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Superoxide Dismutase 1 Knock-down Induces Senescence in Human Fibroblasts*

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Reactive oxygen species (ROS) such as superoxide radicals are responsible for the pathogenesis of various human diseases. ROS are generated during normal metabolic process in all of the oxygen-utilizing organisms. The copper-zinc-containing SOD (SOD1) acts as a major defense against ROS by detoxifying the superoxide anion. In model organisms, SOD1 has been shown to play a role in the aging process. However, the exact role of the SOD1 protein in the human aging process remains to be resolved. We show that SOD1 RNA interference (RNAi) induces senescence in normal human fibroblasts. This premature senescence depends on p53 induction. In contrast, in human fibroblastic cells with inactivated p53, the SOD1 RNAi is without effect. Surprisingly, in cancer cells (HeLa), the SOD1 RNAi induces cell death rather then senescence. Together, these findings support the notion that in normal human cells the SOD1 protein may play a role in the regulation of cellular lifespan by p53 and may also regulate the death signals in cancer cells.

ROS[1] are generated by normal metabolic processes in all of the oxygen-utilizing organisms. It is estimated that around 1% of the total oxygen consumed in the mitochondria becomes the superoxide anion (1). Damage induced by ROS includes DNA mutation, protein oxidation, and lipid peroxidation. ROS may contribute to the development of various diseases, such as cancer, diabetes, atherosclerosis, inflammation, and premature aging (2, 3).

The superoxide dismutases (SODs) constitute a family of antioxidant enzymes that catalyzes the conversion of superoxide anions to oxygen and hydrogen peroxide (for recent review on SODs, see Ref. 4). They include the manganese-containing SOD (SOD2) in the mitochondria (5) and the copper-zinc-containing SOD (SOD1) in the cytoplasm (5) with a small fraction in the mitochondria intermembrane space (6). >90 different mutations in the *SOD1* gene have been found to associate with amyotrophic lateral sclerosis, a disease that causes the degradation of motor neurons (7).

The free radical theory of aging states that ROS generation during metabolism can cause damage to cellular constituents (8). During the life of a cell or an organism, SODs and catalase detoxify ROS. However this process is not perfect and ROS species can cause cumulative damage, which causes the physiological decline characteristic of aging (for review, see Ref. 9). In yeast survival, the stationary phase appears to be limited by ROS because deletion of either SOD1 or SOD2 shortens survival time (10). In Drosophila, overexpression of the human SOD1 extends the fly life span by 40% over controls (11).

After a finite number of divisions, primary human cells in culture enter into a state of replicative senescence in which they are growth-arrested and resistant to mitogenic stimulation. Senescence is considered a mechanism to suppress tumorigenesis because it inhibits cell proliferation (12). Oxidants appear to be important in the development of the senescent phenotype. Cells grown in low oxygen tension exhibit a prolonged life span (13), whereas cells grown in high oxygen concentration have a reduced life span (14). The physiological response to oxidative damage appears to trigger growth arrest via activation of the p53 tumor suppressor, which up-regulates cyclin-dependent kinase inhibitors (15, 16).

One of the features associated with cancer cells is acquisition of the potential to undergo an indefinite number of cell divisions. This feature commonly described as cellular immortalization requires the loss of molecular mechanisms that normally mediate the induction of replicative senescence (reviewed in Refs. 17 and 18). Replicative senescence can be substantially delayed by abrogation of endogenous p53 function through antisense or dominant-negative mutants (19, 20). It is not surprising that the loss of p53, usually in combination with additional genetic alterations, promotes cellular immortalization (21–23).

To directly explore the role of the SOD1 protein in human cellular senescence, we have investigated the effect of SOD1 RNAi on the life span of normal human fibroblasts. We report that SOD1 RNAi induces senescence in WI38 cells. This premature senescence is associated with p53 induction. In human fibroblastic cells in which p53 is not active, the SOD1 RNAi fails to induce senescence. Surprisingly, in cancer cells the SOD1 RNAi induces cell death. Together, these findings support the notion that in normal human cells SOD1 plays a role in delaying p53 induction of cellular senescence and can also regulate death signals in cancer cells.

EXPERIMENTAL PROCEDURES

Plasmids—RNAi plasmids were constructed using pSUPER as the backbone plasmid. pSUPER was obtained from Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). SOD1 RNAi was constructed by cloning the sequence 5′-GGCCTGCATGGATTCCATG-3′ into the pSUPER plasmid. This sequence was determined

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[1] The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismustase; RNAi, RNA interference; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.

to be unique to the *SOD1* gene by BLAST search of GenBank™ data base. pEGFP was obtained from Clontech (Palo Alto, CA).

Cells and Transfections—WI38 and WI38-SV40 cells were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. WI38 cells were maintained in minimum Eagle's medium containing 10% fetal calf serum. Cells were grown in a humidified incubator containing CO_2 (5% v/v) at 37 °C.

HeLa and WI38 transfection were performed using jetPEI (Qbiogene, Carlsbad, CA) and FuGENE 6 (Roche Applied Science), respectively. WI38 cells were co-transfected with pSUPER-SOD1 RNAi and the pBABE-puro as a selection marker. After 24 h, the transfected cells were selected with the puromycin for the following 4 days.

Western Blotting—Cells were lysed in protein sample buffer (50 mM Tris-HCl (pH 6.8) containing 2% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromphenol blue), vortexed, boiled for 5 min, and centrifuged at 17,000 × *g* for 5 min. Extract corresponding to equivalent cell numbers were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and incubated for 20 min with a blocking solution (2.5% lowfat milk, 0.1% Tween 20 in PBS). Membranes were incubated with a p53-specific monoclonal antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-SOD1 antibody FL-154 (Santa Cruz Biotechnology), and as a loading control anti-Actin C-4 (Sigma). Secondary antibodies were goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:10,000). Chemiluminescent signal was generated by incubation with the ECL reagent (Amersham Biosciences).

Immunofluorescence Microscopy—WI38 cells grown on coverslips were transfected and prepared for epifluorescence microscopy. Cells were washed twice with cold PBS and then incubated in 4% paraformaldehyde at room temperature for 10 min. The fixed cells were washed three times with PBS and incubated for 10 min in 0.5% Triton X-100. Cells were washed carefully and incubated for 60 min with a monoclonal antibody against p53 (DO-1) and then with a Alexa594-conjugated chicken anti-mouse secondary antibody. Coverslips were mounted with VectaShield mounting media containing 4′,6-diamidino-2-phenyindole, dilactate (Vector, Burlingame, CA). Epifluorescence microscopy was performed with a Nikon microscope.

β-Galactosidase Staining—Cells on coverslips were washed in PBS and fixed for 5 min in 2% formaldehyde, 0.2% glutaraldehyde. Fixed cells were washed with PBS and incubated at 37 °C (no CO_2) with fresh senescence-associated β-galactosidase stain solution (sodium phosphate buffer (pH 6.0) containing 1 mg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml 40 mM citric acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Staining was detected by light microscopy following the overnight incubation.

Death Analysis—HeLa cells (5×10^5/6-cm dish) and WI38 cells (1.2×10^5/3.5-cm dish) were transfected with a plasmid encoding EGFP with the indicated combinations of expression plasmids. Cultures were analyzed with fluorescent microscope, and transfected cells were identified by the presence of green fluorescence. Apoptotic cells were identified by their round morphology in contrast to the spread-out non-apoptotic cells. Apoptotic cells were counted, and their percentage from the total population of fluorescent cells was calculated.

RESULTS

Because SOD activity decreases with age (24), we investigated whether levels of the protein decreased with increasing population doubling in WI38 cells. To this end, WI38 cells were subject to immunoblotting with an anti-SOD1 antibody. SOD1 protein levels did not vary between population doubling 33 and 49 (Fig. 4*A*).

To determine the functional effect of SOD1 in cellular aging, we investigated the result of decreasing SOD1 levels within cells by RNAi. Three weeks after transfection, cells with the empty pSUPER vector (control) replicated normally (Fig. 1*B*). In contrast, the SOD1 RNAi-transfected cells no longer divided. In addition the SOD1 RNAi morphology was aberrant and cells had an increased cell area and flattened appearance (Fig. 1*A*). Moreover, the cells stayed flattened and did not divide for 100 days. This phenotype is identical to that observed during classical senescence (25, 26).

We next addressed the possibility that the senescence-like phenotype of the SOD1 RNAi cells was because of mortality incurred during transfection and selection. WI38 cells were transfected with the SOD1 RNAi or the control vector together with an EGFP-expression plasmid. Transfection with either SOD1 RNAi or control plasmids did not result in detectable death during the first 2 days (Fig. 1*F*, *days 1* and *2*). Three days post-transfection, a minimal fraction of cells did exhibit death-associated morphological features. However, we could not find any significant difference between the SOD1 RNAi and the control plasmid-transfected cells (Fig. 1*F*, *3 days*). Thus the senescence induced by SOD1 RNAi was not an immediate effect of transfection.

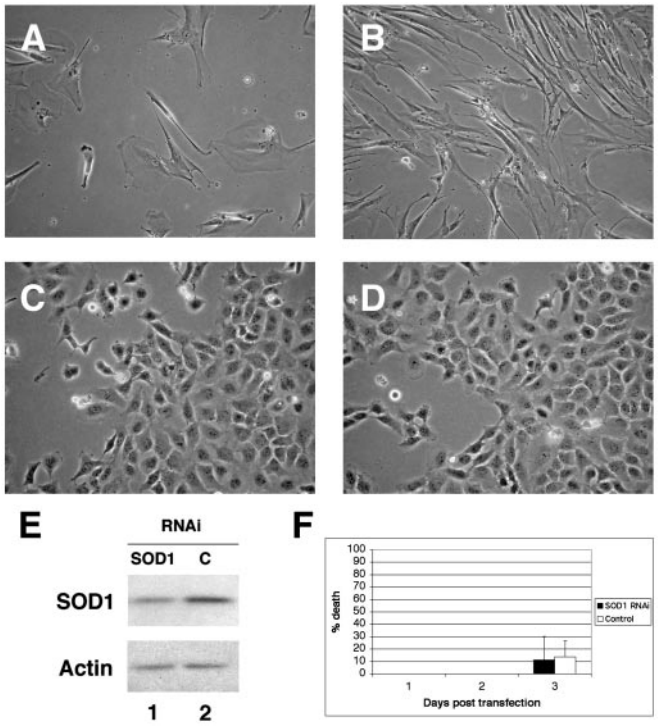

FIG. 1. **SOD1 RNAi creates a morphological change in Wi38 cells.** *A* and *B*, WI38 cells at population doubling 37 were transfected with SOD1 RNAi (*A*) or RNAi vector control (*B*). Cells were co-transfected with pBabe-puro, and 24 h post-transfection, cells were selected with puromycin for a period of 4 days. The photographs were taken 21 days after the transfection. *C* and *D*, WI38 SV40 cells were transfected with SOD1 RNAi (*C*) or RNAi vector control (*D*). Cells were processed as in *A* and *B*. The photographs were taken 14 days after the transfection. *E*, WI38 SV40-transformed cells were transfected and selected as described in *C* and *D*. Cells were extracted and subjected to Western blot analysis for SOD1 and actin as a loading control. *F*, WI38 cells were transfected with EGFP plasmid in combination with SOD1 RNAi or control plasmids. At the indicated time, the cultures were observed by fluorescent light microscopy and the percentage of EGFP-positive cells exhibiting round, shrunken morphology indicative of cell death was determined (*F*). The S.D. is indicated.

To confirm that the flattened and enlarged cells are senescent, we checked the activity of acidic β-galactosidase in WI38 SOD1 RNAi cells. This marker is facilely expressed in senescence cells of many types (27). WI38 SOD1 RNAi cells exhibited significantly stronger activity of senescence-associated β-galactosidase than the control cells (Fig. 2, *A* and *B*). A quantitative analysis indicated that almost 100% of the SOD1 RNAi cells were senescent, whereas in the control culture, only 20% of the cells reached senescence (Fig. 2*C*). Thus SOD1 RNAi induces premature senescence in human fibroblasts.

We next investigated the molecular mechanism of the SOD1 RNAi-induced premature senescence. As mentioned above, a reduction in SOD1 levels is known to induce DNA damage and thereby activate the p53 tumor suppressor protein (for review

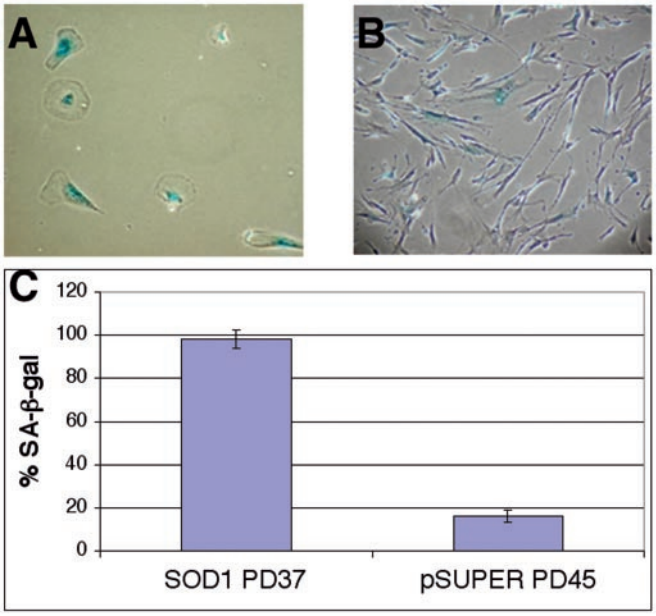

FIG. 2. **SOD1 RNAi induces premature senescence in WI38 cells.** WI38 cells were transfected and selected as described in Fig. 1. After 60 days, the cells were stained with senescence-associated β-galactosidase (*β-gal*). Following an overnight incubation, 5 fields from each culture were photographed. *A*, SOD1 RNAi. *B*, vector control. *C*, graph of the senescence associates β-galactosidase positive cells in each culture. The S.D. is indicated.

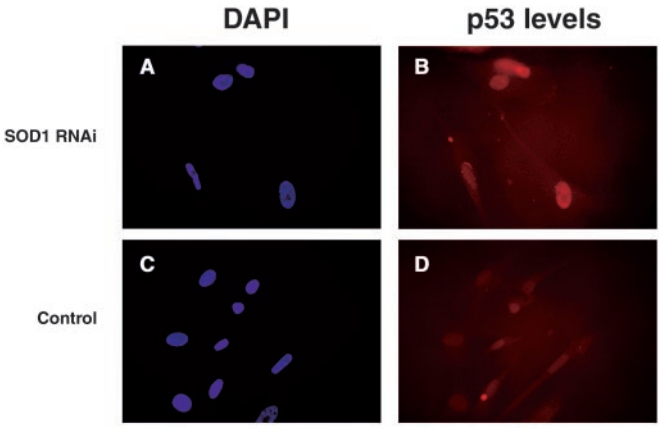

FIG. 3. **p53 levels are increased by SOD1 RNAi WI38 cells.** WI38 cells were transfected on coverslips and selected as described in Fig. 1. Three weeks later, the cells were fixed and stained with XXXXXX (*DAPI*) (*A* and *C*) and anti-p53 antibody (*B* and *D*). The picture were taken at the same exposure time.

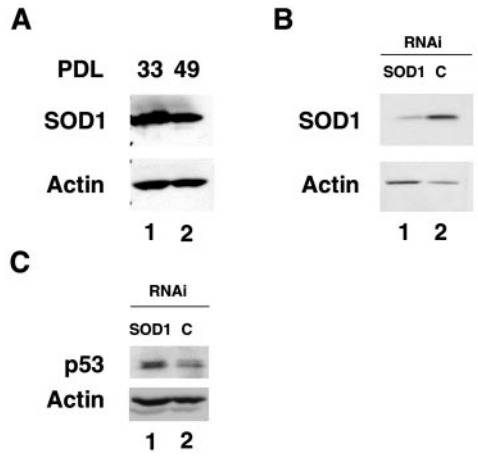

FIG. 4. **SOD1 and p53 levels in WI38 and HeLa cells.** *A*, WI38 cells at relatively low population doubling (*PDL*) (*lane 1*) and senescent WI38 cells (*lane 2*) were extracted and run in a Western blot analysis for SOD1 and actin as a loading control. *B*, HeLa cells were transfected and selected as described in Fig. 1. 48 h post-selection, cells were extracted and subjected to Western blot analysis for SOD1 and actin as a loading control. *C*, HeLa cells were transfected and selected as described in Fig. 1. 48 h post-selection, cells were extracted and subjected to Western blot analysis for p53 and actin as a loading control.

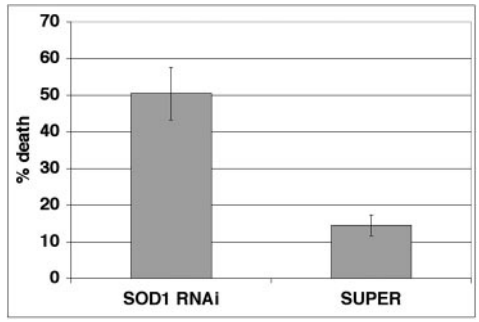

FIG. 5. **SOD1 RNAi induce cell death in HeLa cells.** HeLa cells were transfected with plasmid encoding EGFP in combination with SOD1 RNAi or pSUPER control plasmids. 48 h later, the cultures were observed by fluorescent light microscopy and the percentage of EGFP-positive cells exhibiting round, shrunken morphology indicative of cell death was determined. The S.D. is indicated.

see Ref. 28). To explore whether SOD1 RNAi induced senescence via p53, we investigated whether p53 could be responsible for the SOD1 RNAi-induced premature senescence of the WI38 cells. Cells were seeded on coverslips, transfected with the SOD1 RNAi and control plasmids, and selected with puromycin. After 3 weeks, the cells were fixed and stained with the anti-p53 monoclonal antibody (DO1). In contrast to control cells, SOD1 RNAi-transfected cells displayed the p53 protein in the nucleus (Fig. 3, *A* and *B*) and at elevated expression levels (Fig. 3, *B* and *D*). Therefore, the premature senescence induced by SOD1 RNAi correlates with the induction of the p53 protein.

To establish a causal link between p53 and SOD1 RNAi-triggered senescence, we employed WI38 cells that were transformed with the SV40 large T antigen. SV40 large T antigen interacts with and negatively regulates the p53 protein. WI38 SV40 cells were transfected with the SOD1 RNAi, and the cellular morphology was examined. After 14 days, the SOD1 RNAi did not induce senescence and cells appeared identical to those with the pSUPER control (Fig. 1, *panels C* and *D*). Western blot analysis verified that SOD1 RNAi reduced SOD1 protein levels in WI38 SV40 cells (Fig. 1*E*). In conclusion, the inhibition of p53 by SV40 abolished the induction of senescence by SOD1 RNAi in WI38 cells.

It was of interest to compare the early senescence of primary fibroblast to a response to SOD1 RNAi in cancer cells. Accordingly, HeLa cells were transiently co-transfected with SOD1 RNAi or control plasmids along with the pBABE-puro plasmid. As shown in Fig. 4*B*, SOD1 RNAi dramatically decreased the steady-state levels of the SOD1 protein in transfected cells. Moreover, in SOD1 RNAi-transfected cells, p53 protein level was induced (Fig. 4*C*). The induction of p53 by SOD1 RNAi in HeLa cells was comparable to WI38 cells (Fig. 3).

What Is the Biological Outcome of the p53 Induction by SOD1 RNAi in HeLa Cells?—Interestingly, we did not see any staining for β-galactosidase that would have been indicative of senescence in the transfected HeLa cells (data not shown). However, SOD1 RNAi transfection did result in 50% cell death (Fig. 5). In contrast, transfection with pSUPER resulted in little cell death (Fig. 5). Thus SOD1 RNAi appears to cause death in cancer cells (Fig. 5) but senescence in normal cells (Fig. 2).

DISCUSSION

SOD1 activity is required for the efficient maintenance of cellular integrity as reflected by the number of human diseases associated with the SOD1 protein (4). In the present study, we report that SOD1 RNAi induces premature senescence in normal human fibroblastic cells. This observation supports a role for the SOD1 protein in the life span determination of human cells. It is noteworthy that SOD1 regulates the life span of other organisms such as yeast and Drosophila (10, 11).

In humans, SOD activity decreases with age (24). In this study, we did not find any evidence for the SOD1 protein reduction as cells approached senescence (Fig. 4*A*). This paradox may be resolved in several ways. First, during senescence, SOD1 activity may decrease even though the protein levels do not change. Second, the reduction of SOD activity may be because of a decrease in levels or activity of SOD2, not SOD1. Third, a change in SOD1 levels that occurs in human aging may not happen during cellular senescence.

SOD activity is elevated in tumor tissues (29, 30). Therefore, SOD1 overexpression may play an important role as a defense mechanism in cancer. We found that SOD1 RNAi induces premature senescence in normal human fibroblasts while inducing death in cancer cells. The senescent induction observed in this study is p53-dependent. WI38 SOD1 RNAi-transfected cells displayed the p53 protein in the nucleus at elevated expression levels (Fig. 3). Moreover, inhibition of p53 by SV40 large T antigen abolished the induction of senescence by SOD1 RNAi in those cells (Fig. 1, *C* and *D*). In HeLa cells, the biological outcome was completely different. SOD1 RNAi induced cell death. Similarly to WI38 cells, the SOD1 RNAi caused an increase in the p53 protein levels in HeLa cells (Fig. 4*C*).

Our findings may have implications for cancer therapy. When cancer cells are treated with chemotherapeutic and radiotherapeutic agents, the normal cells that surround the cancer tissue can also be affected. In the long term, mutations that occur in the surrounding tissue may cause secondary tumors. It is possible that treatment with SOD1 RNAi or other inhibitors of the enzyme will induce death in the cancer cells but at the same time trigger senescence in normal cells. The outcome might be the inhibition of secondary tumor formation.

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