Triggering Senescence Programs Suppresses Chk1 Kinase and Sensitizes Cells To Genotoxic Stresses

Vladimir L. Gabai, Cornelia O’Callaghan-Sunol, Le Meng, Michael Y. Sherman, and Julia Yaglom

Department of Biochemistry, Boston University Medical School, Boston, Massachusetts

Abstract

Depletion of the major heat shock protein Hsp72 leads to activation of the senescence program in a variety of tumor cell lines via both p53-dependent and p53-independent pathways. Here, we found that the Hsp72-depleted cells show defect in phosphorylation and activation of the protein kinase Chk1 by genotoxic stresses, such as UVC irradiation or camptothecin. Under these conditions, phosphorylation of Rad17 was also suppressed, whereas phosphorylation of p53 at Ser15 was not affected, indicating a specific defect in phosphorylation of a subset of the ATR kinase substrates. Similarly, suppression of Chk1 activation was seen when senescence signaling was triggered by direct stimulation of p53, depletion of Cdc2, or overexpression of the cell cycle inhibitors p21 or p16. Thus, defect in Chk1 activation was not a consequence of the chaperone imbalance, but rather a downstream effect of activation of the senescence signaling. Inhibition of Hsp72 was significantly enhanced. Thus, activation of the senescence signaling causes a defect in the DNA damage response manifested in increased sensitivity to genotoxic stresses. [Cancer Res 2008;68(6):1834–42]

Introduction

Treatment of various types of tumors often relay on the efficiency of radiation and chemotherapy. However, many cancer cells eventually develop resistance compromising the benefits of these therapies. Because most of anticancer drugs represent genotoxic compounds, cellular DNA repair systems counteract anticancer activities of these drugs (1). Response to genotoxic insults involves sensing of DNA damage by a class of protein kinases, including ATM, ATR, and DNA-PK, followed by activation of Chk1 and Chk2 kinases that cause temporal cell cycle arrest, as well as promote assembly of DNA repair complexes at the damaged sites at chromosomes (see refs. 2–4 for review). Thus, Chk1/Chk2-mediated temporal cell cycle arrest is critical to allow successful completion of DNA repair (3, 4). In fact, loss or inhibition of Chk1 leads to inability to shutdown the cell cycle progression and, accordingly, allows cells to progress into mitosis before completion of DNA repair (5). This, in turn, results in high incidence of mitotic catastrophe and increased sensitivity of cancer cells to various genotoxic drugs (see, e.g., refs. 6, 7). These data suggested that Chk1 could be a target for design of sensitizers to conventional chemotherapy. In fact, specific Chk1 inhibitors have been developed and are currently in clinical trials as an additional modality in combination with conventional drugs (8).

Overexpression of the recombinant heat shock proteins (Hsp) Hsp27 or Hsp72 was shown by several groups to protect cells from genotoxic stresses, including radiation and topoisomerase inhibitors (9–11). Furthermore, we have previously shown that down-regulation of these Hsps in certain cancer cell lines enhances their sensitivity to doxorubicin, radiation, and other drugs (12, 13). Therefore, Hsps seem to play an important role in tumor resistance to radiation and chemotherapy; however, the mechanisms of protection remain to be poorly understood. Various tumors were shown to express Hsps at significantly higher levels compared with normal tissues, and levels of Hsps seem to inversely correlate with the positive outcome of the disease (14, 15). It was suggested that Hsp27 and Hsp72 could guard transformed cells from the apoptotic death during tumor development (15), as well as protect them from chemotherapy and radiation-triggered killing. However, no clear correlation was found between the expression of apoptotic markers and chemoresistance and radioresistance in most common solid tumors (see, e.g., ref. 16). Moreover, it seems that apoptosis plays the major role in cell demise only in hematologic cancers, whereas in epithelial tumors (e.g., breast or prostate), clinically relevant doses of radiation or genotoxic drugs do not cause significant apoptosis. Previously, we have shown that high expression levels of Hsp72 and Hsp27 in various cancer cells efficiently suppress the default senescence program, thus promoting proliferation of cells. Accordingly, specific short hairpin RNA (shRNA)–mediated down-regulation of Hsp72 or Hsp27 resulted in a mixed cell population, wherein some cells became senescent, while the rest of the population continued to divide, although with significantly reduced rates (13). Here, we have found that activation of senescent signaling by various means, including chaperone depletion, leads to a general defect in activation of the protein kinase Chk1 in response to DNA-damaging stimuli. This suppression seems to play the major role in the increased resistance of these cells to genotoxic insults.

Materials and Methods

Cell cultures, treatments, and reagents. HEK293, HCT116, MCF10A, PC-3, and IMR90 cells were from American Type Culture Collection. HCT116 p53−/− cells were kindly provided by Dr. B. Vogelstein; HeLa cells were a kind gift of Dr. M. Borelli. HEK293 and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS); HCT116 cells were grown in McCoy 5× medium with 10% FBS; PC-3 were grown in RPMI 1640 supplemented with 10% FBS; MCF10A were cultivated in DMEM/F12 medium supplemented with 5% horse serum, hydrocortisone (500 ng/mL), insulin (10 μg/mL), and epidermal growth factor (20 ng/mL); IMR90 cells were cultivated in MEM with 15% FBS.
γ-Irradiation of cells was performed using 137Cs source (GammaCell 40) at 64 rad/min. UVC irradiation was performed with UV Stratalinker 1800 from Stratagene. Camptothecin was from Biomol, Nutlin-3A was from Cayman Biochemical, and all other reagents were from Sigma.

**Recombinant retroviral vectors.** For knockout experiments, we used RNAi-Ready pSIREN-RetroQ vector from BD Biosciences, containing a puromycin resistance gene for selection of stable transfectants. The following sequences were selected as targets for RNA interference:

- shHsp72: GAA GGA CGA GTT TGA GCA CAA
- shHsp27: ATC CGA TGA GAC TGC CAA
- shp21: CGG CGA CTG TGA TGC GCT AAT
- shCd2c: GGA ACT TCG TCA TCC AAA TAT

As a control shRNA, we used shRNA to firefly luciferase: GTG GAT TTC GAG TCG TCT TAA T or retroviral vector without an insert ("empty" virus). Babo-p16 and LNCX2-p21 retroviral vectors were kindly provided by Dr. A. Meriin (Boston University).

Retroviruses were produced by transfection of 293T cells with plasmids expressing retroviral proteins Gag-Pol, G (VSVG pseudotype), EGFP (kindly provided by Jeng-Shin Lee, Harvard Medical School), or our constructs. At 48 h after transfection, supernatants containing the retrovirus were collected and frozen at −70°C. Cells were infected with twice diluted supernatant and 10 μg/mL polybrene overnight and washed, and selection with puromycin (0.5 μg/mL) was started 48 h after infection.

**Dephosphorylation assay.** Cells were washed with PBS and left in PBS supplemented with 5 μM rotenone and 10 mM L-2-deoxyglucose to prevent further phosphorylation as described previously (17).

**Immunoblotting and antibodies.** Cells were lysed in lysis buffer [40 mM/L HEPES (pH 7.5), 50 mM/L KCl, 1% Triton X-100, 1% PBS, Na2VO4, 50 mM/L glycerophosphate, 50 mM/L NaF, 5 mM/L EDTA, 5 mM/L EGTA, supplemented with protease inhibitor cocktail from Roche Diagnostics]. Protein concentration in lysates was measured by Bio-Rad protein assay reagent, and lysates were diluted with a lysis buffer to achieve equal protein concentrations in all samples. Quantification of blots was performed using Quantity One software (Bio-Rad). phChk1/Chk1 ratio was calculated by normalizing phChk1 and Chk1 levels to actin.

Hsp72 and Hsp27 were detected with corresponding antibodies from Stressgen. Other antibodies used were β-actin from Sigma, Dc2, phosphorylated Chk1 Ser345, phosphorylated Chk2 Thr68, phosphorylated Ser215 p53, and phosphorylated Rad17 (Ser537) antibody (Cell Signaling), Chk1 (Santa Cruz), and p16 and p21 (BD PharMingen).

Fluorescence-activated cell sorting analysis. Cells were grown to 60% to 70% confluency, fixed in 63% ice-cold ethanol/PBS overnight at −20°C, stained with 50 μg/mL propidium iodine in the presence of 100 μg/mL RNase A, and analyzed using Becton Dickinson FACScan Cytometer.

**Clonogenic assay.** Cells were counted and plated on 60-mm Petri dishes. After 10 days, the formed colonies were stained with 0.5% crystal violet and counted. Survival was calculated as a percentage of viable cells.

**Results**

Down-regulation of Hsp72 impairs Chk1 activation upon UVC irradiation and camptothecin treatment. We have previously reported that depletion of Hsp72 in human colon tumor cell line HCT116 activates the p53-p21 pathway leading to a default senescence, as manifested by the appearance of heterogeneous cell population where ~40% of cells acquired flat enlarged morphology and became β-gal positive, while the rest of the cells continued to divide (13). Because ATM/Chk2 and ATR/Chk1 pathways are among the major mediators of p53 activation, we investigated their role in p53 stimulation upon chaperone depletion. Accordingly, HCT116 cells were infected with shRNA retrovirus directed against Hsp72 (sh72) and selected with puromycin, as described previously (see Materials and Methods). This method allows depletion of Hsp72 by 90% to 95% (Fig. 1A, left; ref. 13). As controls, we used HCT116 cells infected with retrovirus expressing empty vector or shRNA directed toward unrelated sequence (see Materials and Methods; both control retroviruses did not affect expression of Hsp72, and for the rest of the experiment, we used “empty” vector as a control; ref. 13). On day 7, postinfection control and sh72 HCT116 cells were subjected to UVC irradiation at 50 or 100 J/m2 to stimulate ATM/Chk1 or γ-irradiation at 5 Gy to stimulate ATM/Chk2. Activation of the kinases was assayed by immunoblotting of cellular lysates with antibodies against activated forms of Chk1 (phChk1<sup>Thr68</sup>) and Chk2 (phChk2<sup>Thr383</sup>) at indicated time points. As seen in Fig. 1B, activation of Chk2 upon γ-irradiation in sh72 HCT cells was similar to that in control cells (Chk1 was not activated under these conditions; data not shown). On the other hand, upon UVC irradiation, depletion of Hsp72 led to ~2-fold suppression of Chk1 activation (Fig. 1C and Supplementary Fig. S1A). Under these conditions, no ATM activation was seen, as judged by immunoblotting with phosphorylated ATM (Ser1981) antibody (not shown). Therefore, unexpectedly, Hsp72 depletion did not activate but rather suppressed the ATR/Chk1 pathway. Similar effect on suppression of Chk1 upon Hsp72 depletion was seen with HeLa cells (Supplementary Fig. S1B).

Chk1 could be activated by various genotoxic treatments, including a common chemotherapeutic drug camptothecin, which inhibits topoisomerase I. Therefore, to test whether the defect in Chk1 signaling was specific for UVC irradiation, we treated sh72 and control HCT116 cells with various doses of camptothecin and assayed Chk1 activation at the indicated time points. Similar to UVC irradiation, Chk1 activation in response to camptothecin was suppressed in sh72 cells at all treatment regimens (Fig. 1D). Furthermore, hydroxyurea-induced CHK1 phosphorylation was also inhibited in these cells (not shown), suggesting that Hsp72 depletion leads to a general defect in Chk1 signaling.

Hsp72 down-regulation did not lead to any significant difference in either Chk1 expression levels or its stability after genotoxic stresses (Supplementary Fig. S2A and B). Therefore, the observed suppression of Chk1 phosphorylation upon various genotoxic stresses could be mediated either by a failure of the ATR kinase to carry out Chk1 phosphorylation or accelerated dephosphorylation of phosphatases. To distinguish between these possibilities, we have assayed the rate of phosphorylation of Chk1 after UVC irradiation. After activation of Chk1 after UVC irradiation, further phosphorylation was blocked by rapid ATP depletion, as described previously (17), and levels of phosphorylated Chk1 were measured at various time points. Chk1 was losing phosphate groups relatively rapidly, and as seen in Supplementary Fig. S2C, the rates of dephosphorylation in Hsp72-depleted and control cell were similar. Therefore, it seems that inhibition of Chk1 phosphorylation upon UVC irradiation in Hsp72-depleted cells results from suppression of Chk1 phosphorylation by ATR rather than up-regulation of phosphatases. Accordingly, phosphorylation of a different ATR substrate, Rad17, in sh72 HCT116 cells was also reduced upon both UVC irradiation and camptothecin treatment (Fig. 2A). On the other hand, it has been previously shown that the ATR kinase is required for DNA damage-induced CHK1 phosphorylation, and it seems that inhibition of Chk1 phosphorylation upon UVC irradiation in Hsp72-depleted cells results from suppression of Chk1 phosphorylation by ATR rather than up-regulation of phosphatases.
other hand, phosphorylation of another ATR substrate, p53, at Ser15 upon UVC irradiation was similar in control and sh72 cells (Fig. 2B). These data indicate that Hsp72 depletion specifically suppresses phosphorylation only of a subset of the ATR substrates, including Chk1 and Rad17. It is likely that Hsp72 depletion does not compromise activation of the ATR kinase by DNA-damaging stresses UVC and camptothecin but affects ATR interactions with a subset of its substrates.

**Induction of p53/p21-dependent senescence program leads to inhibition of CHK1 signaling.** As mentioned above, depletion of Hsp72 stimulates the p53 pathway. Because depletion of a distinct chaperone Hsp27 also leads to activation of the p53 system

---

**Figure 1.** Down-regulation of Hsp72 or Hsp27 inhibits activation of CHK1 upon UVC irradiation or camptothecin treatment. A, levels of Hsp72 or Hsp27 in sh72-infected or sh27-infected cells, respectively. HCT116 cells were infected with retroviral vectors expressing Hsp72 (sh72) or Hsp27 (sh27) shRNAs and, briefly selected on puromycin. On day 7 of postinfection, levels of the Hsps were determined by immunoblotting with corresponding antibodies. B, down-regulation of Hsp72 does not affect γ radiation–induced CHK2 activation. HCT116 cells were infected with sh72 retroviral vector as in A, subjected to γ radiation (5 Gy), and Chk2 activation (Thr68 phosphorylation) was assayed by immunoblotting of cellular lysates at the indicated time points. C, down-regulation of Hsp72 or Hsp27 suppresses Chk1 activation upon UVC irradiation. HCT116 cells were infected with sh72 or sh27 retroviral vectors as in A, subjected to 100 J/m² UVC radiation, and levels of Chk1 activation (Ser345 phosphorylation) were determined by immunoblotting of cellular lysates at the indicated time points. D, down-regulation of Hsp72 suppresses Chk1 activation upon camptothecin treatment. HCT116 cells were infected with sh72 retroviral vector as in A and treated with 0.5 μmol/L camptothecin, and levels of Chk1 activation were determined as in C.

---

**Figure 2.** Down-regulation of Hsp72 inhibits phosphorylation of Rad17 but not p53 upon UVC radiation or camptothecin treatment. A and B, HCT116 cells were infected with sh72 retroviral vector as in Fig. 1A, treated with UVC radiation (200 J/m²; A and B) or 0.5 μmol/L camptothecin (CPT; A), and the levels of Rad17 phosphorylation (Ser645; A) or p53 phosphorylation (Ser15; B) were determined by immunoblotting of cellular lysates at the indicated time points.
and can trigger the senescence in a fraction of population, we investigated effects of Hsp27 depletion on activation of Chk1 in response to UVC irradiation. Hsp27 depletion was achieved by expression of the corresponding shRNA using a retroviral vector (see Materials and Methods). With this method, we observed ~60% depletion of Hsp27 (Fig. 1A, right). Importantly, under these conditions, activation of Chk1 by UVC was also suppressed, indicating that effects on Chk1 were not specific to Hsp27 depletion (Fig. 1C).

Chk1 is a known Hsp90 client protein, and its folding involves Hsp90, Hsp70, Hop, p23, and other chaperones and their cofactors (20, 21). Therefore, the observed suppression of Chk1 activation upon genotoxic stresses could result from a chaperone imbalance. Alternatively, it could be a specific consequence of induction of the senescence signaling program in transformed cells. To distinguish between these two possibilities, we assayed Chk1 activation in a transformed cell line PC-3, which has inactivated p53 and, as we found previously (12), does not undergo senescence upon depletion of Hsp72. Noteworthy, in these cells, depletion of Hsp72 did not lead to suppression of Chk1 activation upon UVC irradiation (Fig. 3A). These data argue against the direct effect of the Hsp72 depletion on Chk1 activity.

One of the major mediators of the senescence program stimulated by p53 activation is a cell cycle inhibitor, p21. Accordingly, senescence program in PC-3 could be reactivated upon a retrovirus-mediated transduction of p21. We observed that activation of Chk1 kinase upon UVC irradiation was suppressed by 90% in PC-3 cells overexpressing p21 (Fig. 3B). As with HCT116 cells, no difference in the levels or stability of Chk1 protein was observed (Supplementary Fig. S2A and data not shown). Similarly, in HCT116 cells, induction of the senescence signaling program mediated by p21 overexpression also led to a strong suppression of Chk1 signaling after UVC irradiation (Fig. 3C).

Next, we have investigated effects of direct activation of p53 on Chk1 signaling. Accordingly, HCT116 cells were treated with nutlin-3, a specific HD2M antagonist that up-regulates p53 and causes p21 accumulation (Fig. 3D). Three days after the beginning of the nutlin-3 treatment, ~50% of HCT116 cells acquired typical senescent morphology and became β-gal positive (not shown). The rest of the population, however, continued to divide, thus closely resembling the phenotype achieved by the chaperone depletion. As seen in Fig. 3D, nutlin-3 treatment strongly suppressed activation of Chk1 by UVC irradiation. These results indicate that suppression of Chk1 signaling represents a general consequence of activation of the p53/p21-dependent senescence signaling program and is not specific to chaperone depletion.

**P53 activation and p21 accumulation are not essential for suppression of CHK1 signaling upon UVC irradiation.** Because Hsp72 depletion activates senescence via both p53-dependent and p53-independent pathways, we investigated activation of Chk1 by UVC in sh72 p53/-/ HCT116 cells. As seen in Fig. 4A, Chk1 activation by UVC was ~2-fold suppressed in Hsp72-depleted p53/-/ HCT116 compared with p53/± HCT116 cells with normal levels of Hsp72, indicating that this phenomenon does not require p53 and could be a downstream effect of activation of the senescence signaling program. Similar to wild-type HCT116 cells, suppression of Chk1 signaling in p53/-/ HCT116 did not result in accelerated Chk1 dephosphorylation or any differences in Chk1 levels or stability, indicating a defect in the ATR-dependent phosphorylation of Chk1 (not shown).

To directly address the importance of p21 for Chk1 suppression, we used wild-type HCT116 cells where we have simultaneously depleted both Hsp72 and p21 with the corresponding shRNA retroviruses. As seen in Fig. 4B, double infection with sh72 and shp21 viruses strongly reduced p21 levels (compare lanes 3 and 4), and no accumulation of p21 was observed upon Hsp72 depletion (compare lanes 2 and 4). Despite that, a fraction of cells displayed typical senescent morphology and became β-gal positive, indicating that depletion of Hsp72 under these conditions triggered a p21-independent senescence pathway. We observed that inhibition of Chk1 activation by UVC irradiation in the double (sh72 + shp21)–depleted cells was still significantly suppressed compared with control, shp21-depleted cells, although the extent of suppression was 30% less prominent than in cells with normal levels of p21 (Fig. 4C). From these experiments, we have concluded that p21 accumulation is sufficient but not essential for impairment of normal Chk1 signaling.

Beside the p53-p21 pathway, cell senescence could be triggered by activation of the p16-Rb pathway. Interestingly, overexpression of p16 using retrovirus delivery system markedly slowed down the growth of HCT116 cells and evoked senescent phenotype in 40% of cells (not shown). As with p21 overexpression, we have observed that p16 overexpression also led to inhibition of Chk1 signaling (Fig. 5A). Similar results were obtained when senescence signaling was triggered via independent pathway through retrovirus-mediated depletion of Cdc2 (Fig. 5B). Of note, p21 levels were not affected by either p16 overexpression (not shown) or Cdc2 down-regulation (Fig. 5B). Therefore, activation of various senescence signaling pathways (p53/p21, p16 overexpression or cdc2 down-regulation) prevents proper Chk1 activation upon genotoxic stress.

To further characterize a novel phenomenon of Chk1 suppression, we investigated whether activation of the senescence signaling in nontransformed cells also impairs Chk1 signaling. Indeed, we have observed suppression of Chk1 signaling in immortalized breast epithelium MCF10A cells, where p53-dependent senescence was triggered by nutlin-3 treatment or by overexpression of p21 (Fig. 5C). Moreover, Chk1 signaling was also suppressed in UV-C-irradiated normal human fibroblasts, IMR90, undergoing replicative senescence compared with their young counterparts (Fig. 5D). Thus, stimulation of the senescence signaling in normal cells leads to similar defects in Chk1 signaling as in transformed cells.

**Suppression of Chk1 signaling is not mediated by cell cycle abnormalities.** ATR/Chk1 pathway is activated preferentially during S-phase (4). Therefore, suppression of Chk1 activation could be due to difference in the cell cycle distribution associated with senescent population. To directly investigate the role of cell cycle distribution, we assayed Chk1 activation upon UVC irradiation specifically in S-phase cells. Control and sh72 HCT116 cells were incubated with nocodazole for 15 h, and cells in both groups that had reached M phase and became rounded were separated from the rest of the population by the shake-off. Shaken-off sh72 and control HCT cells were then plated into fresh media and grown for additional 13 h to progress into S phase (Supplementary Fig. S3A). As expected, that because control cells grow faster than sh72 HCT cells 13 h after plating into the drug-free media, some of the control cells have progressed into G2 phase, whereas a majority of sh72 HCT116 cells were still in S phase (Supplementary Fig. S3A). At this point, cells were either UV-C-irradiated or subjected to camptothecin treatment, and activation of Chk1 was assessed as described before. As seen in Fig. 5B, activation of Chk1 in response to both treatments was strongly reduced in the S-phase sh72 HCT cells compared with the S-phase control cells. This result indicates that defect in Chk1 signaling was
not due to the reduction of the S-phase population but rather represented a general feature of cells with activated senescence signaling programs.

**Activation of the senescence signaling results in deficient S-phase checkpoint upon UVC radiation.** Activation of Chk1 is critical for ability of cell to halt the cell cycle progression, providing time needed for the DNA repair. Therefore, the observed defect in Chk1 activation could compromise ability of cell to activate checkpoint upon genotoxic stress. To address this question, we have investigated whether Hsp72 down-regulation abrogates proper checkpoint activation in HCT116 cells. Accordingly, sh72 and control HCT116 cells were treated with nocodazole to synchronize cells in mitosis. After 15 h of treatment with nocodazole, all control cells became rounded, indicating that they were arrested in mitosis. In sh72 HCT cells, ~70% of the cells became rounded, whereas the rest of the population remained flat, enlarged, and firmly attached to the plate. This heterogeneity reflects the presence of mixed populations of senescent growth arrested and dividing cells in the Hsp72-depleted cultures. Of note, senescent cells in G2 phase and dividing cells in M phase could not be distinguished by fluorescence-activated cell sorting analysis, as both of them have 4n DNA content (Fig. 6B, left). Nocodazole-synchronized cells were then released into fresh, drug-free media and allowed to resume growth for the next 10 h, at which point most of the sh72 and control cells progressed into S phase (Fig. 6B, middle). G1-S phase synchronized cells were either subjected to UVC irradiation or left untreated. As expected, UVC irradiation blocked cell cycle progression of control cells, and 8 h after UVC irradiation, cells remained in G1-S phases. In contrast, a population of sh72 HCT116 cells failed to maintain arrest. Accordingly, 8 h after UVC irradiation, more than half of sh72 cells have progressed into G2 phase (see Fig. 6B, right). This experiment indicates that inhibition of Chk1 signaling upon UVC irradiation prevents proper activation of inter-S-phase checkpoint in Hsp72-depleted cells.

**Down-regulation of Hsp72 leads to aneuploidy and sensitizes tumor cells to UVC radiation and camptothecin treatment.** The defect in the cell cycle control can potentially lead to chromosome instability and aneuploidy. To directly investigate this

---

**Figure 3.** Activation of senescence signaling but not the depletion of chaperones leads to suppression of the CHK1 signaling. A, depletion of Hsp72 in PC-3 prostate carcinoma does not affect UVC-induced Chk1 activation. PC-3 cells were infected with sh72 retroviral vector as in Fig. 1A and subjected to UVC radiation (200 J/m²), and the levels of Chk1 activation and Hsp72 expression were determined by immunoblotting of cellular lysates at the indicated time points. B and C, expression of p21 in PC-3 (B) or HCT116 cells (C) suppresses UVC-induced Chk1 activation. PC-3 or HCT116 cells were infected with p21-expressing or empty retroviral vectors, selected for 7 d with neomycin, and UVC irradiated (200 J/m²). The levels of phChk1 (left) and p21 (right) were determined by immunoblotting of cellular lysates with the corresponding antibody. D, HDM2 antagonist nutlin-3 suppresses UVC-induced Chk1 activation. HCT116 cells were treated with 5 μmol/L nutlin-3 for 72 h and exposed to UVC radiation (200 J/m²), and the levels of phCHK1, p53, and p21 were determined by immunoblotting of cellular lysates with the corresponding antibody. C, control; N, nutlin-3.
question, we assessed whether activation of the senescence signaling program by the Hsp72 depletion could affect the ability of cells to maintain the proper chromosome number under conditions of genotoxic stress. Accordingly, we prepared metaphase spreads from UVC-irradiated control and Hsp72-depleted cells followed by the telomere-FISH analyses. We did not detect significant difference in number of chromosome or chromatid breaks in metaphase spreads from sh72 and control cells after UVC irradiation. However, there was significant increase in the number of metaphase spreads with less than normal content of chromosomes in sh72 cells compared with control cells (Supplementary Fig. S3 B).

Indeed, if, in control and sh72 cells without treatment, the percentage of aneuploid cells was the same (25+/-8%), in sh72 cells after UV (50 J/m²), it increased to 63+9% comparing to 14+9% in UV-treated control cells ($P < 0.05$ by Student's $t$ test). These data indicated that activation of the senescence signaling program is accompanied by the chromosomal instability, suggesting that sensitivity of cell to genotoxic stresses could be enhanced (see model in Fig. 6D).

To test this hypothesis, sh72 and control HCT116 cells were subjected to UVC irradiation at 50 or 100 J/m² or treated with various doses of camptothecin, and the colony formation was monitored. As reported previously, down-regulation of Hsp72 reduced clonogenic survival of HCT116 cells even without the genotoxic treatment, apparently because it induced senescence in a fraction of the population of cells. Therefore, to adequately compare the sensitivity of cells to genotoxic stresses, we have normalized background levels of clonogenic survival in control and Hsp72-depleted cells (see Materials and Methods). As seen in Fig. 6C, Hsp72 depletion significantly sensitized cells to UVC or camptothecin treatments. Similar sensitization to UVC irradiation was observed in Hsp27-depleted cells (Fig. 6C). Thus, elevated endogenous levels of Hsp27 and Hsp72 in the colon carcinoma HCT116 cells contribute to resistance of these cells to genotoxic stresses by maintaining efficient DNA damage response. Noteworthy, in PC-3 cells where Hsp72 depletion does not activate the senescence signaling program and does not cause suppression of Chk1 (Fig. 3A), almost no sensitization to UVC was observed (Supplementary Fig. S4). Therefore, stimulation of the senescence program in tumor cells, for example, by down-regulation of chaperones, could serve as a new promising approach toward improving chemotherapeutic drug–induced elimination of tumor cells (Fig. 6D).

Discussion

Previously, we have reported that depletion of Hsp72 in certain cancer cell lines leads to activation of the p53-dependent and p53-independent senescence signaling pathway and results in a mixed cell population, wherein ~40% of cells cease dividing and become senescent, whereas the rest of the population continues to divide with reduced rates (13). This population, however, becomes more sensitive to various DNA-damaging stimuli. With some of these stimuli, like doxorubicin or H₂O₂, increased sensitivity was associated with enhanced senescence (13). However, others, like UVC irradiation, did not cause appearance of senescent cells.¹ Here,
we report that depletion of Hsp72 leads to a general defect in DNA damage response associated with suppression of phosphorylation of Chk1, Rad17, and possibly certain other targets of the ATR kinase. This suppression, however, did not result from a general inhibition of the ATR activity, because phosphorylation of its distinct substrate p53 at Ser15 was normal (see Fig. 2). Possibly, in Hsp72-depleted cells, genotoxic insults lead to normal activation of the ATR kinase; however, ATR interactions with a subset of its substrates seem to be jeopardized. Indeed, its interactions with various substrates require distinct mediator proteins (22, 23).

Originally, we hypothesized that a chaperone imbalance that results from the Hsp72 depletion specifically or nonspecifically cause suppression of the Chk1 activation. According with this idea, we have shown that depletion of another chaperone Hsp27 has similar consequences, both in terms of suppression of Chk1 activation and increased sensitivity to the genotoxic stimuli. Furthermore, this hypothesis was in line with previous observation that Hsp90, Hsp70, and other chaperones and cochaperones play a role in folding and stability of Chk1 (20, 21). Further experiments, however, indicated that these effects did not represent a specific response of cells to the chaperone imbalance, but rather resulted from activation of senescence signaling programs. In fact, activation of diverse p53-dependent and p53-independent senescence signaling pathways by various means led to suppression of Chk1 activation in response to UVC irradiation or camptothecin treatment, whereas no suppression of Chk1 signaling was observed in PC-3 cells where Hsp72 depletion does not activate the senescence program (Fig. 6D).

It was reported that activation of Chk1 mainly occurs during S phase of the cell cycle (24). Therefore, the observed suppression of Chk1 activation could be related to decrease in the fraction of S-phase cells upon activation of senescent program. However, in S-phase synchronized population of dividing sh72 HCT116 cells activation of Chk1 kinase was significantly inhibited upon both

![Figure 5. Induction of p53/p21-independent senescence signaling in transformed and normal cells results in inhibition of Chk1 activation upon UVC-irradiation.](image-url)
UVC irradiation and camptothecin treatment, although we did not detect significant differences in the levels of total Chk1 protein (not shown). Furthermore, the degree of inhibition in the S-phase population was similar to that in total population (compare Figs. 1 and 6). These results indicate that suppression of Chk1 after activation of the senescent signaling program applies equally to fully senescent growth-arrested cells and cells that still continue to divide slowly.

Activation of Chk1 kinase plays a critical role in proper execution of inter S-phase checkpoint. In fact, temporal cell cycle arrest mediated by Chk1 is important to allow cells to repair DNA and thus be able to enter unperturbed mitosis (25). Therefore, failure to activate Chk1 could be the major factor in increased sensitivity of cells with activated senescence signaling programs to genotoxic stimuli. Accordingly, inhibition of Chk1 with specific chemical inhibitors leads to premature mitosis and often results in
mitotic catastrophe, thus increasing sensitivity of cancer cells to some antineoplastic drugs, such as camptothecin (7, 8). In line with these data, we show that suppression of Chk1 in cells with activated senescence signaling clearly hinders execution of the inter–S-phase checkpoint after UVC irradiation. Moreover, we have observed that stimulation of senescent program greatly potentate sensitivity of cells to UVC irradiation and camptothecin exposure (Fig. 6D). On the other hand, other factors like inefficient phosphorylation of Rad17 could also contribute to the enhanced sensitivity.

There is a growing body of evidence suggesting that cellular senescence is accompanied by accumulation of DNA damage (26). Furthermore, it is commonly accepted that the main reason for the replicative senescence is chromosomal instability caused by defective telomeres (27). The cause of DNA damage in other types of senescence is poorly understood. Based on data presented in this work, we hypothesize that the important reason for DNA abnormalities detected in senescent cells could be suppression of Chk1, which leads to inefficient checkpoints and further promote DNA instability, forming a positive feedback loop (Fig. 6D). Thus, stimulation of senescence signaling could serve as a novel promising method of sensitization of cancer cells to conventional chemotherapeutic drugs aimed at DNA damage.

Acknowledgments

Received 9/26/2007; revised 12/18/2007; accepted 12/18/2007.

Grant support: National Cancer Institute Public Health Service CA844012 (M. Sherman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Sonia Franco for her help with FISH assay and Dr. A. Merin for plasmids and helpful discussion.

References


Cancer Res 2008; 68: (6). March 15, 2008 1842 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 10, 2017. © 2008 American Association for Cancer Research.
Triggers Senescence Programs Suppresses Chk1 Kinase and Sensitizes Cells To Genotoxic Stresses

Vladimir L. Gabai, Cornelia O'Callaghan-Sunol, Le Meng, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/6/1834

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/03/13/68.6.1834.DC1

Cited articles
This article cites 27 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/6/1834.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/68/6/1834.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.