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Cells with dysfunctional telomeres are susceptible to reactive oxygen species hydrogen peroxide via generation of multichromosomal fusions and chromosomal fragments bearing telomeres

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ABSTRACT

During genotoxic stress, reactive oxygen species hydrogen peroxide (H_2O_2) is a prime mediator of the DNA damage response. Telomeres function both to assist in DNA damage repair and to inhibit chromosomal end-to-end fusion. Here, we show that telomere dysfunction renders cells susceptible to H_2O_2, via generation of multichromosomal fusion and chromosomal fragments. H_2O_2 caused formation of multichromosomal end-to-end fusions involving more than three chromosomes, preferentially when telomeres were erosive. Interestingly, extensive chromosomal fragmentation (yielding small-sized fragments) occurred only in cells exhibiting such multichromosomal fusions. Telomeres were absent from fusion points, being rather present in the small fragments, indicating that H_2O_2 cleaves chromosomal regions adjacent to telomeres. Restoration of telomere function or addition of the antioxidant *N*-acetylcysteine prevented development of chromosomal aberrations and rescued the observed hypersensitivity to H_2O_2. Thus, chromosomal regions adjacent to telomeres become sensitive to reactive oxygen species hydrogen peroxide when telomeres are dysfunctional, and are cleaved to produce multichromosomal fusions and small chromosomal fragments bearing the telomeres.

1. Introduction

Cellular oxidative stress involving production of reactive oxygen species (ROS) damages various cellular components including DNA, proteins, and lipids, and triggers signaling cascades leading to cell death caused by both apoptosis and necrosis [1,2]. Among the various ROS, H_2O_2 is of critical importance, because H_2O_2 plays diverse roles in various pathological and physiological processes [3]. In response to anticancer agents including ionizing irradiation, H_2O_2 is involved in DNA damage signaling and causes both single- and double-stranded DNA breaks [4]. Thus, signaling pathways (including apoptosis and those involved in senescence) are activated during the H_2O_2-mediated DNA damage response [5]. H_2O_2 can be directly generated in cells via enzymatic reactions involving NADPH oxidase, and DuOXs [6,7]. Inhibition of such reactions reduces the extent of H_2O_2 generation and the level of subsequent apoptotic cell death. Therefore, it has become widely accepted that a close relationship exists between cell death, on the one hand, and H_2O_2 generation, on the other, in response to genotoxic stress.

However, responses to H_2O_2 have been examined principally in cells with intact functionally active (thus not erosive) telomeres. Under conditions of telomere erosion, the response to H_2O_2 remains poorly understood. Telomere erosion occurs when telomere length becomes critically shortened or when telomere-binding proteins are inert [8,9]. The ends of chromosomes are normally capped with a specialized structure composed of telomere repeat

Abbreviation: mTERC, mouse telomerase RNA component.

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sequences and telomere-binding proteins [10,11]. This capping prevents chromosomal end-to-end fusion and is essential for maintenance of chromosomal integrity [9–11]. When telomere capping is attenuated, telomere uncapping becomes predominant, leading to loss of telomere function and an increase in chromosomal instability. The presence of erosive (thus dysfunctional) telomeres renders cells susceptible to DNA-damaging agents including ionizing radiation, and microtubule-disrupting agents such as paclitaxel [12–14]. Therefore, telomere length is viewed as a useful predictor of chemo- and radio-sensitivity.

As anticancer agents that are capable of inducing ROS synthesis exert cytotoxic effects both directly on DNA, and indirectly via generation of ROS, it was important to explore whether ROS might contribute to telomere dysfunction-mediated increases in chemo- and radio-sensitivity. In the present study, we added exogenous H_2O_2 to telomerase-deficient cells derived from mTERC−/− (mouse telomerase RNA component-deficient) mice. Interestingly, when telomere erosion was in play, H_2O_2 caused the generation of many small chromosomal fragments containing telomeres, and also triggered multichromosomal fusions involving more than three chromosomes. Cells in which telomeres were dysfunctional were thus rendered susceptible to H_2O_2.

2. Materials and methods

2.1. Cell culture and reagents

Myc/Ras-transformed early (G2) and late generation (G6) mTERC−/−p53−/− MEFs were derived from mice doubly null for mTERC/p53 [12,15,16]. Pooled and clonally established populations of such transformed MEFs were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS in a humidified incubator containing 5% (v/v) CO_2 at 37 °C. *N*-acetylcysteine (cat. A-8199) and H_2O_2 (cat. 216763) were purchased from Sigma (St. Louis, MO).

2.2. Chromosomal fusion and fragmentation

H_2O_2-induced chromosomal fusions and fragmentations were analyzed in metaphase spreads of cells exposed to H_2O_2, using a previously reported procedure [17]. Briefly, 72 h after H_2O_2 exposure, metaphase chromosomes were prepared from cells treated with 0.1 μg/ml colcemid for 2 h. Cells enriched in metaphase chromosomes were swollen in hypotonic 0.075 M KCl for 20 min, and next fixed in methanol/acetic acid (3:1, v/v). Fixed cells were dropped onto slides and baked at 60 °C for 3 h. Such metaphase slides were either stained with Giemsa, or labeled using a telomere-specific probe. Multichromosomal fusions and chromosomal fragments were quantitated using metaphase spreads stained with Giemsa. To detect telomere signal, metaphase slides were fixed in 4% (v/v) formaldehyde, hybridized with a telomere-specific probe [$(CCCTAA)_3$] labeled with a fluorescent marker (PerSeptive Biosystems Inc., Framingham, MA), and counterstained with 4′,6′-diamidino-2-phenylindole.

2.3. Cell cycle analysis

Cell cycle distribution was determined by measuring the DNA content of cells stained with propidium iodide. Briefly, cells were fixed in 70% (v/v) cold ethanol at 4 °C for at least 12 h, washed with PBS, and incubated with 100 μg/ml DNase free-RNase A in 0.1% (v/v) Triton X-100. The nuclei were stained with 200 μg/ml propidium iodide for 30 min at room temperature. Data from 10,000 FACS-sorted cells (presented in histogram format) were analyzed using CellQuest software (BD Biosciences, San Jose, CA) and WinMDI 2.0 software.

2.4. Cell survival assay

Upon exposure to H_2O_2, cell survival was evaluated by measuring the colony-forming abilities of single cells. Briefly, cells were seeded at a low density of 2400 cells per 100 mm culture dish. After exogenous addition of H_2O_2 to adherent cells, such cells were cultured for 7–8 days and the resulting colonies visualized by staining with 0.1% (w/v) crystal violet for 30 min. The survival rates of cells exposed to H_2O_2 were determined by calculation of colony percentages compared to that of control cells not exposed to H_2O_2.

3. Results

3.1. Cells with dysfunctional telomeres are sensitive to H_2O_2

To assess the cellular response to H_2O_2 when telomeres were erosive and thus dysfunctional, we employed Myc/Ras-transformed telomerase-deficient MEF cells derived from early-(G2) and late-generation (G6) telomerase-deficient mice doubly null for both mTERC and p53 [12,15,16]. Transformed G2 and G6mTERC−/−p53−/− MEF cultures, prepared via pooling of transformed foci, are established cell lines in which telomere lengths and chromosomal end-to-end fusions have been characterized [12,13]. Because of the presence of critically shortened telomeres, G6 cells fail to cap chromosomal ends and accumulate chromosomal end-to-end fusions [12,13]. Thus, the telomeres of transformed G6mTERC−/−p53−/− cells are erosive and functionally inactive. However, restoration of telomerase activity via mTERC-reconstitution rescues telomere function by protecting against chromosomal end-to-end fusion [12,13]. Generally, human cancer cells have shorter telomeres than do normal cells [18], and a minimum telomere length is necessary to ensure capping of the chromosomal end [19,20]. Therefore, Myc/Ras-transformed G6mTERC−/−p53−/− cells, and such cells after m-TERC reconstitution, mimic human cancer cells, and permit the contributions of telomerase activity to cell behavior to be assessed under conditions of short telomere length.

Thus, we exogenously added H_2O_2 to G6mTERC−/−p53−/− cells with erosive dysfunctional telomeres, and to such cells in which the telomeres had been rendered robust via mTERC-reconstitution, and compared cell survival rates. Upon exposure to H_2O_2, Myc/Ras-transformed G6mTERC−/−p53−/− cells exhibited greatly reduced survival compared to that of mTERC-reconstituted cells (Fig. 1A). Specifically, mTERC-reconstitution caused 2.7-, 3.9-, 10.1-, or 34.3-fold rises in the survival rate upon exposure to 50, 100, 200, or 400 μM H_2O_2, respectively. However, early-generation G2mTERC−/−p53−/− cells did not show any change in the extent of survival after mTERC-reconstitution (Fig. 1B). G2mTERC−/−p53−/− cells maintain long telomeres and are devoid of chromosomal fusions even in the absence of telomerase activity [14,16]. Thus, when telomere erosion is in play, cells become extremely sensitive to H_2O_2. Further, telomere length rather than telomerase activity *per se* is important in the development of hypersensitivity to H_2O_2.

3.2. When telomeres are erosive, H_2O_2 causes formation of multichromosomal fusions and small chromosomal fragments bearing telomeres

H_2O_2 elicits a DNA-damage response in a variety of cells and organisms [1–5]. Therefore, we analyzed metaphase spreads to determine whether, under conditions of telomere erosion, H_2O_2

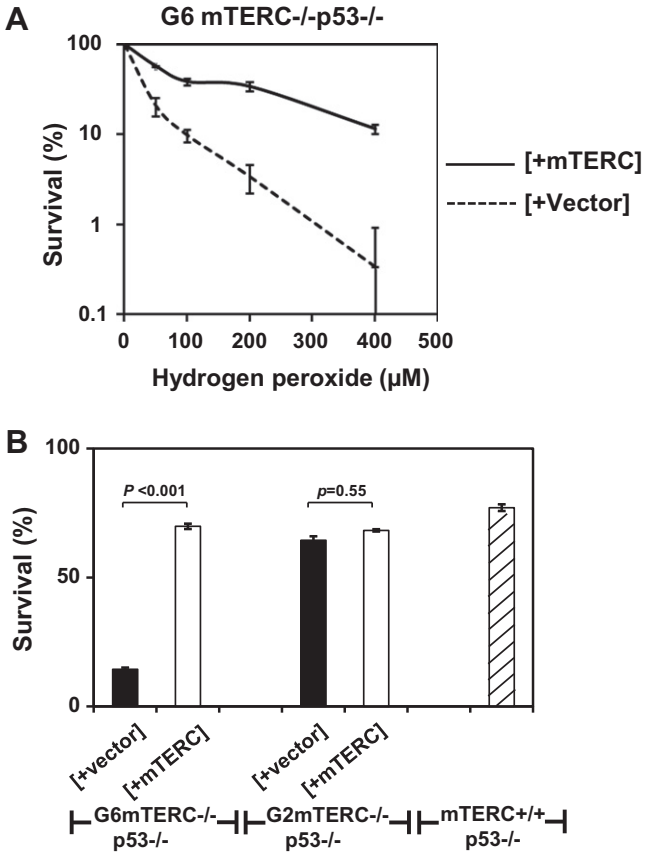

Fig. 1. Cells with dysfunctional telomeres are very susceptible to H_2O_2. (A) Myc/Ras-transformed G6 mTERC−/−p53−/− MEFs with (○) and without mTERC reconstitution (●) were exposed to H_2O_2 at various levels and cell survival rates were compared. The G6 cell lines were prepared by mixing individually isolated clones (five or 10 clones with or without mTERC-reconstitution, respectively). (B) Myc/Ras-transformed G6 and G2 mTERC−/−p53−/− MEF cultures with (open bars) or without (closed bars) mTERC reconstitution, and Myc/Ras-transformed mTERC+/+p53−/− MEF cells (hatched bar), were exposed to 50 μM H_2O_2, and survival rates were determined. The mTERC±/±p53−/−, G2, and G6 cell lines were prepared by mixing individual clones that formed in culture dishes. The data represent means ± SDs of values from triplicate samples analyzed in a single experiment. All experiments were performed three times and the data were reproducible. The *p* values were calculated using the *t*-test.

influenced chromosomal integrity. Upon exposure of G6mTERC−/−p53−/− cells to H_2O_2, the typical chromosomal aberration noted was multichromosomal fusion involving more than three chromosomes; sequential chromosomal end-to-end fusion was in play (Fig. 2A). No telomere signals were detected on the multichromosomal fusion (lower panel of Fig. 2A), indicating the lack of telomere signal at the fusion points. Rather, the small chromosomal fragments bore such signals (arrows in the lower panel of Fig. 2A). Chromosomal fragments were evident in the metaphase spreads showing multichromosomal fusions (Fig. 2A and D). These findings indicate that multichromosomal fusions are generated by H_2O_2-mediated cleavage of chromosomes in regions adjacent to telomeres. It is known that H_2O_2 induces telomere shortening [5]. Upon exposure to 400 μM H_2O_2, multichromosomal fusions were detected in 22.4% of metaphase spreads of G6mTERC−/−p53−/− cells (Fig. 2B). Small chromosomal fragments were present (approximately 19.1 per metaphase) in spreads showing multichromosomal fusions (Fig. 2D). In contrast, such fragments were barely evident in metaphase spreads lacking the fusions (Fig. 2D), further supporting the notion that H_2O_2-induced multichromosomal fusion was associated with generation of small chromosomal fragments. As was noted (above) when rescue of survival was studied, mTERC-reconstitution also prevented generation of multichromosomal fusions and chromosomal fragments in G6mTERC−/−p53−/− cells (Fig. 2A–C). As expected from the survival data, G2mTERC−/−p53−/− cells did not exhibit multichromosomal fusions, irrespective of the status of mTERC-reconstitution. Thus, when telomere erosion is in play, H_2O_2-induced multichromosomal fusions, accompanied by production of small chromosomal fragments, is associated with poor survival. Further, telomere dysfunction facilitates H_2O_2-induced chromosomal instability, as evidenced by formation of multichromosomal fusions. Indeed, such fusions were barely evident in G6mTERC−/−p53−/− cells that were not exposed to H_2O_2 (Fig. 2B). Rather, such cells accumulate end-to-end fusions involving two chromosomes [12,16,21].

3.3. H_2O_2 arrests cells with dysfunctional telomeres at the G_2/M phase

As telomere dysfunction rendered Myc/Ras-transformed cells highly susceptible to H_2O_2, we next examined whether, upon exposure to H_2O_2, the observed multichromosomal fusions and the eventual decrease in survival rate were associated with cell cycle distribution. As expected, a greater proportion of G6mTERC−/−p53−/− cells than mTERC-reconstituted cells were found in the G_2/M phase of the cell cycle upon exposure to 100 or 200 μM H_2O_2 (Supplementary Fig. 1). At 400 μM H_2O_2, G6mTERC−/−p53−/− cells were enriched in the sub-G_1 fraction, whereas most mTERC-reconstituted cells were in G_2/M. Therefore, cells with dysfunctional telomeres arrest at the G_2/M phase upon exposure to H_2O_2 and eventually die, as shown by the presence of a cell population in the sub-G_1 phase. Indeed, it is known that H_2O_2 arrests the cell cycle at the G_2/M phase, followed by cell death via either apoptosis or senescence [22].

3.4. *N*-acetylcysteine reverses H_2O_2-induced generation of multichromosomal fusions and chromosomal fragmentation, and rescues survival of cells with dysfunctional telomeres

To explore whether H_2O_2-mediated multichromosomal fusion was directly associated with the observed reduction in survival of telomere-dysfunctional cells, we preincubated such cells with *N*-acetylcysteine, a scavenger of ROS [23] prior to addition of H_2O_2. Such preincubation rescued the survival of G6mTERC−/−p53−/− cells upon exposure to H_2O_2. Pretreatment with 10 mM *N*-acetylcysteine elevated the control survival rates of 14.5%, 2.8%, and 0.15% upon exposure to 50, 200, and 400 μM H_2O_2, respectively, to 82.4%, 37.3%, and 32.4%, respectively (Fig. 3A). This rescue of survival was accompanied by falls in the extent of multichromosomal fusion and chromosomal fragmentation (Fig. 3B and C). Specifically, the level of H_2O_2-induced multichromosomal fusion was reduced to almost that seen in the absence of H_2O_2 (Fig. 3B). Also, the severe chromosomal fragmentation induced by exposure to H_2O_2 disappeared (Fig. 3C). As expected, no noticeable change in the extent of such fragmentation was evident in metaphase spreads lacking multichromosomal fusions (Fig. 3D). Although the survival rate of mTERC-reconstituted cells was also increased upon addition of *N*-acetylcysteine, such rises were smaller than were those of G6mTERC−/−p53−/− cells (Fig. 3A). In addition, the *N*-acetylcysteine-mediated rescue of survival in mTERC-reconstituted cells was not associated with multichromosomal fusion, because no detectable change in the extent of such fusion was evident in these cells. Thus, upon exposure to H_2O_2 when telomeres are erosive and dysfunctional, the reduction in survival rate is attributable to an increase in multichromosomal fusion, accompanied by chromosomal fragmentation.

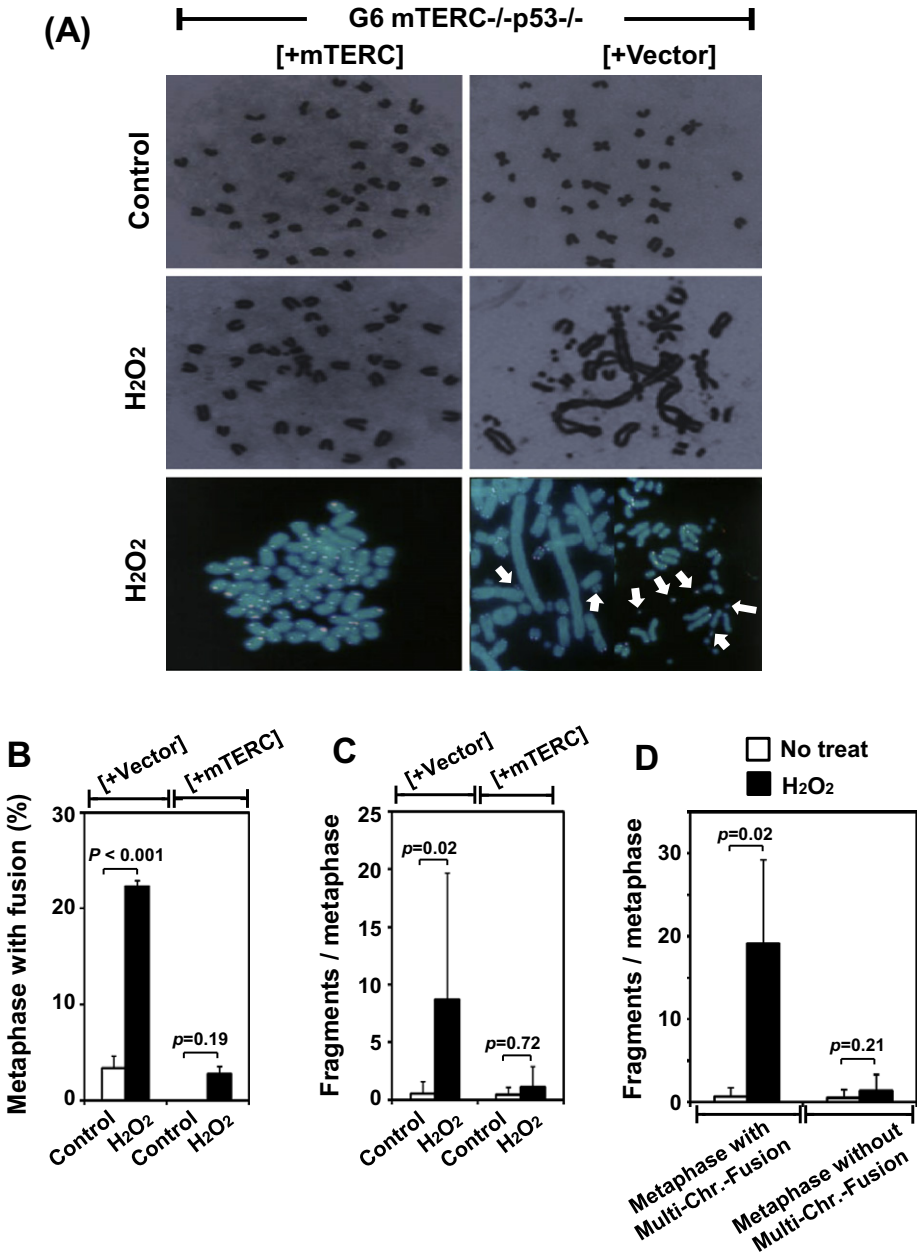

Fig. 2. Telomere dysfunction promotes generation of multichromosomal fusions upon exposure to H_2O_2. (A) Myc/Ras-transformed G6mTERC−/−p53−/− cells carrying the empty control vector [+vector] or a plasmid mediating mTERC reconstitution [+mTERC] were exposed to 400 μM H_2O_2, and metaphase spreads were stained either with Giemsa or with a telomere probe carrying a fluorescent marker, and observed by light (×1000) or fluorescence microscopy (×1000), respectively. (B) The extent of multichromosomal fusion was assessed as the percentage of metaphase spreads containing such fusions. (C and D) Chromosomal fragmentation was assessed as numbers of fragments per metaphase spread. Thirty metaphases were analyzed per sample (B–D). The G6 cell lines were mixed populations of the individually isolated clones described in the legend to Fig. 1A. The *p* values were calculated using the *t*-test. The data shown were reproducible when the results of each of three independent experiments were compared.

3.5. Propagation of telomere dysfunction renders cells more susceptible to H_2O_2

To further explore the susceptibility of cells with dysfunctional telomeres to exogenous H_2O_2, we monitored H_2O_2-induced cell death using clonal populations of G6mTERC−/−p53−/− cells. During long-term passage (up to 220 PD), clonal populations of the G6mTERC−/−p53−/− culture exhibited an increased level of p-to-p arm chromosomal end-to-end fusion, whereas such fusion was barely evident (and certainly did not increase) during passage of clonal populations of the mTERC-reconstituted culture [13]. When we compared cell survival rates upon exposure to H_2O_2 between the same clones at PD 20 and 220, we found that long-term passage led to decreases in the survival rates of the most telomerase-deficient clones (Fig. 4). Only one such clone, clone #5, which did not show an increase in fusion level with increasing passage [13], did not exhibit a fall in survival rate. In fact, this clone was also resistant to irradiation sensitization [13]. However, the other four clones (clones #1, 2, 7, and 10), all of which showed an increase in the extent of fusion with increasing passage, were sensitized to H_2O_2 during the passages. In contrast to the telomerase-deficient clones, mTERC-reconstituted clones exhibited a negligible change (clone #13) or even a rise (clones #16, 17, 19, and 20) in survival rate, to attain the level of wild-type mTERC+/+p53−/− cells.

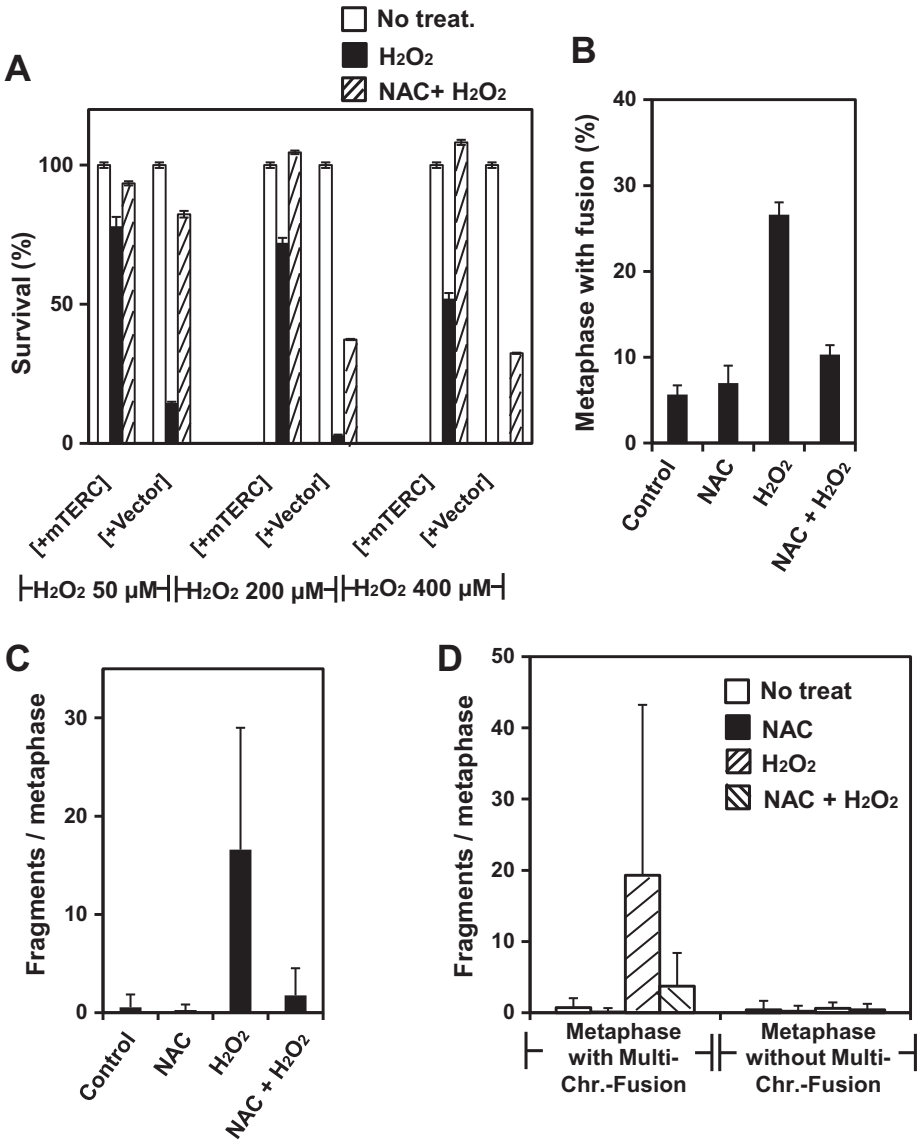

Fig. 3. *N*-acetylcysteine significantly rescues survival, and prevents generation of multichromosomal fusions and chromosomal fragments in cells with dysfunctional telomeres exposed to H_2O_2. (A) G6mTERC−/−p53−/− cells carrying the empty control vector [+vector] or a plasmid mediating mTERC reconstitution [+mTERC] were pre-incubated with 10 mM *N*-acetylcysteine (NAC) for 30 min prior to H_2O_2 exposure, and survival rates were determined 8 days later. (B) The extent of multichromosomal fusion was assessed as the percentage of metaphase spreads containing such fusions. (C and D) Chromosomal fragmentation was assessed as numbers of fragments per metaphase spread. Thirty metaphases were analyzed per sample (B and C). The G6 cell lines were mixed populations of individually isolated clones as described in the legend to Fig. 1A. The data are means ± SDs of triplicate samples analyzed in a single experiment.

Such findings further support the notion that propagation of telomere dysfunction upon passage renders cells more susceptible to exogenous H_2O_2.

4. Discussion

Cells with dysfunctional telomeres are sensitive to anticancer agents [12,14]. Such drugs may act directly or indirectly, via generation of ROS including H_2O_2. Herein, we show that loss of telomere function renders cells susceptible to H_2O_2, resulting in the generation of multichromosomal fusions and chromosomal fragmentation caused by breaks in regions adjacent to the telomeres. Further, such regions are especially vulnerable to H_2O_2.

In the present work, we employed Myc/Ras-transformed G6mTERC−/−p53−/− cells with or without mTERC-reconstitution. In response to H_2O_2, the most pronounced difference between metaphase spreads of the two cell types was the presence of multichromosomal end-to-end fusions involving more than three chromosomes in the former cells. Chromosomal end-to-end fusion occurs when telomeres are erosive and thus dysfunctional [10,11]. Likewise, Myc/Ras-transformed G6mTERC−/−p53−/− cells accumulate chromosomal p-to-p arm fusions [12,13]. However, such end fusion helps the transformed cells to maintain chromosomal integrity, and to grow even under conditions of telomere erosion [12,16]. Therefore, p-to-p arm end-fusion is an adaptation of cells with dysfunctional telomeres to maximize survival. Unlike p-to-p arm end fusions, multichromosomal fusions were barely detected in growing G6mTERC−/−p53−/− cells. Thus, H_2O_2-mediated multichromosomal fusion appears to cause cell death. Restoration of telomere function in G6mTERC−/−p53−/− cells, via mTERC-reconstitution, reduced the level of H_2O_2-induced multichromosomal fusion and chromosomal fragmentation. Thus, in the presence of dysfunctional telomeres, H_2O_2 exposure generates multichromosomal fusions and causes chromosomal fragmentation.

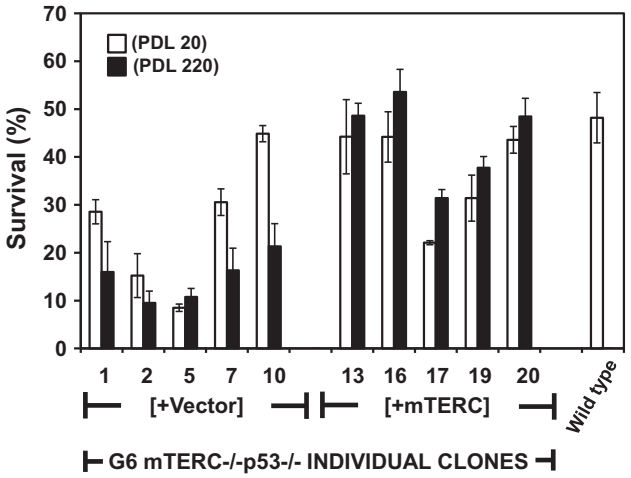

Fig. 4. Reduction in the survival rate of telomere-dysfunctional cells exposed to H_2O_2, as a function of extent of cell division. Individual clones of Myc/Ras-transformed G6 mTERC−/−p53−/− cells, and such cells after mTERC-reconstitution, were exposed to 50 μM H_2O_2, and the survival rates between PDL 20 and 220 were determined. "Wild-type" indicates a population of Myc/Ras-transformed mTERC+/+p53−/− cells pooled at PDL 20; all transformed clones were mixed.

Interestingly, multichromosomal fusions were observed primarily in cells containing small chromosomal fragments, and very seldom in cells lacking such fragments. The chromosomal fusion points lacked telomeres. Rather, the small fragments bore the telomeres. These findings indicate that, when telomeres are dysfunctional, regions adjacent to telomeres (thus presumably subtelomeric in nature) are vulnerable to the effects of H_2O_2, and breakage occurs in such regions to produce small chromosomal fragments containing telomeres. Indeed, it is known that subtelomeric regions are susceptible to double-strand DNA breakage [23]. Telomeres resemble fragile replication sites and thus also become fragile upon depletion of TRF1 [24]. Our current data suggest that H_2O_2 may induce telomere fragility, preferentially in cells in which telomeres are dysfunctional. As a rise in the level of chromosomal end-to-end fusion increases susceptibility to H_2O_2, the shortening of telomeres to a critical extent appears to increase telomere fragility, in turn enhancing susceptibility to H_2O_2. Our present finding that erosive telomeres are fragile upon exposure to H_2O_2 is in line with previous observations to the effect that oxidative stress, including exposure to H_2O_2, promotes senescence, with concomitant acceleration of telomere shortening [25,26]. However, induction of cellular senescence or proliferation by ROS depends on the levels of ROS generated and whether the influence of ROS is transient or chronic. Interestingly, telomere dysfunction decreases mitochondrial biogenesis [27] and enhances ROS generation [28]. Consistently, mitochondrial dysfunction increased ROS generation under conditions of telomere-dependent senescence [29]. Thus, telomere dysfunction-mediated generation of ROS may increase susceptibility to H_2O_2. Our present data, taken together with previous findings suggest that telomeres are sensitive to oxidative stress, and that such sensitization becomes more acute when telomeres are dysfunctional. In the present work, neither fusion nor fragmentation was noted in cells with functional telomeres, for example in G6mTERC−/−p53−/− cells after mTERC-reconstitution.

Cells with dysfunctional telomeres continuously accumulate chromosomal end-to-end fusions, and are sensitive to anticancer agents that induce DNA damage and microtubule disruption [14]. In the present study, we showed that telomere dysfunction mediated sensitization to H_2O_2; this ROS caused multichromosomal fusions and fragmentation of chromosomes via breakage in regions adjacent to telomeres. Thus, anticancer drug-induced generation of ROS including H_2O_2 appears to sensitize cells with dysfunctional telomeres to chromosomal cleavage in regions adjacent to such telomeres.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.086.

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