Human Lung Cancer Cells Endogenously Expressing Mutant p53 Process and Present the Mutant Epitope and Are Lysed by Mutant-specific Cytotoxic T Lymphocytes

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ABSTRACT

The p53 oncoprotein frequently contains somatically acquired missense mutations and is often overexpressed in cancer cells. Missense mutations can give rise to new tumor-specific peptide sequences, which can act as targets for T-cell-mediated immunotherapy. To investigate the ability of human lung cancer cells to adequately process and present a mutant p53-derived CTL epitope, we transfected the human cell line HMy-2.C1R and the p53-null human lung cancer cell lines H358 and H1299 with an expression vector containing a human mutant p53 (135 Cys to Tyr). After transfection with the Kd restriction element, these cells were tested as targets for murine mutation-specific CTLs. We show that these human lung cancer cells effectively process and present this endogenous mutant human p53 epitope, resulting in efficient, mutant epitope-specific lysis by CTLs. In the presence of the appropriate restriction element, human lung cancer cells can be effectively targeted by CTLs specific for somatically acquired, endogenous mutant epitopes, supporting targeted immunotherapy efforts in lung cancer.

INTRODUCTION

The ability of tumor-specific CTLs to lyse tumor cells lies ultimately in their ability to recognize a tumor-associated or specific epitope bound to class I MHC on the tumor cell surface. Mutations in p53 represent one such potential tumor-specific epitope. In cancer cells, p53 is frequently found to be somatically mutated, and the missense mutant protein often accumulates to high levels. These mutations in p53 usually involve alterations at a single codon, leading to inactivation of p53. We have previously demonstrated that murine fibroblasts endogenously expressing a human missense mutant p53 (codon 135, Cys to Tyr) express the mutant epitope on the cell surface and can be lysed by mutation-specific CTLs (1). Furthermore, we have shown that the lytic effectiveness of mutation-specific CTLs is highly variable, depending on the nature of the cells expressing the defined mutant p53 (2). Any immunotherapy targeting mutant p53-derived epitopes requires a T-cell epitope that has to be not only sufficiently immunogenic but also adequately presented by the tumor cells. Therefore, to consider therapeutic attempts to target these epitopes in lung cancer, it is crucial to demonstrate that human lung carcinoma cells maintain the ability to process and present an endogenously synthesized, p53-derived, tumor-specific T-cell epitope. Here, we show that human lung cancer cells possess the ability to process endogenously expressed mutant p53 and to present the mutant epitope to mutation-specific effector cells.

MATERIALS AND METHODS

Cell Lines. HMy-2.C1R and transporter-associated with antigen presentation-deficient cell line T2 were purchased from the American Type Culture Collection (Rockville, MD). Cell lines H358 and H1299 were kindly provided by Dr. A. Gazdar (University of Texas Southwestern Medical Center). The H358 cell line is derived from a non-small cell lung cancer of the bronchioalveolar subtype, and cell line H1299 is derived from a non-small cell lung cancer of the large cell subtype. Both cell lines have homozygous deletions for p53 and express no p53 protein (3, 4).

Mice. Female BALB/c mice, 10–16 weeks of age, were purchased from Harlan-Sprague-Dawley (Indianapolis, IN).

Expression Vectors. The p53 open reading frame from a T1272 human non-small cell lung cancer tumor specimen (containing the point mutation C to Y at position 135) was amplified by cDNA PCR with artificial restriction site ends. The full open reading frame was sequenced in both directions to exclude artifactual PCR-derived mutations. The PCR product was cloned between HindIII and XbaI into pRC/CMV4 (Invitrogen, San Diego, CA). Vector pSVH-2Kd.33, expressing the murine MHC class I Kd, was kindly provided by Dr. P. Kourilsky (Institut Pasteur, Paris, France) and was cloned as described previously (5). Expression vectors for MHC class I D
t (pD
t-1) and L
t (pL
t-444) were kind gifts from Dr. D. H. Margulies (NIH) and contain a selection marker for G418. To perform double transfections, we used the plasmid PGKkphp, which expresses the hygromycin resistance marker and was a gift from G. Donoho (Stanford University, Palo Alto, CA). Expression vec-

1 The abbreviations used are: CMV, cytomegalovirus; HLA, human leukocyte antigen; ER, endoplasmic reticulum.
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Briefly, expression vectors coding only for the segment of p53 from amino acids 128-145 were constructed by cloning p53 oligonucleotides into the pRc/CMV vector either alone or fused in frame to an oligonucleotide coding for the adenovirus E3 leader sequence. The oligonucleotide AGCTA TGAGG TAGGAGT TTATC, for transfectants expressing the epitope alone; or primers T12n-3, GATCA GCGAG CATTT AGGTG for transfectants expressing the minigene following the E3 leader sequence. 

Transfection of Expression Vectors. HMy-2.C1R cells (3 x 10⁶) or H358 lung cancer cells were transfected with pRc/CMV-expressing mutant p53 in the presence of pSVH-2K⁳³ at a ratio of 1:5. A ratio of 1:1 was used for expression vectors coding for D⁺ or L⁺. Cells were grown in G418 (Life Technologies, Inc., Rockville, MD) at concentrations of 400-900 µg/ml and positively selected for expression of MHC class I with streptavidin-coated M-280 magnetic beads (Dynal, Oslo, Norway). For experiments with HMy-2.C1R, the polyclonal, neo-resistant cell population was used. H358 cells were transfected similarly and were cloned by limiting dilution. Cell line H1299 was cotransfected with PGKphp and pSVH-2K⁳³ at a ratio of 1:5 and grown at a concentration of 500 µg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO). Cells were selected for expression of K⁺ with magnetic beads as described above and subsequently cloned by limiting dilution. K⁺-expressing clones were further transfected with pRc/CMV or with pRc/CMV expressing the mutant p53 with substitution of tyrosine for the normal cysteine at codon 135 (T1272). Cells were grown in 500 µg/ml G418. T2 cells were transfected with pRc/CMV and selected at 800 µg/ml G418. All transfections were performed with a Bio-Rad (Richmond, CA) Gene Pulser at 0.24–0.28 kV. Expression of transfectants was assayed immunohistochemically with antibody DO1, recognizing mammalian mutant p53 (Oncogene Science, Uniondale, NY), using standard techniques. Anti-CD8 antibody (Sigma) was used as an isotype-identical, negative-staining control. Expression of the minigenes in transfectants was assayed using reverse transcription-PCR with primers T12n-3, GATCA GCGAG CATTT AGGTG and T12n-5, GAACC CACTG CTFAA CTGGC into pRc/CMV between HindIII and XbaI without the E3 leader sequence.

Fluorescence-activated Cell Sorting Analysis. Transfectants were assayed for expression of MHC class I with surface staining for K⁺, L⁺, or D⁺ using biotin-labeled antibodies SF1-1.1 (K⁺), 34-2-12 (D⁺), and 28-14-8 (L⁺; PharMingen, San Diego, CA) or FITC-labeled, anti-H-2 monotypic antigen antibodies (Boehringer Mannheim, Indianapolis, IN). Expression of HLA was assessed with mouse antibody G46–2.6 (PharMingen) and stained with rabbit antimouse FITC (Sigma).

Peptide Synthesis. Synthetic peptides were prepared using t-Boc solid-phase peptide synthesis on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer and purified as described previously (6). The T1272 peptide sequence was TYSPALNKMFYQLAKTCPVQL, and the V10 sequence (the 10-residue minimal peptide derived from T1272) was FYQLAKTCPV. The control peptides used were FQLAHTCPV (the corresponding normal human sequence, WTVI0) and peptide VSV-8 RGYVYQGL.

CTL Generation. Groups of three mice were immunized with 2 x 10⁶ spleen cells pulsed with 5 µM peptide for 2 h at

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37°C and irradiated with 2000 rads. Four to 6 weeks later, immune spleen cells (3 × 10^6/ml) were restimulated in vitro for 6 days with or without peptide (5 μM); for 4 days in a mixture of Iscove’s modified Dulbecco’s medium and Eagle-Hank’s amino acid medium containing 10% FCS, 2 mM L-glutamine, 5 × 10^{-5} M 2-mercaptoethanol, and antibiotics; and for 2 additional days in the presence of 10% Rat-T Stim without concanavalin A (Collaborative Research, Inc., Bedford, MA). These CTLs have been characterized previously (1, 2). They have a CD8+ phenotype and recognize only mutant and not wild-type peptides. The minimal epitope is contained within the V10 peptide.

**CTL Assay.** The cytolitic activity of the restimulated cells was measured as described elsewhere. Briefly, 51Cr-labeled targets were mixed at appropriate ratios with restimulated effectors in duplicate and incubated at 37°C for 4 h. In experiments for recognition of peptide-pulsed targets, target cells were pulsed with the appropriate peptide: FYQLAKTCPV (V10), a T-cell epitope generated by a point mutation at position 135 from C to Y; or as a control, the wild-type peptide FCQLAKTCPV (WTV10), at 5 μM. Peptide-pulsed targets were washed before use in this assay. Transfectants were assayed for lysis by K^d-restricted CD8+ murine effector cells in the presence of nonlabeled T2 (an) at a ratio of 1:1. The supernatant was harvested with the Skatron (Lier, Norway) harvesting system, and radioactivity was counted in a gamma counter (LKB, Gaithersburg, MD). The percentage of specific lysis was calculated using the equation: 100 × [(experimental release - spontaneous release)/(maximal release - spontaneous release)].

**RESULTS**

**Expression of MHC Class I on Cancer Cells.** Both lung cancer cell lines (H358 and H1299) expressed HLA (data not shown), indicating that the processing and transport functions required to load class I molecules with the stabilizing peptide were intact in these cells. Expression of murine MHC class I was observed after transfection (Fig. 1).

**Expression of p53 in Transfected Lung Cancer Cells.** Transfected p53-null lung cancer cell lines acquired positive immunohistochemical staining after transfection with mutant...
p53. H358 transfectants revealed predominantly nuclear staining (Fig. 2A), whereas H1299 cells showed significant staining in the cytoplasm as well (Fig. 2B). The strength of this staining was comparable to that observed in unmanipulated tumor cells expressing mutant p53 (data not shown).

Assessment of Lysis by Mutant-specific CTLs. To assess for the ability of the endogenously expressed p53 epitope to be presented to mutation-specific CTLs, we first tested transfectants of the cell line HMy-2.C1R, which is known to efficiently process endogenously expressed antigens (7). Transfectants were readily lysed by mutation-specific CTLs (Fig. 3), demonstrating that the human processing and presentation machinery can present this epitope.

Lysis of Lung Cancer Cells Expressing Mutant p53. H358neo transfectants expressing K$^d$ and pulsed with the mutant peptide were readily lysed by peptide-specific CTLs, but not if pulsed with control peptides (Fig. 4). Transfectants expressing D$^4$ could not serve as targets (data not shown), demonstrating the predicted class I restriction. If these p53-null human lung cancer lines were cotransfected with K$^d$ and the pRe/CMV-p53 expression vector, resulting in endogenous expression of the intact mutant p53, significant lysis by mutation-specific effector cells was observed (Fig. 5). H1299 transfectants, which expressed the appropriate restriction element, and either the whole open reading frame of p53 or a minigene were also generated. These minigene expression constructs contained the human mutant p53 sequences from codons 125 to 145, either alone or fused in frame to an ER-targeting sequence (the adenovirus E3 protein leader sequence). The minigene product containing the E3 leader bypasses processing and ATP-dependent transport into the ER (8). Fig. 6 shows that lysis of the endogenously expressed, mutant p53-derived epitope from the intact open frame is comparable to the lysis observed for transfectants expressing only the minigene epitope fused to the E3 leader sequence.

**DISCUSSION**

The effectiveness of targeted immunotherapy ultimately depends on the ability of the effector cells to recognize a tumor-associated epitope (9). Point mutations somatically acquired in tumor cells may allow the generation of mutation-specific effector cells, which would be highly tumor restricted. Several epitopes have been described that are effectively recog-
Fig. 6  Lysis of endogenously expressed p53 in lung cancer cell line H1299. A, double transfectants expressing the restriction element H-2Kd and the whole open reading frame of mutant p53 are lysed by mutation-specific CTLs. B, lysis of cells transfected with pRe/CMV alone in the presence of H-2Kd. C, lysis of H1299-Kd cells. H1299 targets are prone to high nonspecific lysis despite the normal expression of HLA and the presence of MHC class I. D, transfectants expressing the same epitope targeted to the ER are lysed to a degree comparable to transfectants expressing the whole open reading frame, as shown in A. E, transfectants expressing the minigene without targeting to the ER.

nized by cytotoxic T cells and are frequently expressed in cancer (10–14). The evidence is accumulating that oncoproteins can be adequately processed and presented as immunodominant sequences to CTLs in a complex with class I MHC. Direct physical evidence for oncoproteins being presented as peptides on MHC class I has been established by mild acidic elution from MHC class I and high-performance liquid chromatographic analysis in the case of the akt oncogene (15). Human tumor-specific CTLs also can recognize a peptide derived from the HER-2neu oncogene product from codon 654–662, which is presented in the context of HLA 2.1. This oncoprotein-derived peptide has also been eluted from ovarian cancer cell lines (16). In murine model systems, peptide immunization is able to prevent tumor growth in ras-expressing tumor cells, and ras mutation-specific CTLs have been found in a human cancer patient (17–19). Immunization of mice with the whole mutated ras protein resulted in induction of ras-specific CTLs and prevented growth of mutant ras-expressing murine tumors (18). Similarly, induction of mutation-specific CTLs from p53 can give rise to protective immunity in mice (20).

With respect to tumor-suppressor genes, no evidence has been presented up until now that human cancer cells maintain the ability to present epitopes derived from missense mutant p53 or to be lysed by p53 mutation-specific effectors. The immunogenicity of sequences surrounding point mutations from ras and p53 in cancer patients, however, has been reported (21, 22). Transfectants and murine tumors with abnormal p53 may present peptides surrounding point mutations, but the ability to be lysed by cytotoxic T cells may critically depend on the cell background and their ability to process and present the epitope (2). This has been observed for mutant ras-specific and HLA-A*0201-restricted T-cell clones, which were unable to lyse ras-transfected 11B3 cells (23), whereas ras-transfected fibroblasts efficiently presented the mutant ras-derived epitope (19). Thus, even if oncoprotein-specific CTLs can be detected in murine or human systems, it is important to demonstrate that the common human solid tumors expressing these oncoproteins are capable of presenting them to effector CTLs, as we demonstrate here for lung cancer cells.

Certain lung cancer cell lines have been found to have defects in processing and presenting endogenously expressed antigens (24). However, in the transfected human lung cancer cell lines used in the present study, an endogenously expressed epitope surrounding a tumor-specific point mutation derived from human p53 can be efficiently processed and presented to CTLs. Thus, if these lung cancer cells expressed a mutant p53, which bound to the expressed human MHC molecules, they would be expected to be efficient targets for induced or spontaneous mutant p53-specific CTLs, supporting ongoing efforts to use this phenomenon therapeutically. Because both lung cancer and missense mutations in p53 in lung cancers are very common events, this has significant therapeutic implications.

There are many mechanisms, however, by which human cancers can avoid this host defense mechanism. We have previously reported that in human lung cancer, there is an unexpectedly low frequency of missense mutations in p53 which occur in the context of a sequence that would be expected to bind to the class I MHC molecules expressed on the tumor cells (25). This suggests that there may be a negative selection for malignant precursor cells that acquire mutations in p53 effectively presented by their MHC. We also showed in this study that when binding is predicted, homozygous deletion of the restriction element can occur, allowing the cells to effectively avoid CTL-mediated lysis. Gross defects in class I peptide assembly can also arise somatically in lung cancer via mutations in β2-microglobulin.9 This also results in the inability to present endogenously derived peptides. The efficiency of processing and presentation of different mutants of p53 may also be variable. The potential therapeutic utility of this process, therefore, may be limited to that subset of tumors that both express and

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process the appropriate epitope and express the necessary restriction element. These data demonstrate that the intracellular machinery needed to process and present endogenous mutant p53 is often intact in human lung cancer cells.

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REFERENCES

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