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In this study, we have investigated the mechanisms used by wild-type p53 (wtp53) to potentiate tumor cell susceptibility to CTL-mediated cell death. We report that wtp53 restoration in a human lung carcinoma cell line Institut Gustave Roussy (IGR)-Heu, displaying a mutated p53, resulted in up-regulation of Fas/CD95 receptor expression associated with an increase of tumor cell sensitivity to the autologous CTL clone, Heu127. However, when IGR-Heu cells were transfected with Fas cDNA, no potentiation to Heu127-mediated lysis was observed, indicating that induction of CD95 is not sufficient to sensitize target cells to CTL killing. Importantly, our data indicate that the effect of wtp53 on the Fas-mediated pathway involves a degradation of short cellular FLICE inhibitory protein resulting in subsequent caspase 8 activation. Furthermore, we demonstrate that wtp53 restoration also resulted in CTL-induced Bid translocation into mitochondria and a subsequent mitochondrial membrane permeabilization leading to cytochrome c release. These results indicate that tumor cell killing by autologous CTL can be enhanced by targeting degradation-independent mechanisms via restoration of wtp53, a key determinant of apoptotic machinery regulation. The Journal of Immunology, 2005, 174: 871–878.

The CD8+ CTLs have been described to play a crucial role in host defense against malignancies in both mouse and human (1). However, it is also becoming clear that neoplastic cells can escape or resist to the immune response at multiple levels. Accumulating evidence suggests that in vivo tumor cell growth is not only influenced by CTL tumor cell recognition, but also by the susceptibility of the tumor cells to host-mediated immune response, including killer cells. It is established that an-tumor CTL response is regulated at several effector-target inter-action levels involving both intracellular and extracellular stimuli (2). In this regard, we have previously shown that p53 is a key determinant able to improve the effectiveness of CTL response involving at least in part induction of CD95 expression in tumor cells (3).

It is well established that the tumor suppressor p53, activated in response to DNA damage, induces cell cycle arrest and apoptosis through transcriptional activation of its target genes, hence playing a central role in tumor suppression. P53 has a major function in transducing stress to the apoptotic machinery of the cell, consistent with the importance of p53 status as a determinant of cellular response to DNA damaging drugs (4–7). Inactivation of p53 has been identified in many types of tumors particularly in non-small cell lung carcinoma, one of the most common cancers in which p53 mutation has been frequently identified. The resultant mutant proteins are frequently functionally compromised for apoptosis induction. Such a high mutation frequency suggests a strong selection for loss of wild-type p53 (wtp53)3 function during tumorgenesis. The ability of p53 to regulate the cell cycle has been reported to contribute to drug resistance and to apoptosis induced by many anticancer agents (7). However, it is not known how p53 contributes to the immune responses that lead to tumor cell lysis by killer cells. The direct role of p53 in the control of tumor susceptibility to CTL-mediated lysis is still poorly documented and so far, the link between p53 status and tumor susceptibility to CTL mediated killing remains to be elucidated.

In T cell-mediated cytotoxicity process, two major pathways are engaged following TCR recognition of Ag/MHC complexes expressed on target cells. The first one is a secretory pathway involving receptor-triggered exocytosis of preformed secretory granules containing granzymes and perforin (8, 9). The second is based on receptor-induced surface expression of death receptor ligands on effector cells, which cross-links the corresponding receptors (Fas, TRAIL receptors, TNFR I-p55) on target cells (10). Fas (CD95) pathway plays a major role in CTL-mediated cell death. Upon engagement of Fas by its ligand FasL, Fas-associated death domain engages caspase 8, which in turn, autoactivates itself and cleaves downstream substrates directly via activation of the caspase cascade (mitochondria independent) or indirectly via mi-tochondria-dependent mechanisms. These two mechanisms corre-spond to the predominant arms of CD95 signaling, allowing effector caspases 3, 6, and 7 activation and are thought to operate distinctly in two distinct types of cells. Cells using the former pathway are known as type I cells, whereas those using the mito-chondrial pathway are known as type II cells (11). The basis of this cellular preference in type II cells seems to be depending on the relative stoichiometry of cleaved caspase 8, and on the cleavage of...
Bid protein into truncated Bid, which links caspase 8 to mitochondrial pathway (12). Because type II cells depend on the mitochondrial branch, apoptosis can be blocked by ectopic overexpression of Bcl-xL or Bcl-2 (13).

Several endogenous inhibitors of Fas-mediated apoptosis have been identified, including the cellular FLIP (cFLIP). cFLIP is over-expressed in various tumors and has been shown to be implicated in tumor resistance to T cell-mediated apoptosis in vivo. This caspase homologue was identified as a potent inhibitor of apoptosis (IAP) induced by all death receptors (Fas, TRAIL receptors, TNFR I-p55) and was described to allow tumor escape from the immune response in mouse tumor models (14). Multiple splice variants of cFLIP have been reported, but so far only two, designated cFLIP short and cFLIP long, could be detected at the protein level (11). The mechanism of cell death inhibition by cFLIP is not completely elucidated, but it was suggested that cFLIP, as a competitive inhibitor, precludes recruitment of procaspase 8 to the death-inducing signaling complex (DISC) and thereby prevents its activation (15).

The aim of the present study was to determine the molecular basis of p53-induced sensitization of target cells to CTL-mediated killing. We first demonstrated that restoration of Fas on target cells was not sufficient to induce potentiation of tumor cells to CTL killing. Indeed, we have shown that restoration of wt p53 function resulted in Fas expression induction accompanied with cFLIP short inhibition and caspase 8 activation. Conversely, we showed, for the first time, that functional interaction between CTL and tumor target cells expressing wt p53 resulted in the activation of mitochondria leading to Bid translocation, cytochrome c release, and DNA fragmentation. Together, our results emphasize that in addition to its role in controlling irradiation and drug responses, p53 also plays a key role in the control of tumor susceptibility to CTL-mediated killing and that its mutation may be associated at least in part with tumor escape from the immune response. Our studies point to the importance of wt p53 function in sensitizing tumor cells, displaying mutated p53, to CTL response and suggest that the status of the p53 gene may be an important determinant in the efficacy of antitumor killer cells and may take place in immunotherapeutic strategies design.

Materials and Methods

Antibodies

Monoclonal Abs directed against Fas (UB2 and ZB4, mouse IgG1; 7C11, mouse IgM) and against caspase 8 (5F7, mouse IgG2b) were purchased from Immunotech. Anti-caspase 3/PP32 (mouse IgG2a) was purchased from BD Biosciences. Anti-p21WAF1/CIP1 (Ab1) and anti-p53 (Ab2) were purchased from Oncogene Research Products. Anti-survivin (C19), anti-Bcl-xL, (S18), anti-Bid (FL195), anti-Bax (N20), anti-actin (C11), anti-cFAP2 (H85), and anti-Bcl-100 (100) Abs were purchased from Santa Cruz Biotechnology. Anti-cFLIP (NF6) was purchased from Apotex. Anti-cytochrome c was purchased from BD Pharmingen and anti-Hsp70 from StressGen Biotechnologies.

Tumor cell line and CTL clone

The Institut Gustave Roussy (IGR)-Heu tumor cell line was established from a patient suffering from a large-cell carcinoma of the lung (16) and was cultured at 37°C (5% CO2) in DMEM:F12 1:1 with GlutaMax (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies) and 1% sodium pyruvate (Invitrogen Life Technologies), 10% human sera (PromoCell) and was cultured at 37°C (5% CO2) in complete medium (53x52).

Construction of recombinant adenovirus vector expressing wt p53 (Adwtp53) and adeno virus infection of cells

Adwtp53 was produced by in vitro homologous recombination in 293 cells as described (18). Efficiency of Adwtp53 to transfer and direct expression of wt p53 was analyzed on p53 negative SAOS cells using specific mAb 36 h after infection with Adwtp53. For infection of tumor cell lines, the medium of subconfluent cells grown in 10 cm2 culture dishes was removed and 50 PFU per cell of either Adwtp53 or empty adenovirus, used as control, was added on 2 ml of medium. After 2 h incubation at 37°C, 3 ml of complete medium were added to the cells. Cells were cultured for 24 h. The expression of functional p53 in Adwtp53 infected cells was then analyzed using Western blot analysis of p21WAF1/CIP expression and cell cycle progression.

Flow cytometry analysis

Cells (3 x 106) were incubated with UB2 anti-Fas or isotypic control Abs for 60 min at 4°C, followed by FITC-conjugated goat anti-mouse Ab. They were analyzed on a FACSCalibur flow cytometer, and data were processed using CellQuest software (BD Biosciences).

Determination of cell viability with SubG1 assay

Tumor cell (5 x 106) were seeded in flat-bottom 96-well plates and then infected with Adwtp53 or empty virus for control. After 24 h, 7C11 anti-Fas mAb were added for 6 h. Apoptotic cell death induced by anti-Fas mAb was analyzed by measuring proportion of subG1 cells. Briefly, cells were harvested 24 h after infection, washed and fixed in 70% ethanol. Before flow cytometry analysis, cells were washed with PBS and stained with 1 ml of propidium iodide (20 μg/ml) containing 100 μg/ml RNase and 20 μM EDTA. DNA content was determined by a FACSCalibur flow cytometer (BD Biosciences) and the proportion of cells in a particular phase of cell cycle was determined by CellQuest software.

Western blot analysis

Total cellular extracts were prepared by lysing cell in ice-cold buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Equivalent protein extracts (50 μg) were denatured by boiling in SDS and 2-ME, separated by SDS-PAGE and transferred onto Hybond membranes (Amersham Biosciences). Ponceau red staining of the membranes assessed the efficiency of the electrotransfer. Blots were blocked overnight with TBS containing 5% nonfat dry milk and probed with appropriate Ab for 1 h. After washing, blots were incubated with appropriate secondary Ab-HRP conjugated. The complexes were detected using ECL detection kit (Amersham Biosciences).

Cytotoxicity assay

The cytotoxic activity of clone Heu127 was measured by a 6 h lactic dehydrogenase release assay using a Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) on 5 x 104 target cells/well. Functional effects of ZB4 anti-Fas neutralizing mAb was tested by preincubating target cells for 2 h at 37°C before the assay. Experiments were performed in quadruplicate and percentage lysis was determined by 490 nm OD measurement as described in the manufacturer notice.

Detection of caspases 3, 7, and 8 activation

Caspase activity was measured by flow cytometry using a CaspaTag Caspase 8 (LETD) Activity kit (Intergen) and a CaspaTag Caspase 3 and Caspase 7 (DEVD) Activity kit (Chemicon International). Briefly, cells were infected by Adwtp53 or empty virus during 24 h and then treated with 7C11 anti-Fas mAb for 4 h or incubated with Heu127 CTL clone for 30 min (E:T ratio 3:1). After treatment, the cells were washed, and 105 tumor cells/ml were incubated with FAM-Peptide-FMK for 1 h at 37°C under 5% CO2. Cells (3 x 103) were seeded in flat-bottom 96-well plates and then infected with Adwtp53 or empty virus during 24 h and then treated with 7C11 anti-Fas mAb for 4 h or incubated with Heu127 CTL clone for 30 min (E:T ratio 3:1). After treatment, the cells were washed, and 105 tumor cells/ml were incubated with FAM-Peptide-FMK for 1 h at 37°C under 5% CO2. After staining, the cells were washed two times and fixed with formaldehyde-based fixative solution provided in the kit. Flow cytometry analysis was done using a FACS Calibur flow cytometer (BD Biosciences).

Confocal scanning immunofluorescence microscopy

Cells cultured on coverslips were infected for 24 h and then coincubated with H127 CTL clone (E:T ratio 1:1) for 1 h. Cells were washed with PBS, fixed with paraformaldehyde (4% w/v in PBS) for 1 h and then permeabilized with SDS (0.1% w/v in PBS) for 10 min. Nonspecific sites were blocked with FCS 10% in PBS for 20 min before the staining with anti-cytochrome c mAb (mouse IgG1; BD Biosciences) and anti-Bid (FL195) pAb (rabbit; Santa Cruz Biotechnology); all were detected by an Alexa Fluor 546 (red) goat anti-mouse or an Alexa Fluor 488 (green) goat anti-mouse IgG1 secondary Ab and visualized with a Zeiss LSM 510 confocal microscope.
anti-rabbit Abs (Molecular Probes). Nucleus staining was performed with TO-PRO-3 (Molecular Probes). The coverslips were mounted on glass slide using a drop of Vectashield hard set (Vector Laboratories). The fluorescence was examined under LSM 510 confocal microscope (Zeiss). Z-projection of slices was realized using LSM Image examiner software (Zeiss). A punctuate cytoplasmic staining pattern indicates mitochondrial localization of cytochrome c or Bid, when a diffuse staining indicates cytoplasmic localization.

Detection of mitochondrial transmembrane potential disruption
Mitochondrial transmembrane potential ($\Delta \psi_m$) disruption was measured using MitoTracker red (chloromethyl X-rosamine (CMX-Ros); Molecular Probes). Briefly, IGR-Heu cells were infected with 50 PFU of control adenovirus (empty virus) or Adwtp53 for 24 h and treated or not with 1 $\mu$g/ml 7C11 anti-Fas mAb during 6 h or incubated with Heu127 CTL clone for 45 min. Changes in $\Delta \psi_m$ were determined by staining the cells with 50 nM CMX-Ros in culture medium for 5 min at 37°C in a humidified atmosphere containing 5% CO2. Cells were then washed in PBS and analyzed by flow cytometry. The decrease in $\Delta \psi_m$ is seen as a decrease in CMX-Ros fluorescence.

Results
Adwtp53 infection of Fas expressing target cells increase their lysis by autologous CTL and anti-Fas Abs
For this study, we have used the human non-small cell lung carcinoma cell line IGR-Heu, defective for Fas receptor expression and displaying a mutated p53 (codon 132; type AAG > AAT, i.e., Lys > Asp), and the autologous CTL clone Heu127, able to lyse IGR-Heu following recognition of a mutated $\alpha$-actinin 4 peptide in a HLA-A2.1 context (19). As previously described, IGR-Heu cells were lysed by Heu127 CTL clone through the perforin/granzymes pathway (3, 20). Infection of IGR-Heu with Adwtp53 resulted in the restoration of Fas receptor expression and a subsequent increase of tumor cell susceptibility to Fas-mediated apoptosis as shown by the percentage of subG1 cells (Fig. 1A) and also resulted to a potentiation of tumor cell sensitivity to Heu127 CTL-mediated lysis via Fas pathway as demonstrated by Fas neutralization with ZB4 mAb (Fig. 1B). Interestingly, transfection of IGR-Heu with
Fas cDNA and isolation of a Fas positive clone, HF-76, was not associated with a strong increase in its sensitivity to anti-Fas mAb (Fig. 1A) or to CTL-mediated killing (Fig. 1B) as compared with the parental cell line IGR-Heu lacking Fas expression. However, following restoration of wtp53, the HF-76 Fas⁺ clone exhibited a much higher susceptibility to anti-Fas mAb and to the Heu127 CTL clone. These results indicate that the sole induction of Fas expression was not sufficient to lead to an increase of tumor cell susceptibility to the Heu127 CTL clone and that p53 plays an additional role, besides its ability to induce Fas expression, related to an increase of target sensitivity to CTL-mediated lysis.

The effect of p53 on target cell susceptibility to CTL-mediated lysis is associated with a selective degradation of cFLIP short protein

To investigate the mechanisms used by wtp53 to potentiate tumor cell susceptibility to CTL-mediated killing, we analyzed the expression of genes implicated in the control of the Fas-mediated pathway including Bcl-2 family member genes and IAP genes. We first asked whether the regulatory effect of p53 on target susceptibility to the CTL clone involves down-regulation of antiapoptotic genes or in contrast an increase of proapoptotic gene expression. Western blot analysis, shown in Fig. 2A, indicates that infection of IGR-Heu cells with Adwtp53 restores wt53 transactivation activity, as shown by induction of p21 expression, but has no effect on Bcl-2, Bcl-xL, Hsp70, survivin, cIAP2, and Bax expression, excluding an impact of wt53 on Bcl-2 or IAPs family members. We then analyzed the expression of cFLIP, which has been described as a natural inhibitor of Fas-mediated apoptosis. As shown in Fig. 2B, Western blot analysis indicates that both long and short forms of cFLIP were significantly expressed in IGR-Heu cells. Interestingly, when these cells were infected with Adwt53, a dramatic decrease of cFLIP short was selectively observed, whereas cFLIP long protein expression was not affected. Similar results were obtained with HF-76 (data not shown), suggesting that cFLIP short degradation following wtp53 restoration is at least associated with Fas pathway potentiation.

Activation of caspase 8 and caspase 3/7 in Adwtp53-infected IGR-Heu cells after Fas engagement

The recruitment of procaspase 8 to the DISC and its subsequent cleavage and activation is generally thought to initiate the Fas apoptotic cascade and it has been reported that cFLIP precludes the activation. We therefore examined the status of procaspase 8 before and after Fas engagement. Data depicted in Fig. 3A indicate that procaspases 8 and 3 were equally expressed in parental and Adwtp53-infected cells. Fas engagement with anti-Fas mAb resulted in the generation of caspase 8 cleaved intermediate products of 43 and 41 kDa and in cleavage of caspase 3 only in Adwtp53-infected cells. No such cleavages occurred in parental IGR-Heu cells after anti-Fas mAb treatment. In addition, treatment of IGR-Heu cells with the Heu127 CTL clone resulted in the generation of cleaved caspase 8 but not caspase 3. As opposed, treatment of IGR-Heu/Adwtp53 cells with the Heu127 CTL clone resulted in an increase of caspase 8 cleavage as well as cleavage of caspase 3. The specific implication of Fas pathway in the cleavage of caspases after CTL treatment was demonstrated by blocking the Fas receptor with the anti-Fas (ZB4) mAb. To further emphasize the activation of caspase cascade following wt53 infection, cells were treated with carboxyfluorescein-labeled fluoro-methyl ketone peptide inhibitors of caspase 8 (FAM-LETD-FMK) and caspases 3/7 (FAM-DEVD-FMK). When added to a population of cells, the dye enters into each cell and covalently binds to the active caspase heterodimer leading to a green fluorescence signal which directly measures the amount of active caspases. Data depicted in Fig. 3B indicate the presence of active caspase 8 and caspase 3/7 after Fas receptor engagement by anti-Fas mAb only in Adwtp53 infected cells. In addition, treatment of IGR-Heu cells with the Heu127 CTL clone resulted in a weak activation of caspases 8 and 3 suggesting that caspase 8 is partially cleaved but not activated. As opposed, treatment of IGR-Heu/Adwtp53 cells with the Heu127 CTL clone resulted in an increase of caspase 8 and 3 activation, mediated by the Fas pathway as shown by blocking the Fas receptor with the anti-Fas (ZB4) mAb. Thus, these data suggest that the restoration of wt53 function in IGR-Heu cells induces cFLIP short degradation and therefore potentiates the cleavage and activation of caspase 8 and then caspase 3 activation by Fas receptor triggering.

FIGURE 2. Restoration of wt53 function in IGR-Heu cells does not affect Bcl-2 and IAP family members but induces selective cFLIP short degradation. A, Western blot analysis of p53-induced or repressed genes in IGR-Heu cells is shown. At 24 h after infection of IGR-Heu cells with Adwt53 or empty virus, cells were lysed and were subjected to Western blot analysis using anti-Bax, anti-Bcl-2 anti-Bcl-xL, anti-Hsp70, anti-survivin, and anti-cIAP2 Abs. Anti-p21 mAb was used as wt53 transactivation activity control and anti-actin pAb as protein level control. These data are representative of two independent experiments. B, Analysis of cFLIP protein expression after infection of IGR-Heu cells with Adwt53. Modulation of cFLIP short and cFLIP long short by p53 was analyzed by Western blot 24 h after infection of IGR-Heu cells with 50 PFU per cell of Adwt53 or empty virus for control. Actin was used as protein level control. These data are representative of two independent experiments.
Sensitization of wtp53-infected cells to Fas-mediated apoptosis involves mitochondrial membrane permeabilization

It has been shown that death receptors can use the mitochondrial pathway to induce an apoptotic signal in type II cells (21). Evidence has also been provided indicating that activation of caspase 8 and mitochondria were directly connected by truncated-Bid protein to induce the release of apoptotic factors from the mitochondria and the full activation of the effector caspase 3. To determine the possible involvement of mitochondria in the sensitization of Adwtp53-infected cells to Fas-mediated apoptosis, cells were infected with Adwtp53 or empty virus, target cells were treated with the 7C11 anti-Fas mAB for 4 h or incubated with the Heu127 CTL clone for 30 min. Total cell lysate (50 μg) were subjected to Western blot analysis using anti-caspase 8, anti-caspase 3, or anti-actin mAbs. Cleavage products observed in 7C11 mAB treated or Heu127 incubated cells correspond to bands of 43 and 41 kDa. Specific implication of the Fas pathway in the cleavage of caspases after CTL treatment was demonstrated after preincubation of IGR-Heu/Adwtp53 cells for 2 h with the anti-Fas (ZB4) blocking mAB. Jurkat cells treated with the anti-Fas mAB for 2 h were used as procaspase 8 and procaspase 3 cleavage positive control. These data are representative of two independent experiments.

Selective Bid translocation and cytochrome c release in tumor cells displaying wtp53 during CTL-target interaction

To determine whether the autologous CTL clone is able to activate the mitochondrial pathway, IGR-Heu cells, infected or not with adwtp53, were incubated with Heu127 during 1 h and subjected to an immunostaining for Bid, cytochrome c, and the nucleus. Data
depicted in Fig. 5 show that Heu127 CTLs induce mitochondrial pathway activation only in Adwtp53 infected cells. Indeed, Bid translocation from the cytoplasm to the mitochondria, cytochrome c release from the mitochondria intermembrane space to the cytoplasm, as well as nucleus fragmentation were observed in wtp53 IGR-Heu expressing cells after CTL recognition. This mitochondrial pathway activation is strictly dependent of Fas receptor triggering by the Fas-positive ligand CTL clone Heu127, as demonstrated by the anti-Fas neutralizing ZB4 mAb. These data suggest that wtp53 is a key regulator of tumor target cell killing by specific CTLs through a regulation of Fas signaling and mitochondrial activation.

Discussion

At present, most immunotherapeutic strategies are essentially focusing on cytolytic effector cells such as specific CTL and NK cells. Accumulating evidence has been provided indicating that tumor cells are able to generate resistance to CTL-mediated cytotoxic pathways. This resistance may be independent from tumor cell recognition by the immune system and may result from limited antitumor effects of specific CTL (22). In this regard, genetic variability or temporal changes in tumor phenotype are two parameters of critical importance that may need to be evaluated to improve tumor sensitivity to cytotoxic cells. During malignant transformation of human tumors, p53 mutations are selected for loss of their apoptotic and tumor suppressor ability, which coincides with loss of their transactivation function (23). Although a number of apoptosis-related genes that are transcriptionally regulated by p53 have been identified, the transcription-independent downstream pathway of p53 remains largely unclear. The main goal of this study was therefore to better examine the influence of p53 on the regulation of tumor cell susceptibility to killer cells and to dissect the molecular mechanisms by which p53 effectively sensitizes tumor cells to CTL-mediated lysis. For this purpose, we have used a model system including a CTL clone and its autologous tumor target that express a mutated p53. This cell line does not express death receptors (Fas, TRAIL receptors, TNFR I-p55) and was sensitive to CTL killing through the exocytosis pathway involving degranulation of perforin and granzyme B.

Multiple molecular mechanisms have been described to account for the loss of Fas function in cancer cells, including down-regulation of transmembrane Fas following promotor methylation, transcriptional repression, histone acetylation, or alternative mRNA splicing to produce a soluble Fas protein lacking transmembrane anchor (24). Fas is a frequent target for inactivation during oncogenesis, and it has been hypothesized that Fas-induced apoptosis plays a crucial role in the biology and treatment of malignant diseases (25). In the present study, we have demonstrated
that besides inducing Fas receptor expression on tumor cells, p53 acts at multiple steps to sensitize target cells to CTL-mediated lysis by modulating the transcription of a number of cellular target genes. Indeed, our data indicate that the induction of Fas receptor expression on target cells is not sufficient to potentiate CTL killing because transfection of Fas cDNA resulting in a strong expression of CD95 by target cells displaying mutated p53 did not improve CTL-mediated cytotoxicity. This suggests that in addition to a p53-dependent regulation of Fas expression, p53 is able to sensitize tumors cells to CTL-mediated lysis by interfering with additional events involved in the regulation of the Fas apoptosis pathway. In light of our findings and given the fact that the level of intracellular cFLIP may regulate the sensitivity of tumor cells to apoptosis triggered by a variety of stimuli, such as Fas ligand, we investigated whether wt53 modulates cFLIP expression in target cells leading to subsequent increase in their susceptibility to CTL. Our data show that both cFLIP long and cFLIP short forms were expressed in parental cells. Interestingly, a marked decrease in cFLIP short protein was observed in Adwt53 infected cells, raising the possibility that the down-regulation of cFLIP short may render target cells more sensitive to CTL killing. Furthermore, no cFLIP long protein degradation was observed after Adwt53 infection, suggesting that the wt53 effect is independent of its transcriptional inhibition activity. These observations are concordant with a previous report of Fukazawa et al. (26) indicating that an accelerated degradation of cFLIP long protein occurred through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. To examine the functional significance of cFLIP short down-regulation for the sensitization of wt53 infected cells to CTL-mediated killing, we analyzed whether such an event was accompanied by the activation of proapoptotic mediators such as caspase 8 believed to play a central role in propagating death receptor-mediated signals. These studies demonstrated a cleavage of the proximal caspase 8 in the DISC of cells displaying wt53. Furthermore, the activation of caspase 8 was found to deliver a signal to mitochondria following cleavage of Bcl-2 family member protein Bid, which translocates to the mitochondria and triggers cytochrome c release. The present study implies that DNA binding domain of p53 is a dual-function domain, mediating both the transactivation of Fas gene and the mitochondrial proapoptotic pathway. This also emphasizes the extranuclear death function of p53 and shows that this function is critically involved in mediating the proapoptotic action of p53 at the mitochondria level.

Thus, Fas receptor and mitochondrial pathways may be interconnected leading to the amplification of CTL-mediated target cell death. These studies demonstrate for the first time a significant role of mitochondria in the control of target cell death by CTL and clearly show that p53 acts at multiple levels to potentiate the susceptibility of tumor cell to CTL killing. Our study, demonstrating the implication of mitochondria in CTL-mediated target cell death, provides a mechanistic basis of transcription-independent apoptosis by p53 and opens a new avenue toward a comprehensive understanding of mutated p53-expressing tumor escape from T cell-mediated cytotoxicity. We propose that p53 plays a linker role connecting death receptors to mitochondria leading to amplification of the apoptotic machinery in tumor cells. Our data support a

**FIGURE 5.** CTL recognition of IGR-Heu target cells induced activation of the mitochondrial pathway only with wt53 infection. CTL-induced translocation of Bid and cytochrome c in Adwt53-infected cells is shown. IGR-Heu cells were infected with 50 PFU of control adenovirus (empty virus) or Adwt53 for 24 h and incubated with the Heu127 CTL clone during 1 h. Immunostaining was performed with anti-Bid pAb (green), anti-cytochrome c mAb (red), and TO-PRO-3 for the nucleus (blue). Note the translocation of Bid from cytoplasm to mitochondria, the release of cytochrome c from mitochondria to cytoplasm, and the fragmentation of the nucleus in Adwt53-infected cells in contact with CTL. Black arrows indicate Heu127 CTL. One representative experiment of three is shown. The confocal scanning fluorescence micrographs are representative for the majority of CTL tumor cell conjugate analyzed. These data are representative of three independent experiments.
model in which p53 plays a role in the cross-talk between the apoptosis pathway involving ligation of the Fas receptor and the mitochondrial pathway involving Bid translocation and cytochrome c release. Although resistance of tumor targets to killer cells is likely to be regulated by multiple factors (27), the data we present suggest that p53 is an important determinant of target sensitivity to CTL-mediated lysis. In this regard, manipulation of p53 status may improve effectiveness of CTL to tumor immunotherapy and the clinical use of compounds able to restore p53 function could be a novel strategy for optimizing killer cell-based immunotherapy of cancer.

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