Inhibition of Telomerase Activity Enhances Hyperthermia-Mediated Radiosensitization

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Abstract

Hyperthermia is a potent sensitizer of cell killing by ionizing radiation (IR); however, hyperthermia also induces heat shock protein 70 (HSP70) synthesis and HSP70 expression is associated with radioresistance. Because HSP70 interacts with the telomerase complex and expression of the telomerase catalytic unit (hTERT) extends the life span of the human cells, we determined if heat shock influences telomerase activity and whether telomerase inhibition enhances heat-mediated IR-induced cell killing. In the present study, we show that moderate hyperthermia (43°C) enhances telomerase activity. Inhibition of telomerase activity with human telomerase RNA-targeted antisense agents, and in particular GN163L, results in enhanced hyperthermia-mediated IR-induced cell killing, and ectopic expression of catalytic unit of telomerase (TERT) decreased hyperthermia-mediated IR-induced cell killing. The increased cell killing by heat and IR exposure in telomerase-inhibited cells correlates with delayed appearance and disappearance of γ-H2AX foci as well as decreased chromosome repair. These results suggest that inactivation of telomerase before combined hyperthermia and radiotherapy could improve tumor killing. [Cancer Res 2008;68(9):3370–8]

Introduction

Telomerase is a unique ribonucleoprotein enzyme that is responsible for adding the telomeric repeats onto the 3′ ends of chromosomes (1). Besides the obvious role of telomerase in telomere synthesis and DNA damage repair (2, 3), functions for telomerase in response to stress are unknown. A common cellular stress is exposure to elevated temperatures (4, 5). Hyperthermia itself has several cellular effects that have been proposed to be synergistic with ionizing radiation (IR)-induced tumor cell killing (5). For example, hypoxic cells are more resistant to IR-induced cell killing in comparison with normoxic cells, although hypoxic cells are no more resistant to heat-induced cell lethality than are normoxic cells (6). Hyperthermic cell stress activates a highly conserved program of rapid alterations in normal cellular metabolism to optimize synthesis of a limited, specific set of proteins known as heat shock proteins (HSP). The most highly induced and conserved HSP in all organisms from Escherichia coli to man is HSP70

The inability of current radiotherapies to control tumor growth is still a daunting clinical problem leading to failure of the overall treatment regimens. Therapeutic agents that are currently used to enhance radiosensitivity lack specificity for tumor cells and, therefore, have therapy-limiting toxic side effects for normal tissue. Cancer treatment could be improved by the development of drugs that target specific pathways that are deregulated in tumor cells. A fundamental unanswered question is if tumor cells can be specifically sensitized to IR by heat or factors exclusively expressed in tumor cells. One such factor, expressed in most tumors and silent in most human somatic cells, is telomerase. Telomerase activity has been found in approximately 85% to 90% of all human tumors but not in the adjacent normal cells (12, 13). Biochemical and genetic studies have established an association between telomere maintenance and extended life span of human cells mediated through the expression of the catalytic subunit of telomerase (hTERT). Because of this, telomerase is an attractive target for inhibition in anticancer therapy. In the present study, we show that acute hyperthermia results in loss of telomerase enzyme activity, whereas moderate hyperthermia slightly enhanced telomerase activity. Whether the increase in telomerase activity by moderate heat shock may contribute to thermoresistant is not known. Because hyperthermia is used clinically in combination with radiation, the present study also tested whether inactivation of telomerase with human telomerase RNA (hTR)-directed antisense agents could enhance hyperthermia-mediated radiosensitization.

Materials and Methods

Human cell lines used were 293, HeLa, GM857, and primary fibroblast cell lines (GM5823, HFF, and BJ) with their corresponding stably transfected hTERT counterparts (GM5823-hTERT, BJ-hTERT, and HFF-hTERT) as described previously (8, 14). Terc-null and Tert-null mouse embryonic cells along with their wild-type counterparts were obtained from Sandy Change (M. D. Anderson Cancer Center, Houston, TX) and Fuyuki Ishikawa (Kyoto University, Kyoto, Japan), respectively, and details about the cell lines and culture conditions have been described previously (15, 16). Mouse embryonic fibroblasts (MEF) with and without Hsp70.1/3 (Hsp70.1/3+/− and Hsp70.1/3−/−) as well as mouse kidney fibroblasts with and without ataxia-telangiectasia mutated (ATM) were maintained as described.
previously (4). A-T1DL cells were maintained as described (17). Heat shock treatments were carried out at 43°C for different time periods as shown in the respective figures. The acute heat shock treatments were carried out at 45°C and higher.

Telomerase inhibitors. Three types of hTR-targeted antisense agents were investigated (Fig. 3A). The first type of agent was the previously reported mixed DNA/locked nucleic acid (LNA) oligomer, d(CAGTTAGGTTAG-TAG), where the LNA component is shown in lower case and which was transcribed into cells via Lipofectamine 2000 (18). The second type of agent investigated was a series of cell-penetrating peptide-peptide nucleic acid (PNA) conjugates, which used the same sequence as the LNA (Supplementary Table S1) and which were transcribed into cells by simple incubation. These peptide-PNA conjugates were synthesized by standard automated Fmoc synthesis on an ABI Expedite 8909 DNA/PNA synthesizer with the thiophosphoramidate GRN163, GRN163L (5’-PL-TAGGGTTAGCAA-3’), and its mismatch mutant (5’-PL-TAGGGTTAGCAA-3’; ref. 19). Lipid modification of GRN163L has been shown to enhance the potency of telomerase inhibition (19). Cells were treated as previously described, showing that the GRN163L mismatch mutant had no effect on telomerase activity (20). GRN163L inhibits telomerase activity in various tumor cell lines with IC50 values of 3 to 300 nM/L without any cellular uptake enhancers and has potent and sequence-specific anticancer activity in vivo in multiple animal models. In addition, GRN163L significantly affects not only the growth of primary tumors but also the spread and proliferation of metastases.

Survival assay. Cells were plated in 60-mm dishes in 5.0 mL of medium, incubated for 6 h, and subsequently exposed to IR as described previously (21). The number of cells plated per dish was selected to ensure that ~50 colonies would survive treatment of a specific radiation dose. Cells were exposed to IR in a dose range of 0 to 6 Gy at room temperature. Cells were incubated for 12 or more days and then fixed in methanol-acetic acid (3:1) before staining with crystal violet. Only colonies containing at least 50 cells were counted.

Telomerase assay. Telomerase activity was determined using telomerase PCR ELISA kit (Roche Diagnostic Corp.) as described (22). Telomerase activity was determined in triplicate, with negative and positive controls run for each experiment. As a negative control, an aliquot of each extract was heat inactivated for 10 min at 95°C. The number of cells plated per dish was selected to ensure that ~50 colonies would survive treatment of a specific radiation dose. Cells were exposed to IR in a dose range of 0 to 6 Gy at room temperature. Cells were incubated for 12 or more days and then fixed in methanol-acetic acid (3:1) before staining with crystal violet. Only colonies containing at least 50 cells were counted.

γ-H2AX immunofluorescence measurements. Cells were cultured on chamber slides, fixed, and immunostained as described previously (5, 23–25). Fluorescence images of foci were captured using a Zeiss Axioskop 2 epifluorescent microscope equipped with a charge-coupled device camera and ISIS software (Metasystems). Optical sections through nuclei were captured, and images were obtained by projection of the individual sections as described recently (23). The results shown are from three independent experiments. Cells with bubble-like appearance or micronuclei were not considered for γ-H2AX analysis.

Chromosomal DNA strand break analysis. Cell-based analysis of individual chromosome damage provides the most sensitive assay for determining the induction of DNA strand breaks after stress. To determine whether telomerase inhibitors enhanced chromosomal break formation, cells were treated with GRN163L before heat shock and IR exposure. Immediately after IR exposure, the cells were treated with colcemid and chromosomal aberrations were assessed by counting chromatid breaks and gaps per cell as described (4, 26–29). Two hundred metaphases were analyzed for each point.

Results

Effect of heat shock on cellular telomerase activity. Heat shock treatment is known to induce HSP70, which interacts with the catalytic unit of telomerase (hTERT), whose expression extends the life span of the cells (14, 30). Therefore, we first determined whether hyperthermia influences telomerase activity in tumor-derived and normal cell lines. Exponentially growing (293, HeLa, and BJ-hTERT) cells were kept at 41°C, 43°C, 45°C, and 47°C for 1 h and then examined for telomerase activity (Fig. 1A; data not shown). Cells treated with heat at 41°C did not show any change in basal (37°C) telomerase activity (data not shown). Maximum telomerase activity was observed in cells heat shocked at 43°C, whereas acute heat shock of 45°C or above resulted in decreased telomerase activity (Fig. 1A; data not shown). HeLa as well as 293 cells showed the maximum enhancement of telomerase activity within 1 h of heat treatment at 43°C (Fig. 1B, a). Such heat-enhanced telomerase activity is statistically significant as determined by Student’s t test (P < 0.05). A modest increase in telomerase activity was observed in ectopically expressed hTERT in cells with ATM function (BJ-hTERT) and without ATM function (GM5823-hTERT). The increase in telomerase activity after heat shock was followed by a gradual decrease in telomerase activity during recovery period (Fig. 1B, a). Heat shock treatment did not induce any telomerase activity in primary fibroblast cells (HFF and GM5823) or in cells with alternative lengthening of telomeres (ALT; GM847). To determine whether the change in telomerase activity was due to altered transcription, we examined the levels of hTERT and hTR RNA by reverse transcription-PCR. We found that heat treatment did not change the mRNA levels of hTERT or hTR (data not shown) and protein levels of hTERT (Fig. 1B, b).

Inactivation of HSP70 slightly reduced the effect on heat shock–enhanced telomerase activity. HSP70 has been shown to interact with hTERT before its assembly with hTR and other telomerase protein components (31). Although the association of HSP70 with TERT is not required for the functional activity of hTERT in vitro (31), total cellular inactivation of HSP70 does result in reduced basal level telomerase activity in mouse embryonic cells (4). To determine whether inactivation of HSP70 influences heat-induced telomerase activity, we compared telomerase activity in MEFs with and without HSP70 (4) after heat shock at 43°C. Although heat shock treatment transiently increased telomerase activity in both Hsp70.1/3+/+ and Hsp70.1/3−/− cells (Fig. 1C), there was a modestly greater enhancement of telomerase activity in Hsp70.1/3−/− cells.

Effect of in vitro heating on telomerase activity. Cells exposed to moderate heat shock (43°C) show a transient increase in telomerase activity (Fig. 1B, a and C). We tested whether the heat-induced increase in telomerase activity is a direct effect of heat on the telomerase complex. Cell extracts were treated with different temperatures (37°C, 43°C, and 47°C) for 1 h and examined for telomerase activity. A significant drop in telomerase activity was observed at 43°C (P < 0.05, Student’s t test) and an even larger decrease was seen at 47°C (P < 0.001, Student’s t test; Fig. 1D, a). We next determined if heat shock treatment of cell extracts followed by incubation at 37°C influences the telomerase activity. Therefore, we immunoprecipitated the telomerase complex from 293 cell extracts by anti-hTERT antibody. Both the cellular extract and immunoprecipitated telomerase complex were heated for 1 h at 43°C and then incubated at 37°C. In contrast to the transient increase in telomerase activity observed in heat shock–treated cells, a modest decrease in telomerase activity was observed in heat shock–treated cell extracts or immunoprecipitated telomerase complex (Fig. 1D, b). Similar results were obtained when ectopically expressed hemagglutinin (HA)-tagged hTERT was immunoprecipitated with anti-HA antibody from BJ cell extracts, heated, and analyzed for telomerase activity (data not
shown). These results suggest that the transient increase in telomerase activity observed in cells after heat shock is not the direct consequences of the effect of heat on the telomerase complex.

**Influence of hTERT on heat- and IR-induced cell killing.**

Ectopic expression of hTERT in telomerase-silent cells is sufficient to override senescence by extending cellular life span (13, 14, 30, 32, 33). This leads to transcriptional alterations in a subset of genes and changes the interaction of telomeres with the nuclear matrix (2, 34). These alterations are associated with a reduction in spontaneous chromosome damage, enhancement of DNA repair kinetics, and increased nucleotide triphosphate levels (2). These effects of hTERT have been reported to occur rapidly before any significant lengthening of telomeres was observed (2, 34). Such functions of telomerase are distinct from its known effect on telomere length and have potentially important biological consequences. The present studies revealed that moderate heating enhances telomerase activity, suggesting that telomerase may have a role in cellular protection during stress conditions. Because heat shock has been shown to activate ATM independent of DNA double-strand breaks (5), we therefore analyzed whether the presence of hTERT with or without ATM has any influence on heat-mediated IR-induced cell killing.

Cells deficient in ATM function are more sensitive to IR-induced cell killing compared with cells with normal ATM function. Both parental and hTERT-expressing fibroblasts with (BJ) and without (GM5823) ATM were exposed to heat at 43°C for 30 min followed by various doses of IR exposure. Cell colonies were scored and plotted against the radiation dose (Fig. 2A). Ectopically expressing hTERT cells with (BJ+hTERT) and without (GM5823+hTERT) ATM exhibited an increased survival after heat shock treatment compared with cells without hTERT expression (BJ) and GM5823. Interestingly, ATM-deficient cells with ectopic expression of hTERT (GM5823+hTERT) had a higher survival after heat and IR exposure than ATM-deficient cells without hTERT expression (GM5823). These results show that expression of telomerase does significantly (P < 0.05, Student’s t test) reduce heat-mediated IR-induced cell killing in both cells with and without ATM, although the effect of heat on cells deficient in ATM was more dramatic. These results further support the argument that the presence of telomerase influences heat-mediated radiosensitization.

**Inactivation of telomerase enhances heat- and IR-induced cell killing.**

We next tested whether genetic inactivation as well transient inactivation of telomerase influences heat-mediated IR-induced cell killing. Because telomerase activation has been linked with extension of cell life span (14, 33), it is possible that inactivation of telomerase may enhance cell death after heat and IR exposure. To determine the role of telomerase activity in cell...
killing, we compared MEFs with deletion of the telomerase catalytic unit (Tert) or RNA component (Terc) to wild-type fibroblasts to determine if telomerase inactivation increased sensitivity to heat and IR exposure. Mouse embryonic cells with and without mTert or mTerc were treated with heat at 43°C for 30 min followed by irradiation with different doses and examined for clonogenic survival (Fig. 2B). Interestingly, cells deficient for Tert or Terc had increased (P < 0.05, Student’s t test) heat-mediated IR-induced cell killing compared with their wild-type control cells. The above results suggest that the presence of Tert or Terc plays a role in heat-mediated IR-induced cell killing, and therefore, we further tested whether transient inactivation of telomerase activity would induce a similar sensitivity. hTR contains an 11-nucleotide-long template region for binding to and extending telomeric substrates that is easily accessible for hybridization with complementary oligonucleotides. Thus, hTR represents an attractive target for oligonucleotide-based anti-telomerase therapies (35). We tested several classes of telomerase inhibitors, including PNA, LNAs, and thiophosphoramidates (GRN163L; Fig. 3A; Supplementary Fig. S1), to determine whether these treatments could inhibit telomerase activity and enhance heat-mediated IR-induced cell killing. A series of cell-penetrating peptide-PNA conjugates specific for hTR (Supplementary Table S1) were synthesized and tested for inhibition of telomerase activity in 293 cells. After 24 h of PNA treatment, we found that 1 μmol/L hTR13-R9 produced the maximum inhibition of telomerase activity, whereas treatment concentrations >1 μmol/L decreased cell viability (Supplementary Fig. S1A; data not shown), in general agreement with another study using cell-penetrating peptide-PNA conjugates (36). We further compared the inhibition of telomerase activity at <1 μmol/L of PNA hTR13-R9, LNA, and GRN163L in cells treated for 24, 48, and 72 h (Fig. 3B; Supplementary Fig. S1B–D). At all time points examined, we found that GRN163L was more effective in inhibiting telomerase activity than PNA or LNA. After 72 h, only GRN163L treatment resulted in almost complete inhibition of telomerase activity, whereas LNA produced 80% inhibition and PNA resulted in 38% inhibition (Fig. 3B).

To determine the effect of PNA-, LNA-, and GRN163L-induced telomerase inhibition on IR-induced and heat + IR–induced cell killing, 293 cells were treated with 1 μmol/L of PNA, LNA/Lipofectamine, or GRN163L for 72 h and then either heated at 43°C for 30 min and irradiated with different graded IR doses or irradiated only. Cells pretreated with GRN163L followed by exposure to heat and IR showed maximum killing, whereas cells pretreated with LNAs were less sensitive and PNA-treated cells were the least sensitive (Fig. 4A). Overall, these results correlate with individual ability of the three agents to inhibit telomerase activity. To determine whether GRN163L treatment enhances heat-mediated IR-induced cell killing in cells without telomerase activity, isogenic cells with respect to telomerase BJ (telomerase negative) and BJ+hTERT (telomerase positive) and ALT cells (GM857; telomerase negative) were treated with 1 μmol/L GRN163L for 72 h and then either first heated at 43°C for 30 min and irradiated with different graded doses of IR or irradiated only. No major difference in the heat-mediated IR-induced cell killing was observed in BJ or GM857 cells (both lack telomerase activity) with or without GRN163L treatment; however, a significant (P < 0.05, Student’s t test) difference in heat-mediated IR-induced cell killing was observed in GRN163L-treated BJ+hTERT cells compared with untreated BJ+hTERT cells (Fig. 4B and C). Such studies suggest that telomerase activity may protect against heat-mediated IR-induced cell killing.

Our previous results have shown that inactivation of Hsp70.1/3 resulted in decreased telomerase activity (4); we therefore tested whether inactivation of telomerase in Hsp70.1/3-null cells would have any additional effect on cell killing after heat shock and IR.
exposure. As we reported previously (4), when heat shock is followed by IR exposure, there is a significant increase in cell killing in both cells with and without Hsp70.1/3; however, Hsp70.1/3-null cells are more sensitive to IR or heat + IR treatments (Fig. 4D).

In IR-exposed cells, there was no effect of telomerase inhibition on cell survival after irradiation in either wild-type or Hsp70.1/3-null cells. In contrast, when cells were first treated with GRN163L followed by heat and IR exposure, there was a modest decrease in

**Figure 4.** Effect of PNA-, LNA-, and GRN163L-induced telomerase inhibition on IR-induced and heat + IR–induced cell killing. **A,** 293 cells were treated with 1 μmol/L of PNA (a), LNA (b), or GRN163L (c) for 72 h and then either heated at 43°C for 30 min and irradiated with different graded IR doses or irradiated only. Clonogenic survival was determined as described previously (26, 41). **B** and **C,** isogenic cells with respect to telomerase (BJ and BJ+hTERT; B) and ALT cells (GM857; C) were treated with 1 μmol/L GRN163L for 72 h and either first heated at 43°C for 30 min and irradiated with different graded doses of IR or irradiated only. **D,** cell with Hsp70.1/3 (Hsp70.1/3+/+; a) and without Hsp70.1/3 (Hsp70.1/3−/−; b) was treated with GRN163L for 72 h and either heated at 43°C for 30 min and irradiated with different graded doses of IR or irradiated only. The means represent the values from four independent experiments.
cell survival in Hsp70.1/3-null cells and a more significant decrease in Hsp70.1/3 wild-type cell survival (P < 0.05, Student’s t test). It is important to note that the overall sensitivity of Hsp70.1/3-null cells to IR exposure or heat + IR exposure was ~9-fold greater, which may account for the lesser effect of telomerase inhibition in Hsp70.1/3-null cells.

Inactivation of telomerase activity results in the delayed disappearance of γ-H2AX foci formed following heat or IR treatment. The above results suggest that telomerase activity influences heat-mediated IR-induced cell killing. We have recently shown that hyperthermia induces γ-H2AX foci formation similar to foci formed in response to IR exposure and that heat-induced γ-H2AX foci formation is dependent on ATM but independent of HSP70 expression (5). Hyperthermia also enhanced ATM kinase activity and increased cellular ATM autophosphorylation. Because telomerase activity has also been shown to influence DNA damage repair (2, 37), we therefore examined whether telomerase activity influences heat shock–induced γ-H2AX foci formation. This was
achieved by examining heat-mediated γ-H2AX foci formation in (a) human cells with and without ectopic expression of hTERT, (b) mouse cells with and without mTert or mTerc, and (c) cells treated or not with GRN163L to inhibit the telomerase activity. To determine the effect of telomerase activity on heat-induced γ-H2AX foci formation, cells were subjected to a 30-min heat shock at 43°C and then allowed to recover at 37°C for various lengths of time. Immediately following heat treatment, the number of γ-H2AX foci increased in control cells to between 34 and 45 per cell and this number declined substantially during the 720-min recovery period at 37°C (Fig. 5A and B; data not shown). There was a modest decrease in γ-H2AX foci formation in telomerase-deficient cells (Fig. 5A and B); however, these foci persisted significantly (P < 0.05, Student’s t test) longer than those formed in cells with telomerase activity. Heat, therefore, leads to γ-H2AX foci formation irrespective of telomerase activity; however, cells deficient in telomerase activity have relatively delayed disappearance of γ-H2AX foci formation. To determine whether hyperthermia also modulates IR-induced γ-H2AX foci, cells were first treated with GRN163L as described above and then heated at 43°C for 30 min followed by incubation at 37°C. Cells with telomerase activity treated with GRN163L formed fewer foci per cell compared with untreated cells (Fig. 5B; data not shown). Interestingly, the appearance of heat-induced γ-H2AX foci in GRN163L-treated cells was influenced by hyperthermia as well as IR exposure (Fig. 5B and C), arguing that telomerase activity does influence heat-induced stress responses. Because GRN163L treatment resulted in the delayed appearance of heat-induced γ-H2AX foci formation, we further evaluated whether GRN163L influences DNA repair after heat and IR exposure. To determine whether GRN163L treatment results in higher residual chromosome damage, we examined cells with and without treatment with GRN163L for chromosome aberrations at metaphase. We did not find any differences in spontaneous chromosome aberration levels in cells with and without treatment with GRN163L for 72 h (Fig. 5D). However, cells treated with GRN163L had higher chromosome aberration levels after heat and IR exposure compared with cells not treated with GRN163L (Fig. 5D).

Discussion

Hyperthermia is a potent radiosensitizer currently under clinical investigation as a means to improve the response to IR-based cancer treatments (3). Hyperthermia itself has several cellular effects that should be synergistic with IR-induced tumor cell killing (30,38). For example, unlike the IR response (6), neither hypoxic nor plateau-phase cells are resistant to heat-induced cell killing (39). Because phase III clinical trials have shown significant benefits from adding hyperthermia to radiotherapy regimens for several malignancies (40), it is important to understand the mechanism leading to enhanced tumor cell killing. Telomerase is present in most tumors and has been shown to interact with HSP70, a protective protein up-regulated by hyperthermia that maintains protein function and inhibits apoptotic cell death under stress conditions. We have reported that loss of HSP70 expression in mouse fibroblasts (Hsp70.1/3−/−) is associated with decreased telomerase activity as well as increased heat shock + IR-induced cell killing (4). These observations are consistent with a previous report that telomerase plays a critical role in cellular metabolism (2). The present studies show that moderate heat shock enhances telomerase activity, and inactivation of telomerase activity before heat shock and irradiation enhances cell death specifically in telomerase-positive cells. This suggests that telomerase activity is part of an unknown mechanism that contributes to the cell-protective stress response.

Previous studies have shown that ectopic expression of hTERT enhances DNA repair and stabilizes the genome (2). In the present study, inactivation of telomerase was found in the present study to delay the appearance and disappearance of γ-H2AX foci following hyperthermia and irradiation, an indication of defective signaling during the DNA damage response. Interestingly, telomerase-positive cells treated with GRN163L show higher chromosome aberrations after heat and IR exposure compared with untreated control cells, whereas cells without telomerase activity did not show any difference in chromosome aberrations between treated versus untreated cells, further supporting a relationship between telomerase inactivation and heat-mediated IR-induced cell killing.

We used multiple approaches to determine the effect of telomerase inactivation on heat- and IR-induced cell killing. Interestingly, heat does not induce any telomerase activity in primary fibroblasts (BJ or GM5823) and, when treated with telomerase inhibitors, did not show any major influence on clonogenic survival after heat shock and IR treatments compared with the telomerase-positive cells (Fig. 2A). Collectively, our data suggest that inactivation of telomerase activity enhances heat-mediated IR-induced cell killing in telomerase-positive cells. Based on our present finding that telomerase has some additional functions for cell survival besides its role in telomere maintenance (2,13), the inactivation of telomerase has a great potential for enhancing heat shock–induced and IR-induced cell killing of telomerase-positive tumor cells.

References


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