unresponsive tumors (21). It should be stressed, however, that in most cancers the DNA damage-p53 pathway is disabled (1, 22, 23). We thus propose that the effect of Top1 on senescence can be largely circumvented if the DNA damage-p53 pathway is attenuated or inactivated, as is generally the case in cancer cells. As an illustration, HDFs immortalized by hTert displayed a decrease in Top1. When large T (a p53 inhibitor) is added, Top1 increases. When an oncogenic Ras is further added, Top1 strongly increases (24). Accordingly, we observed that oncogenic Ras results in Top1 increase (Supplementary Fig. S3B). Ras or other oncogenes are able to induce senescence in normal cells but not in DNA damage response-p53 inhibited cells (3, 5), a response in which Top1 could be involved according to our results. In conclusion, our data reveal a complex role of Top1 during immortalization and transformation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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