Restoration of the Expression of Transporters Associated with Antigen Processing in Lung Carcinoma Increases Tumor-Specific Immune Responses and Survival

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Abstract

A wide variety of human carcinomas have low expression of tumor-associated antigen presentation in the context of MHC class I antigens due to defects in the antigen presentation pathway. This immune evasion mechanism renders many tumors unrecognizable by host immune surveillance mechanisms. The present study examines the expression of HLA, tapasin, transporter associated with antigen processing 1 (TAP1), and β2 microglobulin in human small cell lung carcinoma and non–small cell lung carcinoma. Immunohistochemical staining showed severe impairment of the antigen presentation pathway in all patients. In order to recover tumor immunogenicity, a nonreplicating adenovirus expressing human TAP1 (AdhTAP1) was used to restore the expression of TAP1 in the antigen presentation pathway–deficient mouse lung carcinoma cell line, CMT.64. Infection of CMT.64 cells with AdhTAP1 increased MHC class I antigen surface expression, antigen presentation, and susceptibility to antigen-specific CTLs. Fluorescence-activated cell sorting and ELISPOT analysis showed that AdhTAP1 treatment significantly increased dendritic cell cross-presentation and cross-priming of tumor antigens. Furthermore, ex vivo and in vivo AdhTAP1 treatment significantly retarded tumor growth and increased survival of mice bearing CMT.64 tumors. Fluorescence-activated cell sorting analysis and immunohistochemical staining showed a significant increase in CD8+ and CD4+ T cells and CD11c+ dendritic cells infiltrating the tumors. The results show that TAP should be considered as a part of the immunotherapies for various cancers because it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic composition of the tumor or the MHC haplotypes of the host. (Cancer Res 2005; 65(17): 7926-33)

Introduction

The discovery of HLA class I restricted, tumor-associated, and tumor-specific antigens has reinvigorated the clinical development of immunotherapies for the treatment of carcinomas. The appeal of immunotherapies is the potential to control disseminated, metastatic disease with a minimum of toxic side effects due to the immune system’s exquisite specificity. Many of the therapies involve vaccination with protein/peptide antigens, plasmids, or recombinant virus–encoding genes for antigens, or whole cell vaccines which consist of irradiated autologous or allogeneic tumor cells, or autologous dendritic cells with a variety of modifications (1). These varied approaches aim to stimulate a T cell–mediated antitumor immune response and are based on the hypothesis that the cellular arm of the immune system can control neoplastic disease through immunosurveillance and targeting of tumor-specific antigens. Although conceptually appealing, the success of cancer vaccines and immunotherapies in humans is variable. In most cases, the vaccines are very well tolerated and specific immune responses to particular antigens are made but the response rate of the disease to the therapy is low. The reason for the low response rates are thought to be due to several factors which include low immunogenicity and tolerance to tumor-associated antigens (TAA), immunosuppressive microenvironment in the tumor and antigen loss variants arising in the tumor through immunoselection (2, 3). The antigen loss variants are characterized by the absence or decreased expression of MHC class I antigens on the tumor cell surface. The alterations in the expression of MHC molecules play a crucial step in tumor development due to the role of MHC antigens in antigen presentation to T lymphocytes and the regulation of natural killer cell function. In some cases, the rates of total HLA class I antigen losses are close to 100% (4, 5). These MHC losses can be produced at any step required for HLA synthesis, assembly, transport, or expression on the cell surface. In humans, the loss of HLA expression in a wide variety of carcinomas is often associated with transporter associated with antigen processing (TAP1 and TAP2) down-regulation (6), which is strongly correlated with disease progression and metastasis in patients (7). Loss of TAP activity leads to failure to transport peptides from the cytoplasm to the lumen of the endoplasmic reticulum, and hence, failure of the class I antigen processing pathway (APP). For example, the Buf1280 cell line from a metastatic lesion of a melanoma patient is TAP1-deficient due to a base pair deletion at position 1,489 of the TAP1 gene expression. The impaired TAP1 protein expression results in deficiencies in TAP2 protein expression, peptide binding, translocation, and MHC class I antigen surface expression. Stable TAP1 gene transfer reconstitutes the described defects (8). This phenomenon has also been observed in a human small cell carcinoma cell line deficient for TAP1 and low molecular weight protein 2 (LMP2). Restoration of TAP1 expression alone increased HLA surface expression but restoration of LMP2 alone did not complement surface expression of HLA molecules (9). The mouse lung carcinoma, CMT.64, a cell line derived from a
spontaneously occurring lung carcinoma in a C57BL/6 mouse (10), is characterized by down-regulation of many of the components of the APP. These components include MHC class I heavy chain, β2 microglobulin (β2-m), proteasome subunits (LMP2 and LMP7), TAP1 and 2 (11, 12) and tapasin. Despite these multiple defects, restoration of TAP1 itself leads to partial restoration of MHC class I antigen surface expression (11). Studies in C57BL/6 mice show that the introduction of mixtures of TAP1-positive and TAP1-negative cells produced tumors composed exclusively of TAP1-negative cells, indicating selection and evasion of immune surveillance in cells with the TAP deficiency (13). Mice bearing TAP-deficient CMT.64 tumors are able to clear the tumor load after treatment with replicating vaccinia virus encoding rat TAP1 alone and develop protective immunity upon subsequent challenge with CMT.64 cells (14). In the light of these studies, TAP gene transfer has a number of potential advantages as an immunotherapeutic strategy for the treatment of cancer. Restoration of TAP activity could restore the immunogenicity of the tumor by restoring the presentation of a number of tumor-specific antigens allowing an antitumor immune response that is HLA-specific, tumor-specific, and patient-specific (14).

In this study, we examine the expression of cell surface HLA and components of the APP, which include β2-m, TAP1, and tapasin, in lung carcinomas from patients that have undergone lung resection. We make a replication-deficient (ΔE1/ΔE3) adenovirus vector expressing the human TAP1 (AdhTAP1) that can restore antigen processing and MHC class I antigen surface expression in the CMT.64 carcinoma cell line. We treat mice bearing CMT.64 tumors with AdhTAP1, to ascertain if boosting immune responses are capable of controlling the progression of the disease in vivo.

Materials and Methods

Antigen presentation pathway status in human lung carcinoma. Paraffin-embedded sections of human small cell lung carcinoma (SCLC, n = 9) and non–small cell lung carcinoma (NSCLC, n = 10) tumors were obtained from the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, St Paul’s Hospital, University of British Columbia, Vancouver, BC. The Centre maintains a registry of human lung and tumor samples cross-referenced with relevant clinical information (15, 16). The procedures used to obtain the tissue for the registry were approved by the Institutional Review Board of the University of British Columbia. Lung parenchyma adjacent to the tumor was used as a control for each case. Tumor sections were stained with antibodies directed against HLA class I heavy chain (monoclonal antibody HC-10 recognizing an epitope on β2-m free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and -HLA33 heavy chains as well as on all β2-m-free HLA-B heavy chains; ref. 17), β2-m (mouse anti-β2-m monoclonal antibody purchased from Dako, Hamburg, Germany), TAP1 (mouse anti-TAP1 monoclonal antibody 148.3), and tapasin (monoclonal antibody TO-3, generated from a BALB/c mouse immunized with a tapasin-derived peptide and fusion protein; ref. 17). Staining was developed with the standard streptavidin-biotin-peroxidase technique [LSAB 2 system–horseradish peroxidase (HRP), Dako]. The percentage of stained tumor cells was determined in each lesion and classified according to the HLA workshop criteria as (0, negative): <25% of stained tumor cells (1, heterogeneous): >25 to <75% of stained tumor cells; and (2, positive): >75% of stained tumor cells. Negative controls were done by omitting primary antibodies (17).

Mice, cells, and viruses. C57BL/6 (H-2b) mice, 6 to 8 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed and bred at the Biotechnology Breeding Facility, University of British Columbia. HEK 293 cells (American Type Culture Collection, Rockville, MD), CRES cells (18), CMT.64 cells (10), T1 (ATCC, CRL-1991, a TAP1-negative cell line), and T2 cells (ATCC, CRL-1992, a TAP1-negative cell line) were cultured in DMEM supplemented with 10% fetal bovine serum except T2 cells, which received 20% fetal bovine serum, 2 mmol/L L-glutamine, streptomycin (0.1 mg/mL), and penicillin (100 units/mL). All recombinant adenosviruses were propagated and titrated in HEK 293 cells. Splenocytes were cultured in RPMI 1640, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, 50 μmol/L β-mercaptoethanol, 1 mmol/L sodium pyruvate, 0.1 mmol/L essential amino acids, and 10% fetal bovine serum.

Construction of recombinant adenosivirus vectors. Human TAP1 was amplified from pCEP/hTAP1 (provided by Dr. P. Wang, England) with the following primers, 5′-CAT AGC ATG CAT GGC TAG CTC TAG GTG TCC C-3′, which introduced the Sp6I site, and 5′-GCA ATC TAG ATC ATT CTG GAG CAT CTG CAG G-3′, which introduced the XbaI site. The hTAP1 PCR fragment was digested with restriction enzymes Sp6I and Xba1, and ligated with T4 DNA ligase to the Sp6I and Xba1 sites of padlox (18), a plasmid shuttle vector for making AdhTAP1. Padlox/hTAP1 was isolated and sequenced to ensure sequence fidelity. Padlox/hTAP1, linearized with SfiI, was cotransfected along with PSV DNA (PSV is an E1 and E3 deleted version of Ad5 containing loxP sites flanking the packaging site) into CRES cells using a modified LipofectAMINE protocol (InVitrogen Life Technologies, Carlsbad, CA; ref. 18). After development of confluent cytotoxic effect (7 or 8 days), cells were freeze/thawed thrice and the resultant lysate passaged thrice in CRES cells. Plaques were screened by immunofluorescence assay for the presence of hTAP1. AdhTAP1 was plaque-purified and propagated in HEK 293 cells followed by purification and concentration by CsCl centrifugation. Purified virus was diazylated against 10% glycerol in PBS (pH 7.4) and stored at −80°C. AdhTAP1 was confirmed by PCR and DNA sequencing using hTAP1-specific primers and primers specific to adenosivirus sequences flanking either side of the hTAP1 cDNA (data not shown). Infectious virus titer was determined on HEK 293 cells by plaque assay and absorbance at 260 nm wavelength of light was used to determine viral particle number (19). Typical particle/plaque-forming units (PFU) ratios were 100. Virus multiplicity of infection (MOI) was defined as PFU/cell.

hTAP1 expression in CMT.64 cells. To examine the time course of hTAP expression, CMT.64 cells were infected with AdhTAP1 or PSV at 10 MOI and harvested every day for 7 days. To examine hTAP1 response to increasing doses of AdhTAP1, CMT.64 cells were infected with AdhTAP1 or PSV at 50, 10, 2, 0.4, 0.08 MOI and harvested 2 days after infection. Both time course and dose response studies were analyzed by SDS-PAGE followed by Western blot. The samples were reacted with rabbit anti-hTAP1 antibodies (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) and mouse monoclonal anti-human β-actin antibody (Sigma-Aldrich, Oakville, Ontario, Canada). Goat anti-rabbit IgG (H+L)–HRP and goat anti-mouse IgG (H+L)-HRP (Jackson ImmunoResearch Lab, West Grove, PA) were used as secondary antibodies. The bands were visualized by exposure to Hyperfilm (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) using the enhanced chemiluminescence procedure.

Surface expression of MHC class I. CMT.64 cells were infected with AdhTAP1 or PSV at 20 MOI. Two days after infection, the cells were incubated with anti-MHC class I monoclonal antibodies, y3 (H-2k-specific) and 28.14.8S (H-2d-specific) at 4°C for 30 minutes (14). Bound antibodies were detected by goat anti-mouse IgG–FITC (Jackson ImmunoResearch Lab). Fluorescence-actived cell sorting (FACS) analysis was done in a FACScalibur (Becton Dickinson, Franklin Lakes, NJ).

CTL assay. Cytotoxicity assay was measured in a standard 4 hour 51Cr-release assay. In brief, we established a stable cell line, CMT/VSV-Np, which was CMT.64 cells expressing an H-2Kk restricted immunodominant determinant from vesicular stomatitis virus (VSV) nucleoprotein (amino acids 52-59). CMT/VSV-Np cells were infected with AdhTAP1 or PSV at 30 MOI for 1 day followed by 53Cr (Amersham, Arlington Heights, IL) label. VSV-specific CTL effectors were generated by ip. injection of mice with 5 × 106 PFU VSV. Splenocytes were collected 5 days after infection and cultured in RPMI 1640 complete medium plus 1 μmol/L VSV-Np (52-99; Peptide Synthesis Lab., University of British Columbia) for 5 days. The percentage of killed cells was calculated using the formula: % release = 100

3 Unpublished data.

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ELISPOT analysis of tumor-associated antigen–specific INF-γ-secreting splenocytes. CMT.64 cells (6 × 10^3 cells) were incubated with AdhTAP1 or Ψ5 (25 MOI) or PBS at 37°C for 2 hours followed by irradiation (10,000 rad for 30 minutes). Mice were immunized with three separate i.p. injections of 2 × 10^4 treated cells, with each injection separated by a 7-day interval. Nine days after the last immunization, splenocytes were isolated and cultured in vitro in RPMI 1640 complete medium with CMT.64 tumor-associated antigen (TAA) MUT1 or MUT2 (20 μg/mL peptide) for 14 hours. The CMT.64 TAA, MUT1 (FEQNTAQP), and MUT2 (FEQNTAQ; ref. 20), were made by the Peptide Synthesis Lab. The frequency of MUF-specific IFN-γ secreting cells was determined using an ELISPOT assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Cross-presentation of ovalbumin by dendritic cells. Splenic dendritic cells were isolated using CD11c magnetic beads (Miltenyi Biotech, Auburn, CA) and infected with either 20 MOI of AdhTAP1 or Ψ5 for 2 hours, followed by incubation with ovalbumin 5 mg/mL for 16 hours. After incubation, dendritic cells were washed and Fc receptors were blocked (2-4 μg FcIII/II blocker, BD PharMingen, Mississauga, ON, Canada). Cells were stained with monoclonal antibody 25.D1.16, specific for H-2Kb, followed by phycoerythrin-conjugated rat anti-mouse IgG1 (BD PharMingen). Antibody binding was detected with biotinylated anti-hamster IgG cocktail secondary antibodies and streptavidin-HRP and a 3,3'-diaminobenzidine detection system. All the reagents were purchased from BD PharMingen.

Survival studies in mice. For ex vivo studies, CMT.64 cells were incubated with AdhTAP1 or Ψ5 at 50, 10, and 2 MOI for 2 hours followed by washing in PBS. Infected cells (4 × 10^7) were i.p. injected into mice (10 mice/group) and survival was followed for 90 days. Aliquots of ex vivo infected cells were cultured for 1 week to confirm viability. Mice were killed upon signs of morbidity and this time was recorded as death. For in vivo treatments, tumors were established in three groups of 24 to 28 mice per group by i.p. injection using 4 × 10^5 CMT.64 cells in 500 μL PBS/mouse. On days 1, 3, 5, and 8 after the introduction of CMT.64 cells, mice were injected i.p. with AdhTAP1, Ψ5, or PBS using 1 × 10^4 PFU/mouse/injection in 500 μL PBS, and mouse survival was followed for 90 days. During the experiment, four to eight mice were killed from each group at selected times to observe tumor growth pattern and to measure the number of tumor-infiltrating CD4+ and CD8+ T lymphocytes and CD11c+ dendritic cells.

Tumor-infiltrating lymphocytes and dendritic cells. For detection of tumor-infiltrating lymphocytes subsets CD4+ and CD8+ T cells by FACS, tumors were washed and homogenized into single-cell suspensions and incubated with FITC-conjugated rat anti-mouse CD8α (Ly-2) monoclonal antibody and R-phycocerythrin-conjugated rat anti-mouse CD4 (L3T4) monoclonal antibody (BD PharMingen). For detection of CD4+ and CD8+ T cells and CD11c+ dendritic cells using immunohistochemical staining, 8-μm frozen sections were acetone-fixed and incubated with the following antibodies: rat anti-mouse CD4 monoclonal antibody (RM4-5), rat anti-mouse CD8 (53-6.7), and hamster anti-mouse CD11c (HL3). Rat IgG2a and hamster IgG were used as isotype controls. Antibody binding was detected with biotinylated anti-hamster IgG cocktail secondary antibodies and streptavidin-HRP and a 3,3'-diaminobenzidine detection system. All the reagents were purchased from BD PharMingen.

Results

Antigen presentation pathway in patient samples. Almost all tumors were either negative (Fig. 1A) or showed heterogeneous patterns of expression for components of the APP. Only one lesion was homogeneously positive for the TAP1 protein (Fig. IB) and two lesions homogeneously positive expressed tapasin. In contrast, the adjacent lung parenchyma for all cases stained homogeneously positive for components of APP (Fig. IC). Neither of the TAP1- or tapasin-positive lesions showed positive staining for HMC I or P2a. The grading for each case is summarized in Table 1. Both SCLC and NSCLC were severely limited in the...
hTAP1 lasted at least 7 days and the amount of expression resulted in high transgene expression of hTAP1. The expression analysis showed that CMT.64 cells infected with AdhTAP1 infection with CMT.64 cells infected with AdhTAP1 compared with cells infected with INF-γ (positive control).

AdhTAP1 restores the antigenicity of CMT.64 cells. A cytotoxicity assay was used to determine if AdhTAP1 enhanced the capability of CMT.64 cells to present antigens. CMT/VSV-Np cells were used as targets for VSV-specific effectors. CMT/VSV-Np cells infected with AdhTAP1 or cells treated with INF-γ (positive control) were sensitive to the cytolytic activity of the VSV-specific effectors, whereas CMT/VSV-Np cells alone or cells infected with AdhTAP1 increases MHC class I surface expression in CMT.64 cells. The effect of hTAP1 expression on cell surface MHC class I antigen surface expression (11, 12). This shows that APP component expression in CMT.64 cells is inducible by INF-γ, which leads to the restoration of MHC class I antigens on the cell surface (14). Cell-surface expression of H-2Kb and H-2Db antigens was increased in restoration of MHC class I surface expression on CMT.64 cells when compared with INF-γ treatment (positive control).

Table 1. APP status of human small cell lung carcinoma (SCLC) and non–small cell lung carcinoma

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Stage, N, T</th>
<th>TAP1</th>
<th>HLA I</th>
<th>β2m</th>
<th>Tapasin</th>
<th>Total score</th>
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<tr>
<td>SCLC</td>
<td>51</td>
<td>M</td>
<td>3, 2, 2</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>SCLC, poorly differentiated</td>
<td>71</td>
<td>M</td>
<td>1, 0, 2</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>F</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SCLC</td>
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<td>F</td>
<td>1, 0, 1</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SCLC</td>
<td>69</td>
<td>M</td>
<td>1, 0, 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCLC</td>
<td>69</td>
<td>M</td>
<td>1, 1, 1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SCLC</td>
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<td>F</td>
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<tr>
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<td>Squamous cell carcinoma</td>
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<td>0</td>
<td>1</td>
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<tr>
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<td>68</td>
<td>M</td>
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<td>Well differentiated squamous</td>
<td>79</td>
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<tr>
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<tr>
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<td>58</td>
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<td>4</td>
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NOTE: Grade 0, <25% cells positive; grade 1, 25% to 75% positive cells; grade 2, >75% positive cells. The maximum total score for each case is 8; ND, not determined.

expression of antigen presentation components, however, no correlation appeared between clinical staging scores and the scores for APP components.

**Human TAP1 expression in CMT.64 cells.** Western blot analysis showed that CMT.64 cells infected with AdhTAP1 infection resulted in high transgene expression of hTAP1. The expression of hTAP1 lasted at least 7 days and the amount of expression was dose-dependent (Fig. 2A and B).

**AdhTAP1 increases MHC class I surface expression in CMT.64 cells.** The effect of hTAP1 expression on cell surface MHC class I antigen expression in AdhTAP1-infected CMT.64 cells was investigated (Fig. 2C). FACS analysis showed that normal CMT.64 cells are devoid of surface MHC class I antigen. This is consistent with previous studies demonstrating that multiple APP components are down-regulated in CMT.64 cells, thereby resulting in low surface MHC class I antigen surface expression. Treatment of normal CMT.64 cells with INF-γ causes a very large increase in MHC class I antigen surface expression (11, 12). This shows that APP component expression in CMT.64 cells is inducible by IFN-γ, which leads to the restoration of MHC class I antigens on the cell surface (14). Cell-surface expression of H-2Kb and H-2Db antigens was increased in CMT.64 cells infected with AdhTAP1 compared with cells infected with INF-γ, which showed no increase in cell surface expression of MHC class I antigens. Therefore, expression of TAP1 alone resulted in restoration of MHC class I surface expression on CMT.64 cells when compared with INF-γ treatment (positive control).

**AdhTAP1 restores the antigenicity of CMT.64 cells.** A cytotoxicity assay was used to determine if AdhTAP1 enhanced the capability of CMT.64 cells to present antigens. CMT/VSV-Np cells were used as targets for VSV-specific effectors. CMT/VSV-Np cells infected with AdhTAP1 or cells treated with INF-γ (positive control) were sensitive to the cytolytic activity of the VSV-specific effectors, whereas CMT/VSV-Np cells alone or cells infected with AdhTAP1 (Ad vector control) were resistant to killing (Fig. 2D). These results show that hTAP1 expression and activity caused by AdhTAP1 infection can restore sufficient MHC class I restricted antigen presentation of a specific epitope [VSV-Np (52-59)], rendering these cells susceptible to specific cytotoxic activity.

**AdhTAP1 increases dendritic cell cross-presentation.** The cross-presentation of the H-2Kb restricted ovalbumin epitope, SIINFEKL (ovalbumin 257-264), processed from an exogenous source of ovalbumin, was examined in dendritic cells. The fluorescence specific to H-2Kb/SIINFEKL complexes were measured in dendritic cells infected with AdhTAP1 and compared with dendritic cells infected with INF-γ, both in the presence of ovalbumin. After ovalbumin incubation, mean H-2Kb/SIINFEKL-specific fluorescence was 60% greater in AdhTAP1 infected dendritic cells than in dendritic cells infected with INF-γ (P < 0.01; Fig. 3A). In addition to significant increases in MHC class I cross-presentation of exogenous antigens, AdhTAP1 infection also significantly increased the mean fluorescence attributed to total surface H-2Kb by 31% over that seen in INF-γ-infected dendritic cells (P < 0.01; Fig. 3B).

**AdhTAP1 treatment increases tumor-associated antigen-specific INF-γ secreting splenocytes.** MUT1- and MUT2-specific cellular immune responses in mice immunized with irradiated AdhTAP1-infected CMT.64 cells were measured using an IFN-γ ELISPOT assay. MUT1 and MUT2 are shared Kb-restricted TAA in CMT.64 cells and another spontaneous C57BL/6 lung carcinoma (3LL). Vaccination with synthetic MUT1 or MUT2 induces CTLs that efficiently kill CMT.64-derived clones, protects mice from CMT.64 metastasis, and affords therapy of established CMT.64 metastases (20). Mice vaccinated with irradiated, AdhTAP1-infected CMT.64 cells showed a large increase in the number of both MUT1- and MUT2-specific, INF-γ-secreting splenocytes compared with mice vaccinated with either irradiated PBS treated cells
or irradiated \( \Psi 5 \)-infected CMT.64 cells. These results indicate that AdhTAP1 treatment of CMT.64 cells induced a Th1-type tumor-specific immune response (Fig. 3C).

**AdhTAP1 treatment ex vivo and in vivo increases survival of mice bearing CMT.64 tumors.** We examined if AdhTAP1 infection of CMT.64 cells inhibited tumor formation in mice. *Ex vivo* infection of CMT.64 cells had a significant dose-dependent effect on both median survival time and long-term survival (Fig. 4A). All levels of *ex vivo* AdhTAP1 infection significantly increased median survival time compared with PBS treatment alone in a dose-dependent manner \( (P < 0.01) \). All mice survived the challenge with cells infected with the highest dose of AdhTAP1 (50 MOI) over the course of the experiment (100 days). Mice treated with the vector control \( (\Psi 5) \) also showed significant increase in median survival time for infections at the two higher levels (10 and 50 MOI) compared with PBS treatment alone \( (P < 0.01) \). No significant effect was seen at the lowest level of infection (2 MOI) compared with PBS treatment \( (P = 0.59) \). *Ex vivo* infection of CMT.64 cells with AdhTAP1 significantly increased both median survival time and long-term survival compared with \( \Psi 5 \) treatment \( (P < 0.01) \).

In another set of experiments, AdhTAP1 treatment of established CMT.64 tumors significantly \( (P < 0.01) \) increased median survival time to 68 days compared with 22 and 25 days for PBS and \( \Psi 5 \) treated mice, respectively. There was also a significant effect on long-term survival. Mice treated with AdhTAP1 showed 35% long-term survival without visible tumors \( (>100 \text{ days}) \) in contrast with PBS- and \( \Psi 5 \)-treated mice where 100% of mice died within 43 and 48 days, respectively (Fig. 4B). No significant increase in median survival time was observed with \( \Psi 5 \)-treated mice compared with PBS treatment \( (P = 0.29) \). For the *in vivo* experiments, four to eight mice from each group were examined for patterns in tumor growth 20 days after the last treatment injection. The peritoneal cavities of mice treated with AdhTAP1 were tumor-free or had only a few small tumors \( < 1 \text{ or } 2 \text{ mm in diameter} \). Both the liver and intestine seemed normal upon visual inspection. This was in sharp contrast with mice treated with PBS or \( \Psi 5 \). These mice had large volumes of bloody ascitic fluid \( (2-5 \text{ mL}) \) and countless tumors distributed throughout the peritoneal cavity. Tumors were observed growing on the liver and intestine and were associated with large fibrotic adhesions. Tumors harvested from these mice were examined for tumor-infiltrating lymphocytes and dendritic cell infiltrates by FACS and immunohistochemical staining. Immunohistochemical staining showed that mice treated with AdhTAP1 had more CD4\(^+\) and CD8\(^+\) tumor-infiltrating lymphocytes \( (\text{Fig. 5A and B}) \) and more CD11c\(^+\) dendritic cells \( (\text{Fig. 5C}) \) in the tumor mass than in mice treated with \( \Psi 5 \) \( (\text{Fig. 5D-F}) \) or PBS \( (\text{Fig. 5G-I}) \). FACS analysis showed that mice treated with AdhTAP1 had greater tumor-infiltrating lymphocytes \( (\text{CD8}\(^+\) = 12.4\% \text{ of total cells and CD4}\(^+\) = 7.7\% \text{ of total cells}) \) than tumor-infiltrating lymphocytes in tumors from mice treated with \( \Psi 5 \) \( (\text{CD8}\(^+\) tumor-infiltrating lymphocytes = 2.8\% \text{ of total cells and CD4}\(^+\) tumor-infiltrating lymphocytes = 3.4\% \text{ of total cells}) \).

**Discussion**

The immunohistochemical analysis of antigen-processing components in human lung carcinoma lesions indicates that APP defects are highly prevalent in human lung carcinomas. Thus, NSCLC and SCLC represent very poor targets for HLA class I-restricted cytolytic lymphocytes. Similar conclusions have been reported confirming that both lung cancer diseases are potentially poor candidates for immunotherapy by TAA immunization \( (25) \).

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**Figure 2.** *A*, time course of hTAP1 expression. CMT.64 cells after infection with AdhTAP1 (MOI = 10) is visualized with a Western blot specific for hTAP1 protein. T1 cells are positive controls, T2 cells and CMT.64 cells infected with \( \Psi 5 \) are adenovirus vector controls for hTAP1 expression. \( \beta \)-Actin was used as a control for protein loading. *B*, expression of hTAP1 is dose-dependent. CMT.64 cells were infected at MOI of 50, 10, 2, 0.4, and 0.08 with AdhTAP1 or \( \Psi 5 \) and harvested 48 hours later. *C*, AdhTAP1 infection increases H-2K\(^b\) and H-2D\(^b\) surface expression in CMT.64 cells. \( \Psi 5 \), adenovirus vector control; IFN-\( \gamma \), positive control. *D*, infection of CMT/VS-Np cells with AdhTAP1 restores MHC class I antigen presentation of VS-Np epitope and increases susceptibility to lysis by VS-Np specific effector cells. Targets: CMT/VS-Np [CMT.64 transfected with VS-Np (52-59) minigene] treated with PBS (mock treatment control), IFN-\( \gamma \) (positive control), \( \Psi 5 \) (adenovirus vector control), or AdhTAP1. Effectors: splenocytes from VS-Np-infected mice.
The lack of correlation between clinical staging and APP status suggests that antigen loss variants have been established long before diagnosis and treatment of lung tumors. The results are in contrast with melanoma where significant correlations were found between APP status and disease progression (7). This may be due to earlier detection and diagnosis of primary melanomas, before antigen loss variants predominate. It is noteworthy that all patients analyzed in this study were heavy smokers (data not shown) and strengthens a recent report demonstrating that tobacco extracts inhibit TAP1 protein expression in a dose-dependent manner thereby leading to reduced HLA expression in epithelial cells (26).

In this study, the partial restoration of the APP was attempted by gene transfer in order to increase the immunogenicity of lung cancer cells. Our results show that replication-incompetent adenovirus vectors could infect murine lung carcinoma cells (CMT.64) resulting in high expression of hTAP that persists for more than 7 days. The level of expression is dependent on the dose of AdhTAP1 used to infect the cells. The persistent expression of TAP1 indicates that this high expression in itself is not toxic to the cells. The expression of the TAP1 subunit alone is capable of restoring MHC class I surface expression on the cell surface despite down-regulation of many of the components of the APP, indicating that TAP1 alone is capable of peptide transport activity (12). MHC class I appears on the cell surface after infection with AdhTAP1, but not after infection with $\Psi_5$, indicating that this process is specific to TAP1 expression rather than an effect caused by the vector. This suggests that low but sufficient amounts of $\beta_2$-m and MHC class I heavy chain subunits are present in the endoplasmic reticulum and that these subunits assembled into MHC class I molecules when stabilized and supplied with peptides through the presence and activity of TAP1.

The level of surface expression of MHC class I, as a consequence of TAP1 expression, is capable of presenting sufficient immunogenic peptides to make the cells susceptible to antigen-specific response. This was demonstrated by the increased expression of MHC class I on the cell surface after AdhTAP1 infection compared to control groups (26).

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CTL killing in vitro. Mice challenged with CMT.64 cells infected ex vivo with AdhTAP1 were able to survive the challenge provided that enough cells were infected. It is clear from the ex vivo animal studies that the rate of survival is dependent on viral dose. There is a major dose-dependent effect associated with the presence of TAP1 and a minor effect associated with the adenovirus vector itself. This suggests that a threshold number of cells have to express TAP1 to overcome the lack of immunologic recognition and that the viral vector alone may also stimulate the immune system to generate antitumor responses. Because CMT.64 is not known to express the costimulatory molecules required for the stimulation of a Th1-type antitumor response they would have to be processed by dendritic cells. The dendritic cells would have to acquire, process, and cross-present the tumor antigens in order to cross-prime a cellular antitumor response. The expression of hTAP1 in CMT.64 cells facilitates cross-priming, shown by the hTAPI-dependent increase in MUT1- and MUT2-specific INF-γ-secreting splenocytes. The observed increase in survival of mice receiving CMT.64 cells infected with the highest dose of Ψ5 might be due to stimulation of innate responses mediated by viral gene transcription byproducts (dsRNA) interacting with TLR 3 (27, 28). The virus vector seems to act as an adjuvant by providing a potent “danger signal” to dendritic cells and may contribute to the priming of TAPI-dependent antitumor immune responses.

The administration of AdhTAP1 to mice already bearing disseminated i.p. tumors also increases survival significantly. It is interesting and encouraging that at the doses applied in these experiments, it is remote that sizable numbers of CMT.64 cells were infected in vivo by AdhTAP1. It is clear, however, that the administration of AdhTAP1 stimulated an effective immune response against CMT.64 cells. This provides reinforcement that the response is due not only to increased MHC class I expression on some tumor cells but also due to some other contributing process. Presumably, this other process is at the level of cross-presentation of acquired tumor antigen to MHC class I restricted components by dendritic cells. In vitro, there were significant increases in the cross-presentation of exogenous ovalbumin antigens by dendritic cells infected with AdhTAP1. In vivo, the biological relevance of these increases observed in vitro are corroborated by the increase in IFN-γ-secreting TAA-specific splenocytes in mice vaccinated with irradiated AdhTAP1-infected CMT.64 cells but not Ψ5-infected cells. Further corroboration is provided by the observed increase in CD4+ and CD8+ T cells and CD11c+ dendritic cells within tumors from AdhTAP1-treated mice.

Figure 5. Immunohistochemical staining for tumor-infiltrating CD4+ (A, D, and G) and CD8+ (B, E, and H) lymphocytes and CD11c+ dendritic cells (C, F, and I) in tumors treated with AdhTAP1 or Ψ5 (Ad vector control) or PBS (mock treatment control; 200× magnification).
It is intellectually satisfying that the administration of TAP immunotherapy induces the production of tumor-specific CTLs; however, it should be stressed that in addition to responses against the MUT antigens, there must also be other CTL responses to tumor-specific and tumor-associated antigens that have not yet been identified. TAPI activity in this class of tumors would make a wide variety of antigens available for loading onto nascent MHC class I molecules. The immune response against these antigens must be sensitive to very low levels of MHC class I expression as it is unlikely that a large portion of the introduced CMT.64 cells are infected in vivo by AdhTAPI. In accord is the work by Purbhoou et al. (29) demonstrating that during the effector stage, CTLs were able to detect even one to three peptide-MHC complexes but required about 10 peptide-MHC complexes to achieve stable synapse formation and complete signaling to activate CTL precursors.

This study extends previous reports that have shown that the introduction of TAPI into tumor-bearing mice increases survival. The previous study accomplished this result with replication competent vaccinia vectors (14). In this study, we show that a replication-incompetent adenovirus vector can also be used to establish a specific antitumor immune response against TAP-deficient CMT.64 cells. This is an important step in establishing the potential for TAPI gene transfer as an immunotherapy for the treatment of human cancer because these vectors are well tolerated, have a safe clinical history, and the cyclic guanosine 3′,5′-monophosphate production of these vectors is established. Overall, these studies are encouraging for the clinical implementation of recombinant adeno-virus vectors encoding TAPI and need to be extended to other types of cancer. The results further showed that TAP should be considered for inclusion in cancer therapies, as it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic complement of the tumor or the MHC haplotypes of the host.

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References

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