Ignition of p53 Bomb Sensitizes Tumor Cells to Granzyme K-Mediated Cytolysis[1]

Guoqiang Hua, Shuo Wang, Chao Zhong, Peng Xue, and Zusen Fan[2]

Inactivation of tumor suppressor p53 results in loss of the apoptosis-regulating function of the p53 protein in tumor cells. Restoration of wild-type p53 expression in p53 mutant tumor cells increases tumor susceptibility to CTL-mediated cytolysis. However, the direct role of p53 in regulating tumor sensitivity to NK cell-mediated lysis and the functional relationship between p53 and granzymes in the control of tumor killing are still poorly documented. In this study, we found that p53 can sensitize tumor-killing susceptibility to NK and granzyme K-mediated cytolysis. Granzyme K is constitutively expressed in high levels in NK cells and induces rapid caspase-independent cell death. Granzyme K may exert a critical role in NK cell-mediated tumor clearance. p53 associates with granzyme K and is a physiological substrate of granzyme K. p53 was processed to three cleavage products of p40, p35, and p13 fragments at Lys^{24} and Lys^{305}. These three cleavage products harbor strong proapoptotic activities that amplify the proapoptotic action of p53 to potentiate tumor-killing sensitivity. Therefore, p53 is as a cytotoxic bomb that can be triggered by granzyme K, leading to potentiating killing efficacy. ***The Journal of Immunology,*** **2009, 182: 2152–2159.**

The tumor suppressor p53 plays multiple roles in cell cycle control, differentiation, angiogenesis, genomic stability, and apoptosis (1). Mutations of the *p53* gene are frequently found in >50% of all human tumors, suggesting that loss of this gene represents an important step in the formation of human cancers. p53-deficient mice are extremely susceptible to spontaneous, oncogene, and mutation-induced tumorigenesis (2). Moreover, the ability of p53 to control the cell cycle and apoptosis has been reported to contribute to drug sensitivity induced by many anticancer agents (3). It is well established that tumor suppressor p53 plays a critical role in tumorigenesis.

Transformed cells still need to escape immune surveillance to form tumor cells. Cytotoxic lymphocytes such as NK and CTL can recognize and eliminate the transformed cells mainly through the granule exocytosis pathway, which releases perforin and tumor killer granzymes (Gzms)[3] from the preformed secretory granules into the immunological synapse formed with the target tumor cell (4–6). Thiery et al.(7) reported that the restoration of wild-type p53 expression in p53 mutant tumor cells increases tumor susceptibility to CTL-mediated cytolysis (7). They further showed that p53 is a key determinant in antitumor CTL response that regulates induction of Fas receptor expression, cellular FLICE/caspase 8 inhibitory protein short-form degradation, and Bid translocation to target mitochondria (8). CTL targeting results in p53 accumulation and activation at very early times (9). However, the functional relationship is less defined between p53 and Gzms in the control of tumor-killing susceptibility.

GzmK is a second tryptase among all of the Gzms. Tryptase activity is essential for CTL-mediated tumor clearance. GzmK constitutively expresses high levels in CD56 NK cells, memory CD8 T cells, and CD56 T cells (10). We found that GzmK triggers rapid cell death with ssDNA nicks independently of caspase activation through targeting the endoplasmic reticulum-associated SET protein complex (11, 12). GzmK can also induce rapid reactive oxygen species (ROS) generation and collapse of mitochondrial inner membrane potential leading to release of cytochrome *c* and endonuclease G (13). GzmK-mediated target cell death is consistent with the lysis dynamics of NK cells. It suggests that GzmK exerts an important role in NK cell-mediated tumor clearance. In this study, we found that p53 can sensitize tumor-killing susceptibility to GzmK or NK cell-mediated cytolysis. p53 associates with GzmK and is a physiological substrate of GzmK. p53 was processed to three cleavage products at Lys^{24} and Lys^{305}. These three cleavage products harbor strong proapoptotic activities that amplify the proapoptotic action of p53 to potentiate tumor-killing sensitivity.

Materials and Methods

Cell lines, Abs, and reagents

HCT116 $p53^{+/+}$ cells and the isogenic HCT116 $p53^{-/-}$ cells (provided by Dr. B. Vogelstein, Johns Hopkins Medical School, Baltimore, MD) were maintained in McCoy's 5A medium supplemented with 10%FBS. NK cells isolated from PBMC were cultured in DMEM containing 15% FBS, 15% horse serum, 2 mM L-glutamine, and 100 U/ml recombinant human IL-2. H1299 cells (Chinese Academy of Medical Sciences, Beijing, China) were grown in RPMI 1640 medium supplemented with 10% FBS. HeLa and 293A cells were grown in DMEM supplemented with 10% FBS, 50 μM 2-ME, 100 U/ml penicillin, and streptomycin. Monoclonal and polyclonal Abs for GzmK were generated by our laboratory. Commercial Abs were mouse mAb against hemagglutinin (HA) (BD Pharmingen), β-actin, HRP-conjugated sheep anti-mouse IgG (Santa Cruz Biotechnology), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (Molecular Probes). Bax and

National Laboratory of Biomacromolecules and Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

Received for publication July 15, 2008. Accepted for publication December 3, 2008.

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.

[1] This work was supported by the National Natural Science Foundation of China (Grants 30525005, 30830030, and 30528028), 863 Program (2006AA02Z4C9), 973 Programs (2006CB504303, 2006CB910901), the Innovative Program (KSCX2-YW-R-42), and the Hundred Talents Program of the Chinese Academy of Science (to Z.F.).

[2] Address correspondence and reprint requests to Dr. Zusen Fan, National Laboratory of Biomacromolecules and Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. E-mail address: fanz@moon.ibp.ac.cn

[3] Abbreviations used in this paper: Gzm, granzyme; mGzmK, mutated GzmK; Ad, adenovirus; ROS, reactive oxygen species; HA, hemagglutinin; CMA, concanamycin A; PI, propidium iodide; RT, room temperature; S-AGzmK, Ser-Ala GzmK.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802307

p53 Abs (DO1, FL393) were purchased from Santa Cruz Biotechnology. LipofectamineTM2000 was from Invitrogen. Annexin V-FITC was from BD Pharmingen and the ProLong Antifade kit was from Molecular Probes. ^{51}Cr (as sodium dichromate) was purchased from PerkinElmer.

Isolation of NK cells

PBMC from healthy donors were purchased from the Beijing Blood Center and were purified by Ficoll-Hypaque gradient centrifugation separation. Primary NK cells were enriched from PBMC by positive selection using the human NK cell isolation kit according to the manufacturer's instructions. Briefly, the PBMC were resuspended in PBS buffer containing 0.5% BSA and exposed to anti-CD56 microbeads at 4°C for 15 min. NK cells were then positively selected using the MACS cell separator system (Miltenyi Biotec). The purity of the enriched human NK cells was >90% NK cells as determined by flow cytometry. These enriched human primary NK cells were cultured in IL-2-containing medium.

Pull-down assay

Mutated GzmK (mGzmK) was covalently linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. Equal amounts of Affi-Gel 10 beads coupled to GST protein were used as controls in the assays. HCT116 p53$^{+/+}$ cell lysates were incubated with immobilized mGzmK Affi-Gel 10 overnight at 4°C. The gel was washed four times by brief centrifugation and aspiration. The pull-down proteins separated by SDS-PAGE were analyzed by Western blotting.

Recombinant protein expression

Recombinant GzmK and mGzmK (enzymatically inactive GzmK produced by mutating the active site Ser^{214} to alanine) were produced and purified as previously described. Full-length cDNA coding wild-type human p53 and mutant human p53 (K24A, K305A) was subcloned into pQE30 and expressed in *Escherichia coli* strain M15. Recombinant p53 with 6× His tags were purified through nickel columns as above. The Stratagene QuikChange site-directed mutagenesis kit was used to mutate candidate lysine residues to identify the GzmK cleavage site according to the manufacturer's instructions.

Coimmunoprecipitation and immunoblotting

Abs were preincubated with protein A-Sepharose (Pharmacia) for 1 h at 4°C. Ab-coated beads were washed twice in PBS before being added to recombinant proteins (50 μg/ml) or cytosolic lysates (5×10^6 cell equivalents in 20 μl of Nonidet P-40 lysis buffer) that had been preincubated at 4°C for 2 h. After overnight incubation with shaking at 4°C, the beads were washed extensively in 1% Nonidet P-40/0.1% SDS in PBS and boiled in 2× SDS loading buffer before electrophoresis through SDS-PAGE gels. After transfer to nitrocellulose, blots were probed with the indicated Abs.

Cell-mediated cytotoxicity assay

Tumor cell lysis by NK cells was quantitated in a 4-h ^{51}Cr release assay. Target cells (1×10^6) were labeled with 200 μCi of ^{51}Cr and plated at 1×10^4 cells/well. Effector cells were added at various E:T ratios, and after 4 h of incubation, the supernatant was removed and radioactivity was measured in a gamma counter. The percent cytotoxicity was determined as follows: percent cytotoxicity = [(sample release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release of target cells was measured following treatment with 2% Triton X-100 detergent. For selective inhibition of the perforin/Gzm pathway, NK cells were pretreated for 2 h with 500 nM concentrations of concanamycin A (CMA) and then assayed for cytotoxicity.

Loading GzmK with adenovirus

Cells were washed three times in HBSS and resuspended in loading buffer (HBSS with 0.5 mg of BSA/ ml, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Target cells (2×10^5) in 50 μl of loading buffer were incubated at 37°C for the indicated times with different concentrations of GzmK, Ser-Ala Gzmk (S-AGzmK), and an optimal dose of adenovirus (Ad). Cells were incubated for an additional 15 min in 1 mM PMSF before being lysed for immunoblot.

Cleavage assay

Cell lysates prepared from HCT116 cells treated with Nonidet P-40 lysis buffer (0.5% Nonidet P-40/25 mM KCl/5 mM $MgCl_2$/1 mM PMSF/10 mM Tris-HCl, pH 7.6). Cell lysates (equivalent to 2×10^5 cells) or 1 μM rp53 was incubated with the indicated doses of GzmK or S-AGzmK for the indicated time points in 20 μl of cleavage buffer (50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 1 mM $MgCl_2$). For in vivo cleavage assay, cells loaded with GzmK/Ad were lysed in 0.5% Nonidet P-40 lysis buffer. The reaction samples were terminated in 5× SDS loading buffer and probed by immunoblotting. For in vitro cleavage assay, 0.5 μM rp53 was incubated with different concentrations of GzmK for the indicated times. The reaction products were probed as above.

Flow cytometry analysis

FITC-conjugated annexin V was used to evaluate phosphatidylserine externalization. Target cells treated with S-AGzmK, GzmK, and Ad were stained with annexin V-Fluos and propidium iodide (PI) and analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

TUNEL assay

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, treated tumor cells were washed three times with PBS, fixed with 2% paraformaldehyde in PBS for 1 h at room temperature (RT), and permeabilized with freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Fifty microliters of TUNEL reaction mixture was added into each sample and incubated for 1 h at 37°C in a humidified atmosphere in the dark. Cells were further incubated with 5 μg/ml Hoechst 33342 (Sigma-Aldrich) in PBS for 10 min and visualized through immunofluorescent microscopy.

Transfection of HCT116 (p53$^{-/-}$) and H1299 (p53$^{-/-}$) cells with p53

A full-length p53 DNA construct was synthesized by PCR amplification of PQE-30-p53 and inserted into a pEF PGKpuro or pcDNA3.1 plasmid vector. After verifying DNA sequence and orientation, the p53 construct was transfected into HCT116 or H1299 cells using the Lipofectamine 2000 transfection reagent (Invitrogen). The transfectants were selected in the presence of 2 μg/ml puromycin or 1 mg/ml G418, and the surviving clones were pooled. The expression of p53 protein in the stable transfectants was confirmed by Western blotting analysis.

Laser-scanning confocal microscopy

HeLa cells were plated on 8-well chamber slides coated with rat collagen I (BD Falcon) and transfected with full-length p53 or p53 fragment plasmids. After a 24-h transfection, cells were incubated with 500 nM Mito-Tracker (Molecular Probes) for 30 min according to the manufacturer's instruction. The cells were fixed with 4% paraformaldehyde for 20 min at RT and permeabilized with 0.1% Triton X-100 for another 20 min. The cells were incubated at RT for 1 h with 5 μg/ml anti-HA mAb, 50 μg/ml donkey serum, and 100 μg/ml RNase I. The cells were stained with Alexa Fluor 488-conjugated donkey anti-mouse IgG and soaked for 5 min in PBS containing 0.1 μg/ml Hoechst. The slides were mounted with ProLong Antifade reagent and observed using laser-scanning confocal microscopy (Olympus FV500 microscope).

Plasmids and luciferase assay

Human wild-type p53 encoding pCMV-HA plasmid with an N-terminal influenza HA epitope tag was used to generate different p53 fragments. Different p53 fragments were cloned in pCMV-HA in-frame with HA tag. Luciferase assays were performed following the instructions of the Promega Dual Glo kit. Briefly, 293A cells were cotransfected with p53 or p53 fragment expression vector (pCMV-HA-p53, pCMV-HA-p40, pCMV-HA-p35, pCMV-HA-p13) with promoters (PG13-Luc, Bax-Luc, P21-Luc, MDM2-Luc). Luciferase assay was performed after a 36-h transfection. Cells were harvested and washed once with PBS. Then, they were lysed in a lysis buffer. Luciferase activity was measured in 20 μl of the supernatants using a dual luciferase reporter assay system (14).

Results

GzmK associates with p53

We previously showed that GzmK induces caspase-independent cell death with ssDNA nicking. GzmK proteolyses the nucleosome assembly protein SET and abolishes its nucleosome assembly activity (12). GzmK activates the DNase NM23H1 activity to nick chromosomal DNA via cleavage of its inhibitor SET. To define the substrates of GzmK, enzymatic inactive S-AGzmK(mGzmK) was generated to perform a pull-down assay by using p53-expressed colon cancer cell (HCT116p53$^{+/+}$) lysates. p53 was eluted from

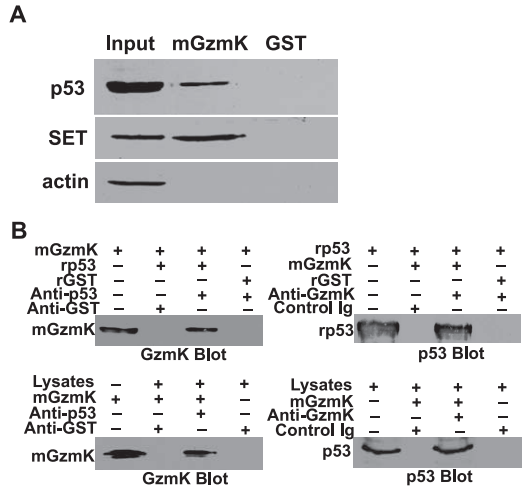

FIGURE 1. GzmK associates with p53. *A,* Inactive mutant S-AGzmK-(mGzmK) can bind to native p53 by the pull-down assay. p53 was expressed in the colon cancer cells (HCT116p53$^{+/+}$). HCT116 p53$^{+/+}$ cell lysates were incubated with immobilized mGzmK Affi-Gel 10 overnight at 4°C and the pull-down fraction was detected by p53 Ab. Its previously identified substrate SET was probed as a positive control. rGST was used as a negative protein control. *B,* p53 binds directly to S-AGzmK. rp53 and S-AGzmK were coincubated in PBS for 2 h at 4°C and then immunoprecipitated with GzmK mAb (*right*), p53 polyclonal Ab (*left*), or a control Ab. The blots were probed by p53 polyclonal Ab (*right*) or GzmK mAb (*left*). Similar results were obtained when S-AGzmK coprecipitated native p53 in HCT116 p53$^{+/+}$ cell lysates.

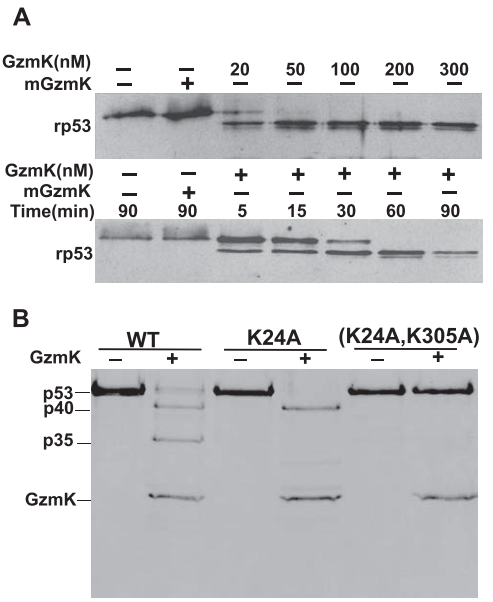

FIGURE 2. GzmK directly cleaves rp53. *A,* rp53 was cleaved by GzmK in a dose- and time-dependent fashion. rp53 was incubated with different concentrations of GzmK at 37°C for 90 min or with 0.1 μM GzmK for the indicated times. The reactions were stopped by adding 5× SDS loading buffer and analyzed by immunoblotting with p53 Ab. *B,* Identification of the GzmK cleavage sites in p53. p53 has two GzmK cleavage sites. Two GzmK target sites, K24 and K305, were identified through mass spectrometry and site-directed mutagenesis. Wild-type (WT) and mutant p53 proteins (K24A and K24A, K305A) were purified and incubated with GzmK for its cleavage confirmation.

immobilized mGzmK Affi-Gel 10 beads and coeluted with a previously identified GzmK substrate SET (Fig. 1*A*). GST Affi-Gel beads did not precipitate p53 or SET. To verify the direct interaction of p53 with GzmK, we performed coimmunoprecipitation experiments with the rp53 and S-AGzmK. Anti-p53 Ab can precipitate mGzmK (Fig. 1*B*). Anti-GzmK Ab was also capable of precipitating rp53. Neither anti-GST Ab or control Ig precipitated mGzmK or rp53. rGST had no interaction with either mGzmK or rp53. The interaction of mGzmK with native p53 was confirmed through preincubation of mGzmK with HCT116p53$^{+/+}$ cell lysates as shown in Fig. 1*B*. Therefore, GzmK directly binds to p53 in vitro and associates with native p53 in vivo.

Gzm K cleaves p53 at Lys^{24} and Lys^{305}

To determine whether p53 is cleaved by GzmK, rp53 was incubated with different doses of GzmK at 37°C for 90 min or with 0.1 μM GzmK for 5–90 min. p53 began to degrade at a low concentration of 20 nM GzmK or an early time of 5 min (Fig. 2*A*). The major p40 band was detected by the DO1 anti-p53 Ab. The inactive S-AGzmK cannot cleave rp53. p53 degradation required enzymatic activity of GzmK. To test the cleavage sites of p53, rp53 was treated with active GzmK and resolved by SDS-PAGE followed by Coomassie staining. rp53 was processed to two major bands, p40 and p35. Cleavage sites were determined at Lys^{24} and Lys^{305} through mass spectrometry and site-directed mutagenesis. We mutated Lys^{24} to alanine (K24A) or both Lys^{24} and Lys^{305} to alanine (K24A/K305A) and expressed their recombinant proteins. These two mutant proteins were treated with active GzmK and visualized by SDS-PAGE. K24A mutant only produced one product, p40 fragment (Fig. 2*B*). Although K24A/K305A cannot be cleaved by GzmK, these data further confirmed p53 was directly processed by GzmK at Lys^{24} and Lys^{305}. Therefore, three cleavage products were generated as p40(1–305), p35(25–305), and p13(306–393). However, the p13 fragment was undetectable. It may be in a low concentration or further degradation by GzmK.

p53 is a physiological substrate of GzmK but not GzmB

To look at whether GzmK can proteolyse native p53, HCT116 p53$^{+/+}$ cell lysates (2×10^5 equivalent) were incubated with 50–300 nM GzmK or with 0.1 μM GzmK for 5–90 min. Native p53 started to be processed at an early time of 5 min and at a low dose of 50 nM GzmK (Fig. 3*A*). After 20 min or 200 nM GzmK, p53 was almost completely proteolysed. The same blot was stripped and probed by β-actin. β-Actin was unchanged as a good control. Similar results were obtained by using another p53-contained human lung adenocarcinoma epithelial cell line A549 cells (Fig. 3*B*). To further verify p53 processing is physiologically relevant, HCT116 p53$^{+/+}$ cells were treated with 1 μM GzmK in the presence of Ad for 1–4 h. p53 was cleaved by 2 h and probed by the DO1 anti-p53 Ab (Fig. 3*C*). The cleavage product p40 was detectable as that of GzmK-processed rp53 or cell lysate p53. SET was cut to produce a 25-kDa product as a previous observation. β-Actin was unchanged as a good loading control. Treating cells with either GzmK or Ad alone or with mutant GzmK plus Ad did not result in the change of p53 (Fig. 3*C*). Two hundred nanomolar GzmK started to cleave p53, which is earlier than SET cleavage. We demonstrated here that p53 cannot be cleaved by GzmB (Fig. 3*D*). Caspase 3 was activated in GzmB-loaded cells, but not in GzmK-loaded cells. Taken together, p53 is a physiological substrate of GzmK, but not GzmB.

p53 cleavage products exert proapoptotic activity

To evaluate whether the p53 processed products have proapoptotic function, we generated expression vectors containing either full-length p53 or the various fragments with N-terminal HA tags as

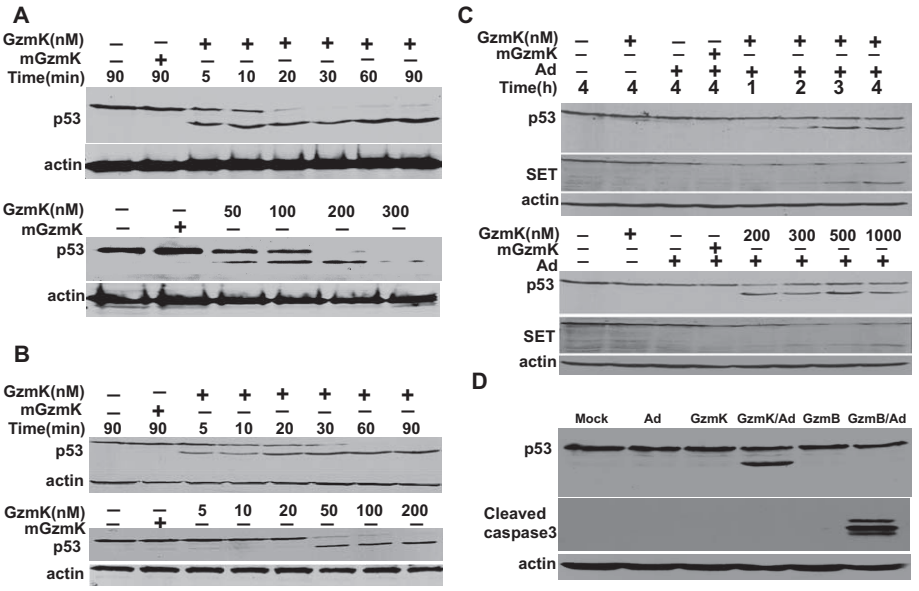

FIGURE 3. GzmK degrades native p53 in cell lysates and loading intact cells. *A,* GzmK cleaves native p53. HCT116 $p53^{+/+}$ cell lysates (2×10^5 cell equivalents) were treated with different doses of GzmK at 37°C for 90 min or with 0.3 μM GzmK for the indicated time points. *B,* GzmK cleaves native p53 in A549 cell lysates. *C,* HCT116 $p53^{+/+}$ cells were treated with the indicated doses of GzmK or mGzmK for 4 h in the presence of Ad (100 PFU/ml) at 37°C or the indicated times with 1 μM GzmK plus Ad. p53 and SET were detected by immunoblotting. *D,* HCT116 $p53^{+/+}$ cells were treated with 0.5 μM GzmB or 0.5 μM GzmK plus Ad. p53 was cleaved by GzmK, but not GzmB. Caspase 3 was probed as a positive control. β-Actin was unchanged as a loading control.

shown in Fig. 4*A*. All fragments, p40, p35, and p13, were expressed in transfected HeLa cells detected by immunoblotting with anti-HA Ab (Fig. 4*B*). After a 24-h transfection, the cells were deprived of serum for another 24-h incubation to initiate apoptosis. Surprisingly, p40, p35, and p13 showed spherical morphology, floating, and other death features through observation by phase-contrast microscopy, which were reminiscent of the full-length p53 (Fig. 4*C*). pCMV-HA-transfected cells were used as a negative control. Then dead cells were stained by PI and followed by flow cytometry. The PI-positive cells for the three fragments were 57.9 ± 3% for p40, 44.6 ± 4% for p35, and 50.5 ± 1% for p13. The PI-positive cells for p53 were 65.3 ± 3%. HA control was 12.9 ± 2%. Together, all three cleavage products have strong proapoptotic activity.

Cellular localization and transcriptional activity of GzmK-processed p53 fragments

We assessed cellular distribution of the p53 cleavage products through confocal microscopy. Full-length p53 and p13 (306–393), which harbor the nuclear localization sequence, exhibited a predominant nuclear staining (Fig. 5*A*). p40 (1–305) and p35 (25–305) were distributed to the cytoplasm and nucleus. Both of them are localized to mitochondria. All three GzmK processed products induce cell death as described above. We next tested whether their proapoptotic activity depends on their transcriptional action. We analyzed several p53 target promoters such as *p21*, *PG13*, *Bax*, and *MDM2* genes through a luciferase reporter

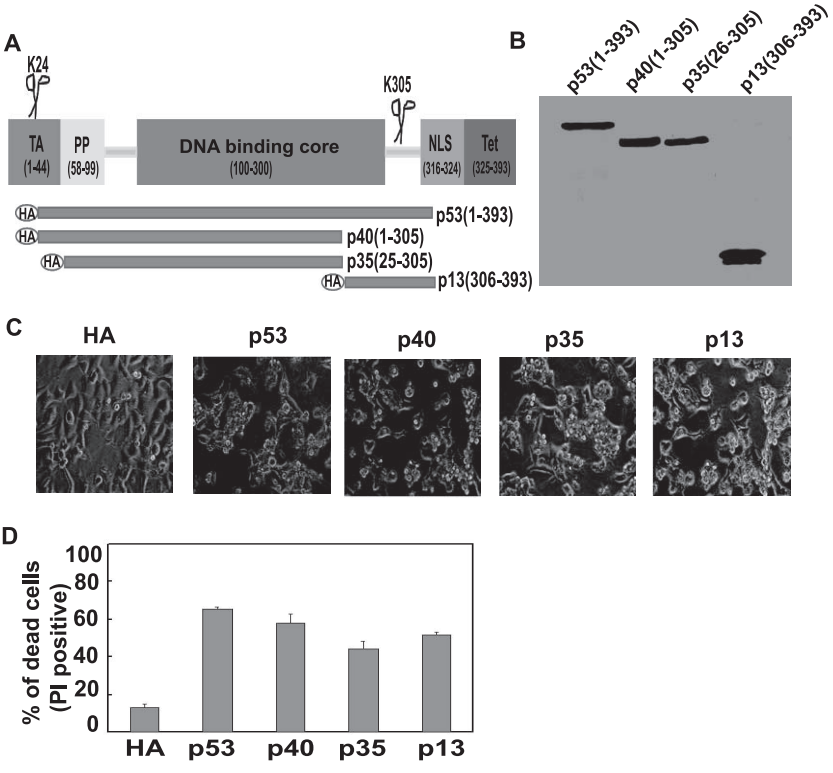

FIGURE 4. p53 fragments exert proapoptotic activity. *A,* Diagram shows the domain structure of the p53 protein and the cleavage sites of GzmK. The constructs generated are schematically represented with a HA tag. TA, Transactivation domain; PP, polyproline domain; Tet, tetramerization domain; NLS, nuclear localization sequence. *B,* p53 or p53 fragments were expressed in HeLa cells by transfection. After a 24-h transfection, the cells were lysed and subjected to Western blotting using HA Ab. *C,* p53 fragments exert proapoptotic activity. 293A cells were transfected with p53 or p53 fragment expression vectors (pCMV-HA-p53, pCMV-HA-p40, pCMV-HA-p35, pCMV-HA-p13, or pCMV-HA). After a 24-h transfection, the cells were deprived serum for another 24 h to induce cell apoptosis. Spherical morphology, floating, and other death features were shown by phase-contrast microscopy. *D,* The dead cells were stained by PI and followed by flow cytometry. pCMV-HA-transfected cells were used as a negative control. The data are representative of at least three separate experiments. The results are calculated as mean ± SD.

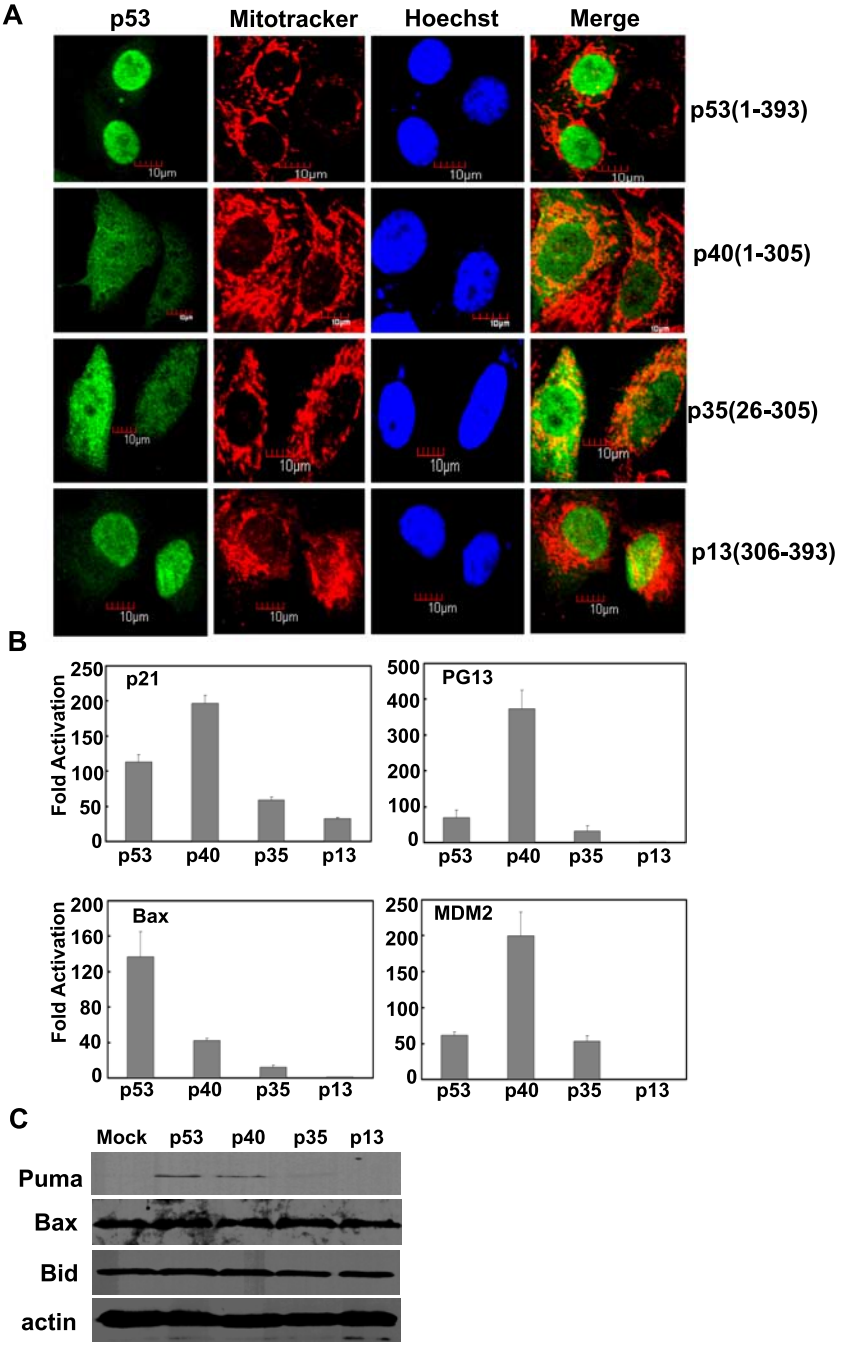

FIGURE 5. Cellular localization and transcriptional activities of GzmK-processed p53 fragments. *A,* HeLa cells were plated in chambers and transfected with p53 or p53 fragments. After a 24-h transfection, the cells were fixed, permeabilized, and probed with HA Ab. MitoTracker was used to probe mitochondria and the nucleus was stained with Hoechst. *B,* The transcriptional activity of full-length p53 and three fragments was assayed through activation of *Bax*, *p21*, *MDM2*, or *PG13* promoters. 293A cells were cotransfected with p53 or p53 fragment expression vectors (pCMV-HA-p53, pCMV-HA-p40, pCMV-HA-p35, or pCMV-HA-p13) with the luciferase-tagged promoters. Luciferase assay was performed after a 36-h transfection. The results represent at least three independent experiments and are calculated as mean ± SD. *C*, Puma was weakly expressed in the cells transfected with p53 and p40 fragments. p53-deficient H1299 cells were cotransfected with p53 or p53 fragment expression vectors as above. Puma, Bax, and Bid were analyzed by immunoblotting. β-Actin was probed as a loading control.

assay. p40 showed strong transcriptional action to p21, PG13, and MDM2 (Fig. 5*B*). Whereas it exhibited weak transcriptional activity to Bax. p35 harbored weak transcriptional activity to all of the detected genes. p13 had no transcriptional action. Puma was only weakly expressed in p53- or p40-transfected H1299 cells (Fig. 5*C*).

p53 sensitizes GzmK-mediated tumor cell death

To determine in vivo effect of p53 on GzmK-mediated killing, HCT116p53$^{+/+}$ or HCT116p53$^{-/-}$ cells were treated with GzmK or Ad alone or GzmK plus Ad at 37°C for 4 h and stained with annexin V/PI. HCT116p53$^{+/+}$ cells were much more sensitive than HCT116p53$^{-/-}$ cells to GzmK-induced lysis (38.5 ± 2% vs 19.2 ± 2%, $p < 0.01$; Fig. 6*A*). GzmK or Ad alone or S-AG-zmK/Ad just got background death. Tumor lysis needs enzymatic activity of GzmK. Time course experiments obtained the similar results as shown in Fig. 6*B*. These data are representative of at least four separate experiments.

To confirm the role of p53 in potentiating GzmK-induced lysis, HCT116p53$^{-/-}$ cells were expressed with wild-type p53 through the pEF-PGK-p53 vector. p53 stable transfectants were screened out and p53 expression was probed with anti-p53 Ab (Fig. 6*C*). p53-rescued HCT116p53$^{-/-}$ cells were incubated with GzmK plus Ad at 37°C for 4 h and followed by TUNEL assay. p53-rescued tumor cells gained more TUNEL-positive cells than their parental p53-deficient cells (48.6 ± 2% vs 29.1 ± 3%, $p < 0.05$; Fig. 6, *D* and *E*). The death rate was similar to that of HCT116p53$^{+/+}$ cells (48.6 ± 2% vs 51.5 ± 2%, $p < 0.05$).

To verify the effect of p53 on GzmK-mediated cell death, an additional p53-deficient human lung cancer cell line H1299 was transfected with pcDNA3.1-p53 vector. p53 stable expression transfectants were screened out through G418 selection (Fig. 6*F*).

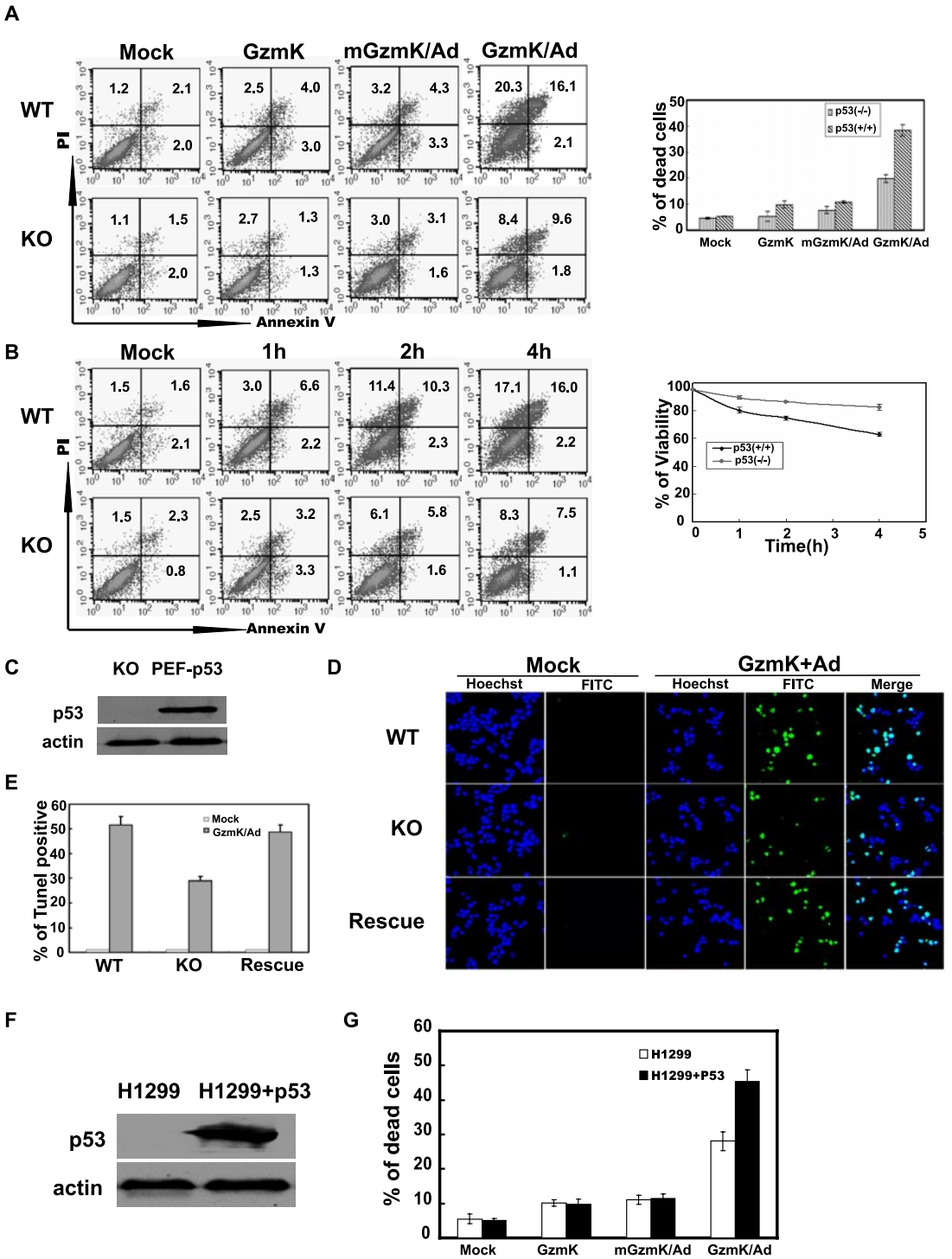

FIGURE 6. p53 sensitizes tumor cells to GzmK-induced cell death. *A* and *B,* HCT116 $p53^{-/-}$ and HCT116 $p53^{+/+}$ cells were treated with 1 μM GzmK or S-AGzmK for 4 h in the presence of Ad (100 PFU/ml) at 37°C (*A*) or the indicated times with 1 μM GzmK plus Ad (*B*). The treated cells were stained with annexin V/PI followed by flow cytometry. The data are representative of at least four separate experiments. Total cell death including annexin V and PI as well as double-positive cells were calculated as shown in the *right panels* (mean ± SD). *C,* p53 was restored in HCT116 $p53^{-/-}$ cells. HCT116 $p53^{-/-}$ cells were transfected with p53 expression vectors (pEF-p53/puromycin). The stable transfectants were selected and verified by immunoblotting. *D,* Rescue of p53 restores the sensitivity of HCT116 $p53^{-/-}$ cells to GzmK-induced death. HCT116 $p53^{-/-}$, HCT116 $p53^{+/+}$, and HCT116 $p53^{-/-}$ cells transfected with p53 (rescue) were incubated with 1 μM GzmK and Ad at 37°C for 4 h and assessed for apoptosis by TUNEL assay. *E,* TUNEL-positive cells were calculated as shown in the mean ± SD for three independent experiments. *F,* p53-deficient H1299 cells ($p53^{-/-}$) were transfected with p53 expression vectors (pcDNA3.1-p53/neomycin). p53-expressed stable transfectants were selected with G418 and verified by Western blotting. *G,* H1299 ($p53^{-/-}$) and H1299 ($p53^{+/+}$) cells were treated with 1 μM GzmK or mutant S-AGzmK for 4 h in the presence of Ad (100 PFU/ml) at 37°C. The treated cells were stained with annexin V/PI followed by flow cytometry. The data are representative of at least three separate experiments and calculated as mean ± SD.

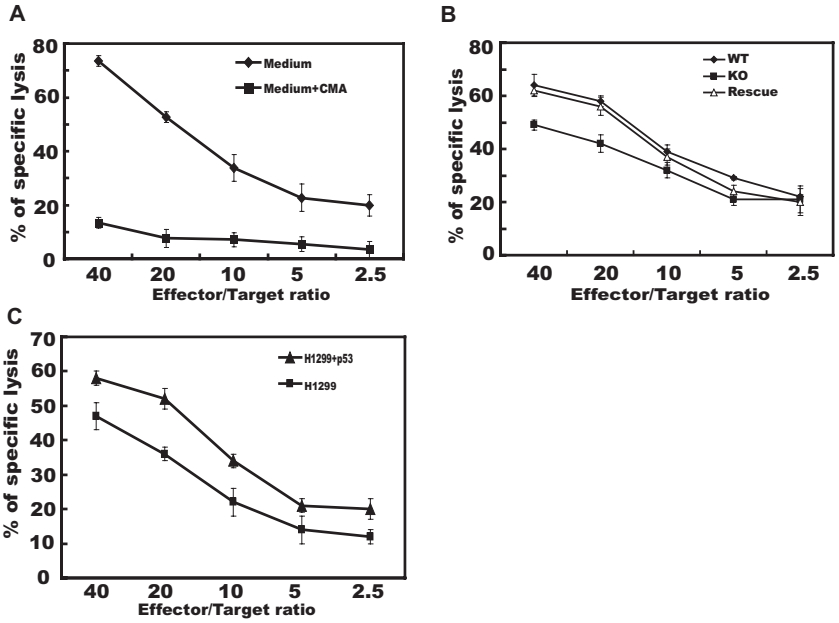

FIGURE 7. p53 potentiates tumor susceptibility to NK cell killing. *A,* NK-mediated cytotoxicity to target tumor cells is mediated by the perforin/Gzm pathway. HCT116 $p53^{+/+}$ cells were incubated with IL-2-activated NK cells isolated from PBMC for different E:T rations at 37°C for 4 h. For inhibition of the perforin/Gzm pathway, IL-2-activated NK cells were pretreated with 500 nM CMA before coculturing with tumor cells. The percentage of lysis was assayed by release of ^{51}Cr. *B,* HCT116 $p53^{+/+}$ or restoration of wild-type tp53 function in HCT116 $p53^{-/-}$ cells potentiate NK-mediated killing. HCT116 $p53^{+/+}$ or p53-rescued HCT116 $p53^{-/-}$ cells (rescue) preloaded with ^{51}Cr were treated with IL-2-activated NK cells at different E:T ratios. Data are the means of at least four separate experiments (mean ± S.D). *C,* p53-expressed H1299 cells were more sensitive to lysis induced by NK cells. p53-expressed H1299 or p53-deficient H1299 cells were treated by IL-2-activated NK cells at different E:T ratios as above.

p53-expressed H1299 cells were much more sensitive to GzmK-induced cytolysis than p53-deficient H1299 cells (45.4 ± 2% vs 28.1 ± 2%, $p < 0.01$; Fig. 6*G*). The data are representative of at least three separate experiments.

p53 potentiates tumor-killing susceptibility to NK cytolysis

We previously showed that GzmK induces a rapid target cell death, which is consistent with the dynamics of NK cell-mediated tumor lysis. To investigate physiological function of p53 to tumor-killing susceptibility, IL-2-activated CD56 NK cells were incubated with HCT116$p53^{+/+}$ or HCT116$p53^{-/-}$ cells followed by a 4-h ^{51}Cr release assay. NK cells exhibited good killing action to HCT116$p53^{+/+}$ cells even at a low E:T ratio, 2.5 (Fig. 7*A*). CMA, an inhibitor of cytotoxic granule exocytosis through chelating free calcium, can block NK cell-mediated cytolysis. It suggests that NK cell-mediated killing is mainly induced by the perforin/Gzm pathway. p53-deficient HCT116$p53^{-/-}$ cells were more resistant to NK cell-mediated lysis (Fig. 7*B*). p53-restored HCT116 $p53^{-/-}$ cells (rescue) were more sensitive to cytolysis induced by NK cells similar to HCT116$p53^{+/+}$ cells (Fig. 7*B*). Similar results were found in p53-rescued H1299 cells (Fig. 7*C*). Taken together, p53 sensitizes tumor lysis susceptibility to NK cells.

Discussion

p53 tumor suppressor has a major function in transducing stress to the apoptotic machinery of the cell, consistent with the role of p53 status as a determinant of cellular response to DNA-damaging drugs (3, 15). Inactivation of p53 has been identified in >50% tumor types and such inactivation will result in loss of the apoptosis-regulating function of the p53 protein. It is well established that p53 mediates tumor growth suppression through cell cycle arrest and apoptosis regulation. Considering the high mutation frequency of p53 in tumor cells, it is believed that p53 has the potential ability to regulate the tumor cell susceptibility to immune killer cells. The role of p53 in the control of tumor susceptibility to CTL-mediated lysis has been reported. Recently, Chouaib's group (7, 8) found that p53 sensitizes tumor susceptibility to CTL killing. However, the direct role of p53 in regulating tumor sensitivity to NK-mediated lysis and the functional relationship between p53 and Gzms in the control of tumor killing are still poorly documented. In this study, we demonstrated that p53 can sensitize tumor killing to NK cytolysis and GzmK-mediated cell death. p53 was processed by GzmK to three cleavage products of p40, p35, and p13, which exerted strong proapoptotic activity to augment cell lysis.

NK cells and CTL are the principal cytotoxic effectors of the antitumor immune response. They kill tumor cells by two major pathways that require direct cellular contact. The first pathway is granule exocytosis, which delivers a membrane-disrupting molecule perforin (pore-forming protein) and proapoptotic serine proteases Gzms. The second one is involved in death receptor-induced surface expression of ligands on killer cells, which cross-links the corresponding receptors of target cells such as Fas ligand and TRAIL and TNFR (16). In our study, we found that the cytotoxic granules exocytosis inhibitor CMA can block NK-induced tumor lysis. It indicates that NK-mediated cytotoxicity is induced by the perforin/Gzm pathway. Our observation is in agreement with CTL-mediated tumor elimination (9).

NK cells are named initially due to their ability to spontaneously kill certain tumor cells in vitro. They are commonly defined as the effectors of the innate immune system, acting as the first line of defense against viral infection and transformed cells (17). GzmK is highly expressed in NK cells (10, 18). We previously demonstrated that GzmK induces rapid target cell death independent of caspase activation (12). GzmK can target the SET complex to degrade its substrates, releasing the NM23H1 DNase activity to induce ssDNA nicks. GzmK also triggers rapid ROS generation and collapse of mitochondrial inner membrane potential (13). Blockade of ROS accumulation suppresses GzmK-induced cell death. Intracellular ROS accumulation is believed to be a key initial step in caspase-dependent apoptosis, including that induced by GzmB and GzmM, and caspase-independent apoptosis that is mediated by GzmA and GzmK (11, 19–22). A recent report showed stress-induced ROS generation initiates a strong p53 activation (23). Meslin et al. (9) just reported that targeting tumor cells by CTL induces p53 accumulation at early times. This observation is consistent with the dynamics of ROS stress induced by CTL. These data suggest that p53 plays a key role in granule-mediated cytolysis. We showed here that HCT116 $p53^{+/+}$ cells are more cytotoxic to NK- and GzmK-induced death than their parental p53-deficient HCT116 $p53^{-/-}$ cells. p53-rescued HCT116 $p53^{-/-}$ cells restore their sensitivity to GzmK-induced cytolysis.

We shed light on the molecular mechanism of p53 to potentiate tumor cell susceptibility through NK-mediated cytolysis. We found that p53 is a physiological substrate of GzmK and is processed at Lys^{24} and Lys^{305} to generate three cleavage fragments (p40, p35, p13). All three of these products exert proapoptotic activity that amplifies the proapoptotic action of p53. p40 and p35 showed a similar distribution pattern in cells and translocated into mitochondria. The p40 fragment without the C terminus of p53 harbor higher transcriptional activity. The last 30 residues of p53 constitute a negative regulatory domain with potent suppression of sequence-specific DNA binding by p53 and transactivation of target genes (24). Deletion of the C terminus by GzmK activates transactivation of target gene expression in transfected cells, which correlated with up-regulation of *p21*, *PG13*, and *MDM2*, three known targets of p53. Our observation is in agreement with an earlier report that showed p53(△C30) upregulates expression of p21 (24–26). However, p40 showed weak transcriptional activity to Bax that remains to be further studied. The p35 fragment has potent proapoptotic activity. Although the transcriptional activity of p35 was lower than the p40 fragment, these data suggest that the N terminus may interact with cellular transcriptional elements and be required for transactivation of p53 target genes. A recent report showed caspase 3-processed p53 fragments p53(1–186), p53 (22–186), and p53(22–393) were localized to mitochondria and caused mitochondrial depolarization (27). Therefore, p40 and p35 might target mitochondria to cause death. We also found that the C-terminal 13-kDa domain localized to the nucleus and bore overt proapoptotic activity. Fine's group (28) reported that the C-terminal peptide can induce target necrosis in multiple mutated p53 human prostate cancer cells. However, p13 had not any transcriptional activity. Thus, proapoptotic activity of GzmK-processed p53 products requires further investigation.

Different types of DNA damage can induce p53 cleavage products. Damaged DNA induces N-terminal cleavage of p53 to generate p50(△N23) and p35(core) protein products (24). The cleaved p53 products exert important roles in cell cycle arrest and apoptosis induction. GzmK induces ssDNA nicks. Whether this DNA damage can enhance p53 processing by GzmK as a positive feedback loop needs to be studied further. p53 can be processed to form various fragments by caspases to amplify the process of apoptosis (27). A previous study showed that p63, a p53 homolog, is a caspase target, and such cleavage results in loss of the transcriptional inhibitor domain at the C terminus of the p63a isoform (29). Melino's group (30) recently reported that p73, a member of the p53 family, is cleaved by caspase 3 and caspase 8 in vitro and in vivo during apoptosis elicited by DNA-damaging drugs and TRAIL receptor ligation. Our observation indicates that p53 is as a cytotoxic bomb that can be triggered by GzmK, leading to potentiating killing efficacy. Therefore, restoration of p53 function in tumor cells might be a potential strategy for cancer immunotherapy by enhancing killing sensitivity of killer lymphocytes.

Acknowledgments

We thank Dr. Bert Vogelstein for providing the HCT116 $p53^{+/+}$, the isogenic HCT116 $p53^{-/-}$ cells, and the PG13-Luc and P21-Luc plasmids. We also thank Dr. Karl-Heinz Scheidtmann for providing Bax-Luc and MDM2-Luc plasmids and Chunchun Liu, Youli Xu, Lei Sun, and Yan Teng for their technical help.

Disclosures

The authors have no financial conflict of interest.

References

1. Lu, X. 2005. p53: a heavily dictated dictator of life and death. *Curr. Opin. Genet. Dev.* 15: 27–33.
2. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356: 215–221.
3. Brown, J. M., and B. G. Wouters. 1999. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res.* 59: 1391–1399.
4. Pipkin, M. E., and J. Lieberman. 2007. Delivering the kiss of death: progress on understanding how perforin works. *Curr. Opin. Immunol.* 19: 301–308.
5. Trapani, J. A., and M. J. Smyth. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nat. Rev. Immunol* 2: 735–747.
6. Russell, J. H., and T. J. Ley. 2002. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 20: 323–370.
7. Thiery, J., G. Dorothee, H. Haddada, H. Echchakir, C. Richon, R. Stancou, I. Vergnon, J. Benard, F. Mami-Chouaib, and S. Chouaib. 2003. Potentiation of a tumor cell susceptibility to autologous CTL killing by restoration of wild-type p53 function. *J. Immunol* 170: 5919–5926.
8. Thiery, J., S. Abouzahr, G. Dorothee, A. Jalil, C. Richon, I. Vergnon, F. Mami-Chouaib, and S. Chouaib. 2005. p53 potentiation of tumor cell susceptibility to CTL involves Fas and mitochondrial pathways. *J. Immunol.* 174: 871–878.
9. Meslin, F., J. Thiery, C. Richon, A. Jalil, and S. Chouaib. 2007. Granzyme B-induced cell death involves induction of p53 tumor suppressor gene and its activation in tumor target cells. *J. Biol. Chem.* 282: 32991–32999.
10. Bratke, K., M. Kuepper, B. Bade, J. C. Virchow, Jr., and W. Luttmann. 2005. Differential expression of human granzymes A, B, and K in natural killer cells and during $CD8^+$ T cell differentiation in peripheral blood. *Eur. J. Immunol.* 35: 2608–2616.
11. Guo, Y., J. Chen, T. Zhao, and Z. Fan. 2008. Granzyme K degrades the redox/DNA repair enzyme Ape1 to trigger oxidative stress of target cells leading to cytotoxicity. *Mol. Immunol.* 45: 2225–2235.
12. Zhao, T., H. Zhang, Y. Guo, Q. Zhang, G. Hua, H. Lu, Q. Hou, H. Liu, and Z. Fan. 2007. Granzyme K cleaves the nucleosome assembly protein SET to induce single-stranded DNA nicks of target cells. *Cell. Death Differ.* 14: 489–499.
13. Zhao, T., H. Zhang, Y. Guo, and Z. Fan. 2007. Granzyme K directly processes bid to release cytochrome c and endonuclease G leading to mitochondria-dependent cell death. *J. Biol. Chem.* 282: 12104–12111.
14. Lohrum, M., and K. H. Scheidtmann. 1996. Differential effects of phosphorylation of rat p53 on transactivation of promoters derived from different p53 responsive genes. *Oncogene* 13: 2527–2539.
15. Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74: 957–967.
16. Chan, C. W., and F. Housseau. 2008. The 'kiss of death' by dendritic cells to cancer cells. *Cell. Death Differ.* 15: 58–69.
17. Fan, Z., P. Yu, Y. Wang, Y. Wang, M. L. Fu, W. Liu, Y. Sun, and Y. X. Fu. 2006. NK-cell activation by LIGHT triggers tumor-specific $CD8^+$ T-cell immunity to reject established tumors. *Blood* 107: 1342–1351.
18. MacDonald, G., L. Shi, C. Vande Velde, J. Lieberman, and A. H. Greenberg. 1999. Mitochondria-dependent and -independent regulation of granzyme B-induced apoptosis. *J. Exp. Med.* 189: 131–144.
19. Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol. Cell. Biol.* 20: 3781–3794.
20. Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green, and R. C. Bleackley. 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J. Exp. Med.* 192: 1391–1402.
21. Martinvalet, D., P. Zhu, and J. Lieberman. 2005. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity* 22: 355–370.
22. Hua, G., Q. Zhang, and Z. Fan. 2007. Heat shock protein 75 (TRAP1) antagonizes reactive oxygen species generation and protects cells from granzyme M-mediated apoptosis. *J. Biol. Chem.* 282: 20553–20560.
23. Karawajew, L., P. Rhein, G. Czerwony, and W. D. Ludwig. 2005. Stress-induced activation of the p53 tumor suppressor in leukemia cells and normal lymphocytes requires mitochondrial activity and reactive oxygen species. *Blood* 105: 4767–4775.
24. Okorokov, A. L., F. Ponchel, and J. Milner. 1997. Induced N- and C-terminal cleavage of p53: a core fragment of p53, generated by interaction with damaged DNA, promotes cleavage of the N terminus of full-length p53, whereas ssDNA induces C-terminal cleavage of p53. *EMBO J.* 16: 6008–6017.
25. Chen, X., L. J. Ko, L. Jayaraman, and C. Prives. 1996. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev.* 10: 2438–2451.
26. Halazonetis, T. D., and A. N. Kandil. 1993. Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J.* 12: 5057–5064.
27. Sayan, B. S., A. E. Sayan, R. A. Knight, G. Melino, and G. M. Cohen. 2006. p53 is cleaved by caspases generating fragments localizing to mitochondria. *J. Biol. Chem.* 281: 13566–13573.
28. Dinnen, R. D., L. Drew, D. P. Petrylak, Y. Mao, N. Cassai, J. Szmulewicz, P. Brandt-Rauf, and R. L. Fine. 2007. Activation of targeted necrosis by a p53 peptide: a novel death pathway that circumvents apoptotic resistance. *J. Biol. Chem.* 282: 26675–26686.
29. Sayan, B. S., A. E. Sayan, A. L. Yang, R. I. Aqeilan, E. Candi, G. M. Cohen, R. A. Knight, C. M. Croce, and G. Melino. 2007. Cleavage of the transactivation-inhibitory domain of p63 by caspases enhances apoptosis. *Proc. Natl. Acad. Sci. USA* 104: 10871–10876.
30. Sayan, A. E., B. S. Sayan, V. Gogvadze, D. Dinsdale, U. Nyman, T. M. Hansen, B. Zhivotovsky, G. M. Cohen, R. A. Knight, and G. Melino. 2008. P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis. *Oncogene* 27: 4363–4372.