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Potentiation of a Tumor Cell Susceptibility to Autologous CTL Killing by Restoration of Wild-Type p53 Function

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Inactivation of p53 has been implicated 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 aim of this study was to investigate the influence of p53 status on the regulation of tumor susceptibility to specific CTL-mediated cell death. For this purpose, we used a cytotoxic T lymphocyte clone, Heu127, able to lyse the human autologous lung carcinoma cell line, IGR-Heu, in a HLA-A2-restricted manner. Direct genomic DNA sequencing revealed that IGR-Heu expresses a mutated p53 at codon 132 of the exon 5 which results in the loss of p53 capacity to induce the expression of the p53-regulated gene product p21^WAF1/CIP1. Initial experiments demonstrated that IGR-Heu was resistant to Fas, TNF, and TRAIL apoptotic pathways. This correlated with the lack of p55 TNFRI, Fas, DR4, and DR5 expression. The effect of wild-type (wt) p53 restoration on the sensitization of IGR-Heu to autologous CTL clone lysis was investigated following infection of the tumor cell line with a recombinant adenovirus encoding the wt p53 (Adwtp53). We demonstrate that the restoration of wt p53 expression and function resulted in a significant potentiation of target cell susceptibility to CTL-mediated lysis. The wt p53-induced optimization of tumor cell killing by specific CTL involves at least in part Fas-mediated pathway via induction of CD95 expression by tumor cells but does not appear to interfere with granzyme B cytotoxic pathway. The Journal of Immunology, 2003, 170: 5919–5926.

In addition to the several known classical strategies used by tumor cells to escape immune surveillance, the phenomenon of tumor resistance to cell death is of major concern and the mechanisms underlying it have yet to be fully elucidated. Thus, the search for new approaches to sensitize resistant cells to killing by cytotoxic effectors remains important in immunotherapy of cancer. CTL play a key role in immunosurveillance against tumors and infected cells (4). Two major pathways, triggered following TCR recognition of target-cell MHC/Ag complex, underlie T cell-mediated cytotoxicity. The first one is a secretory pathway involving receptor-triggered exocytosis of pre-formed secretory granules containing granzymes and perforin. The second is based on receptor-induced surface expression of death receptor ligands on effector cells, which cross-links the corresponding receptors (Fas, TRAIL, receptor (TRAIL-R), and TNF-1-p55 (TNFR1)) on target cells (5, 6). Indeed, stimulation of these receptors with their respective ligand leads to their clustering and the formation of the death-inducing signaling complex. Recruitment and oligomerization of caspase-8 to the death-inducing signaling complex results in its proteolytic activation, which initiates a cascade of caspases, leading to apoptosis (6). Many genes have been reported to regulate these death-associated events (7–9).

The p53 tumor suppressor protein plays multiple roles in cell cycle control, differentiation, genomic stability, angiogenesis, and apoptosis (10, 11). Mutations that inactivate the p53 gene product are frequently found in human cancers (12, 13). In this regard, point mutations of the p53 gene are the most frequent genetic alterations in tumor cells (14, 15). These mutations, resulting in stabilization and accumulation of high level of a defective p53 product, result in loss of the cell cycle-regulating function of the

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3 Abbreviations used in this paper: TRAIL-R, TRAIL receptor; TNFR-I, TNFR 1-p55; GrB, granzyme B; CMA, concanamycin A; LU, lytic unit; wt, wild type; PI, propidium iodide.

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p53 protein and represent a critical step in carcinogenesis. It is clearly established that the p53 protein functions as a sequence-specific DNA-binding factor and can activate genes whose promoters contain a p53 response element. In this context, several genes involved in the regulation of cell death are transactivated by p53 (16–18). The ability of p53 to regulate the cell cycle has been reported to contribute to drug resistance and to apoptosis induced by many anti-cancer agents (19, 20). However, the direct role of p53 in the control of tumor susceptibility to CTL-mediated lysis is still poorly documented.

In the present study, we have investigated the mechanisms by which the restoration of wild-type (wt) p53 function in p53 mutant tumor cells increases their susceptibility to CTL induced cytotoxicity. Our results show that the restoration of p53 function following infection of tumor cells harboring a mutated p53 with a recombinant adenovirus for wtp53 resulted in a significant increase in target cell death. Our findings suggest that such restoration may regulate cell-mediated cytotoxicity and provide new approaches to develop a more effective therapeutic intervention.

Materials and Methods

Abs and reagents

mAbs directed against Fas (UB2 and ZB4, mouse IgG1; CH11, mouse IgM) were purchased from Immunotech (Marseille, France). Anti-TNFRI mAb (clone H398) was purchased from Serotec (Oxford, U.K.), and anti-TRAIL-R mAb (respectively, M271, M413, M430, and M445 directed against TRAIL-R1-R2-R3, and R4) were from Immunex (Seattle, WA). Anti-p21WAF1/CIP was purchased from Oncogene Research Product (Boston, MA), and anti-actin mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified granulocyte M (Grob) was purchased from ICN Pharmaceuticals (Aurora, OH).

Tumor cell line and CTL clone

The IGR-Heu tumor cell line was established from a patient suffering from a large-cell carcinoma of the lung (21). Heu127 CTL clone was isolated from autologous tumor-infiltrating lymphocytes as described previously (22).

Sequencing of P53 gene in IGR-Heu cells

The genomic DNA extracted from IGR-Heu tumor cells was PCR-amplified sequenced from exons 2 to 11 of the p53 gene using the appropriate sense and antisense primers. Following purification, PCR products respectively to each exon were submitted to direct forward and reverse sequencings using Dye Terminator sequencing kit (Applied Biosystems, Courtabouef, France). Gel electrophoresis analyses were done by mean of ABI prism 377 (Applied Biosystems) and sequencing analysis software (Applied Biosystems). The identification mutation was confirmed twice on both strands.

Construction of recombinant adenovirus vector expressing wtp53 (Adwtp53) and expression of functional p53

Adwtp53 was produced by in vivo homologous recombination in 293 cells as described (23). Efficiency of Adwtp53 to transfer and direct expression of wtp53 was analyzed on p53 negative SAOS cells using specific mAb 36 h after infection with Adwtp53.

Adenovirus infection of cells

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.

Flow cytometry analysis

Cells (3 × 10⁶) were incubated with UB2 anti-Fas, H398 anti-TNFRI, M271 anti-TRAIL-R1, M413 anti-TRAIL-R2, M430 anti-TRAIL-R3, M445 anti-TRAIL-R4, or isotypic control mAb 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, San Jose, CA).

DNA content analysis by propidium iodide (PI) staining

Flow cytometry analysis of PI-stained cells was performed to analyze the restoration of wtp53 function in IGR-Heu cells on cell cycle progression. Briefly, cells were harvested 24 h after infection, washed, and fixed in 70% ethanol. For irradiated cells (5 Gy), an additional time of 5 h is necessary before staining. Before flow cytometry analysis, cells were washed with PBS and stained with 1 ml of PI (20 μg/ml) containing 100 μg/ml RNase and 20 mM 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. Apoptotic cell death induced by anti-Fas mAb was analyzed by measuring proportion of subG1 cells using the same staining.

Determination of cell viability

Tumor cells (5 × 10⁵) were seeded in flat-bottom 96-well plates and then infected with Adwtp53 or empty virus for control. After 24 h, CH11 anti-Fas mAb, recombinant TRAIL, or TNF-α were added for 16, 24, or 72 h, respectively. GRB was delivery into cells by BioPORTER Protein Delivery System (Gene Therapy Systems, San Diego, CA) (24). Briefly, BioPORTER reagent is first dissolved in methanol and aliquoted (2.5 μl) into Eppendorf tubes. After complete drying, BioPORTER is formulated with purified GRB (500 ng) (ICN Pharmaceuticals). The hydrated mixture is then added onto cells and incubated for 4 h with the target cells in absence of serum. After this incubation, complete medium was added for 20 h before viability test. Viability of cell was measured with MTT (Sigma-Aldrich, Saint Quentin, France). The percentage of cell viability was calculated as follows: percentage of viability = (A1/A0) × 100, where A1 and A0 represent absorbance obtained, respectively, for treated and untreated cells.

Western blot analysis

Total cellular extract were prepared by lysing cells 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, Orsay, France). The efficiency of the electrotransfer was assessed by Ponceau Red staining of the membranes. Blots were blocked overnight with TBS containing 5% nonfat dry milk and probed with appropriate Ab (anti-p21WAF1/CIP or anti-actin) for 1 h. After washing, blots were incubated with appropriate secondary Ab-HRP conjugated. The complexes were detected using an ECL detection kit (Amersham).

Cytotoxicity assay

The cytotoxic activity of CTL clone Heu127 was measured by a 6 h lactate dehydrogenase release assay using Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Charbonnière-les-Bains, France) on 3 × 10⁵ 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. Inhibition of granulocyte mediated cytotoxic pathway was performed by using effector cell preincubation for 2 h with 100 nM concanamycin A (CMA) (Sigma-Aldrich). Target cells are then added to the wells with CMA-treated effector cells (concentration of CMA during lysis: 50 nM). Experiments were performed in quadraplet and the percentage of lysis was determined by OD₅₇₀ measurement as described in the manufacturer’s instructions. Lytic units (LU) were calculated as described by Pross et al. (25). One LU was defined as the number of effector cells required for 30% lysis of 3 × 10⁵ target cells and the number of LU present in 10⁶ effector cells was calculated using a computer program.

Results

Expression of death receptors by IGR-Heu

We have previously established a tumor cell line (IGR-Heu) and an autologous CTL clone (Heu127) from a non-small cell lung cancer patient. The cytotoxic activity of Heu127 against IGR-Heu was HLA-A2.1 restricted and recognized a mutated peptide of α-actin 4 (26). Data depicted in Fig. 1A indicate that the CD8⁺ clone Heu127 efficiently kills the autologous IGR-Heu target. The inhibition of exocytosis-mediated pathway by CMA resulted in the abrogation of Heu127 CTL clone cytotoxicity indicating that the death receptors pathway is not involved in Heu127-mediated...
lysis and that the observed cytotoxicity is rather mediated by the perforin/granzyme pathway.

To confirm these data, immunofluorescence analysis performed using UB2, M271, M413, M430, M445, H398 mAb specific for Fas, TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2 and TNF-R1 respectively, revealed a lack of these receptors expression on the surface of IGR-Heu cells (Fig. 1B).

Mutation of p53 gene and loss of its transactivation activity in IGR-Heu target

Direct sequencing of IGR-Heu genomic DNA revealed a p53 mutation at codon 132 (Fig. 2A). This mutation, of transversion type (AAG > AAT, i.e., lys > asp), has a dramatic functional impact on p53 function as judged by down-stream p53 inducible genes such as the archetypal cell cycle blocker p21Waf/Cip1 (27). Indeed, as shown in Fig. 2B, the IGR-Heu cells had an undetectable p21 protein level, indicating a total lack of p53 transactivation activity.

The infection of IGR-Heu with Adwtp53 restores p53 transactivation activity

To determine the effect of wt p53 in IGR-Heu cells, we have constructed an adenovirus expressing wt p53 (Adwtp53) (23). To investigate whether infection of IGR-Heu with Adwtp53 leads to p21 induction, Western blot analysis was performed. Fig. 3A shows that infection of these cells with such a vector induced an increase in the p21 protein level, indicating that the infection with Adwtp53 resulted in the restoration of p53 function in IGR-Heu cells.

It is well established that cell treatment with DNA damaging agents results in cell cycle arrest in G1 in cells expressing functional p53 protein. Therefore, we have conducted cell cycle distribution of infected IGR-Heu cells. Data shown in Fig. 3B demonstrate that the number of G1 cells was increased after infection of IGR-Heu cells with Adwtp53 (51 vs 41%). Following gamma irradiation, the G1 arrest in IGR-Heu/Adwtp53 cells was more pronounced (46%) as compared with noninfected IGR-Heu cells (24%), confirming the restoration of wt p53 functional activity.

The restoration of p53 wt activity enhanced tumor target sensitivity to CTL lysis

We have previously shown that the restoration of p53 resulted in the attenuation of tumor resistance to the cytotoxic action of TNF...
To determine whether restoration of the transactivation activity of p53 in IGR-Heu target cells induces an increase in CTL-mediated lysis, the cytotoxic activity of Heu127 clone toward IGR-Heu cells infected with Adwtp53 was examined. As shown in Fig. 5, a significant increase (39.4 vs 15.9 LU) in CTL killing was observed when the IGR-Heu target was infected with Adwtp53 as compared with the parental cell line. To investigate whether Fas induction on IGR-Heu is associated with the sensitization of these cells to CTL response, we used the ZB4-blocking anti-Fas mAb. The incubation of target cells IGR-Heu with ZB4 totally abolished the wtp53-induced increase in Heu127 CTL clone killing (Fig. 5). The GrB-mediated cytotoxicity is independent from the p53 status in IGR-Heu target cells

To gain more insights into the mechanism involved in p53-induced sensitization of target cells, we asked whether p53 influences the GrB pathway since the CTL clone mainly uses GrB pathway to kill IGR-Heu target cells. Data depicted in Fig. 6A demonstrate that the restoration of wtp53 expression and function does not appear to affect IGR-Heu target sensitivity to GrB mediated cell lysis. Indeed, cytotoxic experiments performed using a recombinant GrB delivered into the cells with BioPORTER indicate a similar susceptibility of infected cells and control cells. To confirm these data, we used CMA to inhibit the Ca^{2+}-mediated lysis, involving the perforin/GrB pathway. Data shown in Fig. 6B indicate that such a treatment does not result in a complete inhibition of the lysis of IGR-Heu-infected cells as compared with IGR-Heu parental cells. This results suggest that p53 has no effect on GrB-triggered pathway.

**Discussion**

It is well established that cell proliferation reflects the interplay of cell survival and cell death pathways and that the resistance of tumor cells to cytotoxic treatments involves multiple mechanisms protecting cells from apoptosis (28). Therefore, understanding the molecular basis of the survival pathways and the ways they intersect with the apoptotic cascade and identifying agents that can bypass these signals will likely be necessary for the development of effective immunotherapy. In addition to chemoresistance and radioresistance, tumor cells also generate resistance to CTL-mediated cytotoxic pathways. The most known resistance mechanism involves low expression of tumor Ag which may either not be sufficient to be recognized by the TCRs or insufficient to activate the killing pathways. Another mechanism is associated with the failure to trigger lymphocyte mediated killing process. Nevertheless, tumor resistance may be independent of immune recognition and specific CTLs may have limited antitumor effects (29). This is consistent with the fact that human tumors often do not regress and may continue to grow despite detectable spontaneous immunization-induced immune responses in circulating lymphocytes. The
discovery of TAA has allowed the rational design of antitumor CTL strategies, which kill target tumors expressing the appropriate Ag and MHC class I (30) and at present, most immunotherapeutic strategies are essentially focusing on cytolytic effector cells such as specific CTL and NK cells. In this regard, genetic variability or temporal changes in tumor phenotypes are two parameters of critical importance that may need to be evaluated to improve tumor response to cytotoxic cells.

p53 is a key genetic factor able to influence tumor susceptibility to cytotoxic mechanisms, and it plays a critical role in tumor progression. Mice deficient in p53 develop normally but are highly predisposed to spontaneous tumors (31). The majority of p53 homozygous-null mice develop lymphomas and sarcomas before 6 mo of age and most of them die after 9 mo. In addition to being an ideal target and able to induce tumor growth suppression through cell cycle arrest and apoptosis regulation, p53 has been implicated in the cellular response to DNA damaging radiotherapy and chemotherapy (32, 33). However, the implication of this tumor suppressor gene in the control of tumor susceptibility to CTL killing remains largely unclear. In a previous study, we have shown that the impairment of wt p53 function may contribute to cell resistance toward the cytotoxic action of TNF and provided evidence that the disruption of wt protein resulted in loss of the cell sensitivity to cytotoxic activity of TNF (34).

To delineate the relationship between p53 and the tumor susceptibility to CTL killing, we took advantage of our cell model based on the use of a fresh non-small cell lung cancer cell line as a target and its autologous specific CTL clone. We have demonstrated that the tumor target IGR-Heu has an accumulated mutant p53 molecule displaying a point mutation (K to N) at codon 132. The primary objective of these studies was to determine the influence of p53 status in target cells on CTL-mediated cytotoxicity. Therefore, initial experiments were aimed at restoring wt p53 function in p53 mutant IGR-Heu target cells. Based on the fact that human tumor cells with constitutive NF-κB activity might exhibit a compromised p53 response since p53 and NF-κB inhibit each other's ability to stimulate gene expression (35), we have first demonstrated, using gel shift experiments, that NF-κB was not constitutively activated in our cell model (data not shown). The studies indicate that the infection of IGR-Heu cells with Adwt p53 recombinant virus was efficient in restoring p53 transactivation activity which correlated with a significant increase in target susceptibility to CTL killing. These findings implicate the status of p53 as a potential modulator of target susceptibility to CTL killing. To get more insights into the mechanisms involved in the p53-induced potentiation of IGR-Heu target cell killing, we have examined the consequence of wt p53 restoration and its subsequent transactivation activity on the expression of death receptors which were lacking in parental IGR-Heu cells. While TNFRI and TRAIL-Rs were not induced, a significant induction of Fas was observed following infection with Adwt p53. More importantly, our data indicated that the infected cells became sensitive to killing.

**FIGURE 4.** A, Surface Fas receptor expression on IGR-Heu-infected cells was determined 24 h after infection by indirect immunofluorescence analysis using UB2 anti-Fas mAb (gray). Isotypic control mAb was included (black line). The percentage of positive cells is indicated. B, Sensitivity of IGR-Heu-infected cells to Fas-mediated lysis. Twenty-four hours after the infection of IGR-Heu target cells with Adwt p53 or empty virus, CH11 anti-Fas mAb was added at a different concentration, and cell viability was determined after 16 h of incubation as described in Materials and Methods. C, Analysis of apoptotic cell death by measurement proportion of subG1 IGR-Heu-infected cells. Target cells were treated with 1 μg/ml CH11 anti-Fas mAb during 6 h and stained with PI before flow cytometry analysis.
following triggering by both the agonistic anti-Fas Ab and the specific CTL. It should be noted that the restoration of p53 does not restore only Fas expression but also Fas-mediated apoptotic pathway. We have previously shown that the transfection of Fas cDNA in IGR-Heu cells, despite a significant Fas induction, did not result in the restoration of CTL-induced Fas-mediated target lysis (36) suggesting that p53 acts at the level of Fas expression but presumably at an additional level involving Fas responsiveness. Experiments in progress will attempt to identify the intermediate that connects p53 to the Fas apoptotic machinery. The restoration of p53 transactivation activity did not lead to the expression of other p53 target genes involved in the regulation of apoptotic pathway including Bcl-2, Bcl-X, Bax, and Survivin (data no shown).

The present data point to the importance of the alternate cytotoxic pathway involving Fas/FasL interaction as a potential component involved in the execution of the CTL program and are in agreement with the work of Medema et al. (37) and Djerbi et al. (38) emphasizing the potential role of this pathway in vivo. It should be underlined that some killer cells have a distinct preference for either the death receptor pathway or the granule exocytosis pathway. For instance while CD8\(^+\) CTL can use either mechanism, NK cells and CD4\(^+\), Th2 T cells preferentially use the

**FIGURE 5.** A, Analysis of CTL mediated lysis after IGR-Heu target cells infection with Adwtp53. Cytotoxic activity of Heu127 CTL clone was evaluated using Cytotox 96 non-radioactive assay 24 h after infection of IGR-Heu target cells with Adwtp53 or empty virus. Heu127 CTL were added at the indicated E:T ratio for 6 h of incubation. Specific implication of Fas pathway in CTL lysis potentiation was demonstrated after preincubation of IGR-Heu/Adwtp53 cells for 2 h with anti-Fas (ZB4) blocking mAb. B, Representation of LU present in Heu127 effector cells. One LU was defined as the number of Heu127 effector cells required for 30% lysis of 3 \(\times 10^3\) target cells and the number of LU present in 10\(^6\) effector cells was represented.
perforin/granzyme pathway and CD4\(^+\) Th1 preferentially use the Fas pathway (5).

Since our findings indicate that tumor cells lacking functional p53 are less sensitive to apoptosis induced by CTL, it is tempting to speculate therefore that tumor cells harboring p53 mutations although killed to a certain extent, display a decreased sensitivity to CTL and may escape the host immune system. As a result, patients with tumor expressing a mutated p53 may be immunocompromised and that during immunotherapy the status of p53 merits consideration in designing future clinical trials.

GrB is a major component of granule-mediated cytotoxicity of T lymphocytes. Although it can directly activate caspases, it induces apoptosis predominantly via Bid cleavage, mitochondrial outer membrane permeabilization and cytochrome c release (39). To study the influence of p53 status on the GrB-induced cell death pathway, we used a CTL-free cytotoxicity system, where target cells are treated with purified GrB. Although p53 may act as a determinant of sensitivity for CTL-mediated killing, restoration of this gene does not appear to influence the GrB cytotoxic pathway.

This suggests that the two pathways leading to cytolytic lymphocytes induced apoptosis are under distinct regulatory mechanisms. At present, extensive attempts are made to restore the p53 function in tumor cells by introduction of peptides derived from the C-terminal fragment of p53 (40). In this regard, it has been recently reported by Bykov et al. (41) that a new low m.w. compound named PRIMA-1 restored the sequence-specific DNA binding and the active conformation to mutant p53 proteins in vitro and in vivo. It is tempting to speculate that such tools may help to achieve a significant therapeutic index of cytotoxic cells during tumor-specific CTL immunotherapy. It should be emphasized that intratumor injections of adenovirus vector expressing wt p53 in patients with advanced non-small cell lung carcinoma resulted in an antitumor effect with prolonged tumor stability (42). Whether this anti-tumor effect is accompanied or not with a loco regional antitumor response remains to determined.

Taken together, our data point to the importance of p53 function in sensitizing tumor cells with p53 mutations to CTL response and suggest that the status of the p53 gene, which is mutated in a high percentage of human cancers, may be an important determinant of the efficacy of killer cells based immunotherapy. The clinical use of peptides able to restore p53 function in immunotherapy approaches could be a novel strategy for optimizing the immunotherapy of cancer by increasing the CTL killing potential.

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