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Analysis of the Major Histocompatibility Complex Class I Antigen Presentation Machinery in Human Lung Cancer

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Abstract Tumor cells may alter the expression of proteins involved in antigen processing and presentation, allowing them to avoid recognition and elimination by cytotoxic T cells. In order to investigate whether the major histocompatibility complex (MHC) class I-mediated antigen processing machinery is preserved in human lung cancer cell lines, we examined the expression of multiple components of the MHC class I antigen processing pathway, including transporter associated with antigen processing (TAP), β_2-microglobulin, MHC class I molecules, and chaperones which have not been previously examined in this context. Flow cytometry analysis showed that the cell surface expression of MHC class I molecules was downregulated in all of the cell lines. While some cell lines showed no detectable expression of MHC class I molecules, pulse-chase experiments showed that MHC class I molecules were synthesized in the other cell lines but not transported from the endoplasmic reticulum to the cell surface. Low or nondetectable levels of TAP1 and/or TAP2 expression were demonstrated by Western blot analysis in all of the cell lines, representing a variety of lung tissue types. In some cases, this was accompanied by loss of tapasin expression. Our findings suggest that downregulation of antigen processing may be one of the strategies used by tumors to escape immune surveillance. This study provides further information for designing the potential therapeutic applications such as immunotherapy and gene therapy against cancers.

Key words: Antigen presentation, chaperone, cancer, immune escape, MHC

The immune system can recognize tumor-specific antigens, and CTLs specific for tumor antigens have been detected in animals and patients with tumors [4]. However, the development of tumors in immunocompetent hosts indicates that the immune system is unable to recognize or eliminate many tumors, implying that tumors can evade the immune system. The mechanism by which tumors escape from immune surveillance is only partly understood. One of the possibilities is that tumor cells may alter expression of components of the major histocompatibility complex (MHC) class I antigen processing pathway and potentially interfere with the processing and presentation of tumor antigens.

MHC class I molecules preferably present endogenous proteins to CD8+ cytotoxic T lymphocytes (CTLs) as small antigenic peptides [27]. These peptides are generated by the proteasome-mediated protein degradation in the cytosol [9]. Subsequently, peptides are translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) [13, 24, 28]. TAP is composed of two proteins of the ATP-binding cassette family, which are encoded by two genes (TAP1 and TAP2) located in the class II region of the MHC [11, 16]. Once peptides have gained access to the ER, they are loaded onto the empty MHC class I complex, which are then transported through the secretory pathway to the cell surface.

Assembly of MHC class I molecules is initiated in the ER, where nascent, unfolded MHC class I molecules associate with the ER-resident chaperone calnexin [14]. Calnexin is dissociated upon binding of class I to β_2-microglobulin (β_2-m) [20] and the class I-β_2-m heterodimer then associates with TAP and forms part of a large ER complex, which includes the soluble ER-resident chaperone calreticulin, and the recently identified chaperone tapasin [20, 23]. In mutant cell lines which lack the expression of any of these chaperones, MHC class I assembly and subsequent cell surface expression are impaired [15, 20, 25].

Loss or reduced expression of MHC class I molecules has been observed frequently in malignant cells of different origin [5] and allows such cells to escape the CTL-mediated recognition and elimination. Several mechanisms are responsible for deficiencies in the MHC class I antigen expression, including structural alterations and transcriptional

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and/or post-transcriptional suppression of the heavy or light chain molecules [12, 26]. Recent data suggest that suppression of TAP expression is also associated with a loss of MHC class I surface expression in cervical and prostate carcinomas [6, 22], as well as in adenovirus type 12-transformed embryonal fibroblasts [18]. Thus, functional loss of the antigen-processing machinery can result in a reduced or defective MHC class I surface expression in tumor and in virus-transformed cells. No information is available regarding the expression of chaperones in tumor cells.

The present study was undertaken to examine the expression of components comprising MHC class I antigen processing machinery in a series of human lung cancer cell lines. The expected findings should help us to understand the mechanisms related to the defective presentation of the MHC class I molecules by tumor cells, which may have important consequences for future therapeutic processes such as gene therapy.

MATERIALS AND METHODS

Cell Lines and Cell Culture

A variety of human lung cancer cell (LCC) lines which are representatives of different lung tissues were obtained from the American Type Culture Collection (ATCC). L-132 cells, derived from the fetal lung epithelial tissues, were cultured in DMEM medium (GIBCO, Grand Island, U.S.A.) supplemented with 10% FCS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml), and were used as a positive control for these studies. With the exception of A549 (lung carcinoma), NCI-H358 (bronchioalveolar carcinoma), NCI-H522 (lung adenocarcinoma), NCI-H889 (small cell lung carcinoma), NCI-H1299 (large cell lung carcinoma) were grown in RPMI1640 medium (GIBCO) containing 10% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml). A549 cells were maintained in Hams F12 medium (GIBCO) with 10% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml). All cell lines were grown at 37°C in a 5% CO_2 atmosphere.

Antibodies and Flow Cytometric Analysis

Antibodies used were the following: W6/32 (ATCC) recognizes MHC class I/β_2-m heterodimers and BBM.1 (ATCC) reacts with β_2-m. K455 recognizes class I heavy chain and β_2m in both assembled and nonassembled forms [2]. Rabbit anti-calnexin antibody and rabbit anti-calreticulin antibody were purchased from Affinity Bioreagents Inc. (Golden, U.S.A.) A rabbit anti-peptide antibody to tapasin was a generous gift from Dr. P. Cresswell (Yale University, New Haven). Rabbit anti-TAP1 antibody and rabbit anti-TAP2 antibody have been described previously [1]. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and anti-β-actin antibody were purchased from Sigma.

The membrane expression of MHC class I glycoproteins was determined by flow cytometry (FACScalibur; Becton Dickinson, Mountain View, U.S.A.) after indirect immunofluorescence staining. Cells (1×10^6) were washed with PBS-BSA (PBS containing 0.1% BSA and 0.1% NaN_3), resuspended in 0.1 ml of PBS-BSA, and incubated for 30 min with W6/32 monoclonal antibody. Cells were washed in PBS-BSA and incubated for 30 min in 1:150 dilution of FITC-conjugated goat anti-mouse IgG (Sigma St. Louis, U.S.A.). The cells were washed in PBS and resuspended at $2-5\times10^6$ cells/ml in PBS containing 0.1% NaN_3. Control was stained with a nonrelevant primary antibody and a secondary antibody. A total of 10,000 gated events were collected on a FACScalibur and analyzed by using the CellQuest software (Becton Dickinson, Mountain View, U.S.A.).

Immunoblot Analysis

After being separated by SDS-PAGE, proteins were transferred to nitrocellulose membranes by a semi-dry electrophoretic transfer cell (Bio-Rad, Richmond, U.S.A.). To normalize for cell equivalents and gel loading, β-actin was quantitated on the same gel. The membranes were blocked by incubation in PBS containing 5% (w/v) non-fat dry milk and 0.2% (v/v) Tween-20 overnight at 4°C, and probed with the primary antibody for 1 h, washed and incubated in a 1:2000 dilution of rabbit anti-mouse horseradish peroxidase or goat anti-rabbit horseradish peroxidase secondary reagent (Jackson Immunoresearch Lab, Grove, U.S.A.). Immunoreactive proteins were visualized using the ECL Western Blotting detection kit (Amersham Bucking hamshire, U.K.).

Metabolic Labeling and Immunoprecipitation

Cells were methionine-starved for 30 min in methionine-free medium prior to pulse labeling with 0.1 mCi/ml of [^{35}S]methionine for 15 min (Amersham). The label was chased for various times with DMEM containing 10% FCS. After one wash with ice-cold PBS, the cells were extracted for 30 min at 4°C in 1% Nonidet P-40 (Sigma) in PBS containing 0.2 mM phenylmethyl sulforyl fluoride (Sigma) and 10 mM iodoacetamide (Sigma). After pelleting the debris, lysates were precleared overnight at 4°C with normal mouse serum (Sigma) and protein G-Sepharose (Pharmacia, Uppsala, Sweden). Aliquots were then incubated with the appropriate antibodies for 2 h, followed by 30 min with protein G-Sepharose. The immunoprecipitates were washed four times with 0.1% NP-40 in PBS before being separated by SDS-PAGE, dried, and processed for autoradiography. For endoglycosidase H (Endo H) treatment, immunoprecipitates were digested with 3 milliunits of Endo H (Boehringer Mannheim, Mannheim, Germany) for 16 h at 37°C in the Endo H buffer (50 mM NaOAc, pH 5.6/0.3% SDS/150 mM 2-mercaptoethanol) before analysis by SDS-PAGE.

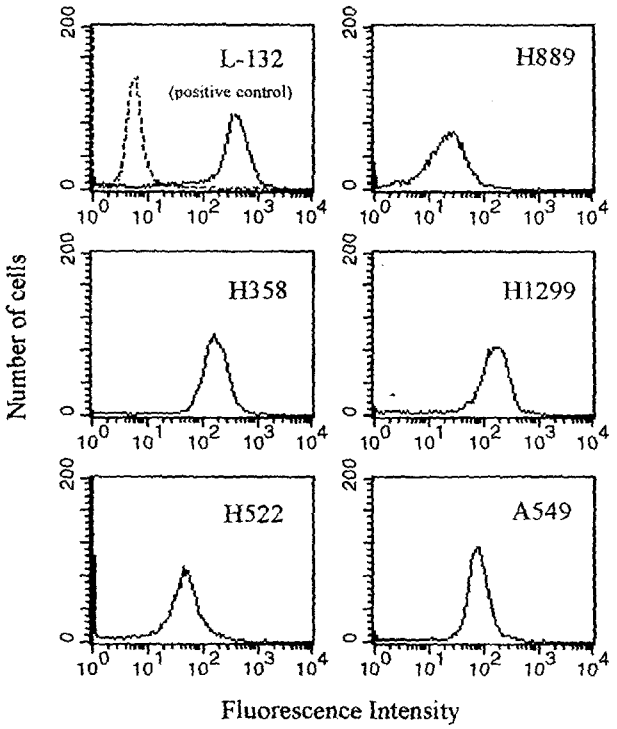

Fig. 1. Downregulation of cell-surface expression of MHC class I molecules in human lung cancer cells.
FACS analysis was done to measure the cell-surface expression of MHC class I molecules in five cultured human lung cancer cell lines (H358, H522, H889, H1299, and A549) and one immortalized cell line derived from normal lung epithelium (L-132). Solid lines indicate staining with human MHC class I-specific antibody (W6/32). The control staining, which was similar for all cell lines, is representatively shown for L-132 cells with the dotted line.

RESULTS

MHC Class I Surface Expression Is Downregulated in Human LCC Lines

MHC class I surface expression was determined by flow cytometry using a monoclonal antibody (W6/32), which recognizes the MHC class I heavy chains associated with β_2-m. Representative histograms from this analysis are shown in Fig. 1. As a positive control, we included L-132 cell lines, which are derived normal fetal lung tissues. L-132 cells synthesized high amounts of MHC class I molecules reactive with W6/32. In contrast, MHC class I surface expression was reduced for all of the cancer cell lines examined. The reduction was particularly significant for H522 and H889 almost by an order in terms of mean fluorescence intensity.

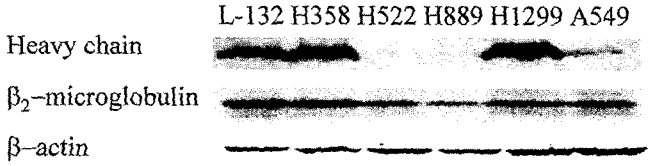

Fig. 2. Western blot analysis of MHC class I heavy chain and β_2-m.
Cell extracts from various lung cancer cell lines were resolved on SDS-PAGE and blotted with K455 or BBM.1 for detection of heavy chain and β_2-m, respectively. To normalize for cell equivalents and gel loading, β-actin was quantitated on the same gel.

Downregulation of MHC Class I at the Cell Surface Occurs at a Post-Translational Level in Some LCC Lines

It was unexpected that reduced expression of MHC class I molecules at the cell surface was observed in all of the LCC lines. This may be due to transcriptional and/or post-translational suppression of the heavy or light chain molecules. In order to discern these possibilities, we examined the expression of these molecules by Western blot analysis. β-actin was included as an internal control to ensure that the equal amount of proteins from each cell lysate was loaded on the gel (Fig. 2, third panel). There were no significant differences in the expression level of β_2-m among LCC lines relative to normal L-132 (Fig. 2, second panel). H522 and H889 showed no detectable expression of MHC class I molecules (Fig. 2, first panel), whereas A549 showed greatly decreased MHC class I expression compared to that of L-132. Non- or reduced expression of MHC class I molecules is likely due to the low transcriptional activity, although we currently have no evidence to support it.

Interestingly, H358 and H1299 gave a MHC class I signal similar to that of L-132 (Fig. 2, first panel). In

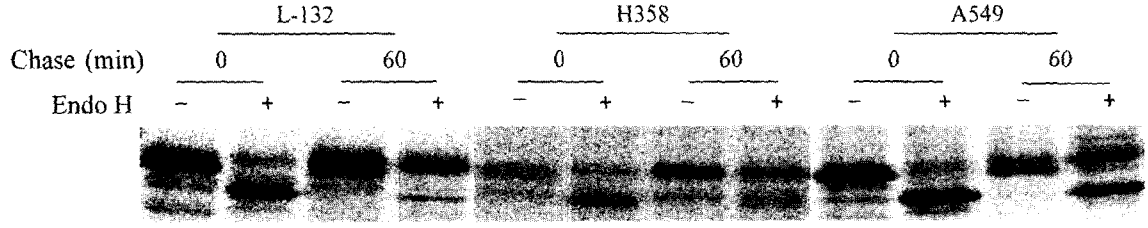

Fig. 3. Assembly and transport of MHC class I molecules in the human lung cancer cell lines.
Detergent extracts from [^{35}S]methionine-labeled cells were immunoprecipitated with W6/32 antibody, and left untreated or digested with Endo H, as indicated, before analysis by SDS-PAGE. Cells were chased for 0 or 60 min after pulse labeling to monitor the intracellular transport of MHC class I molecules. The picture is from three different gels.

conjunction with the data shown in Fig. 1, this indicates that the intracellular transport of MHC class I molecules is inhibited in these cell lines. To further characterize by which specific mechanisms and where MHC class I molecules are retained inside cells, we performed a pulse-chase experiment. MHC class I molecules were immunoprecipitated from detergent extracts from cells pulse-radiolabled with [^{35}S]methionine for 15 min, and chased for up to 60 min. One half of each immunoprecipitate was then digested with Endo H, which cleaves N-linked oligosaccharides in high mannose forms that exist before the modifications associated with transport of the glycoproteins through the *medial*-Golgi complex. As seen in Fig. 3, most of MHC class I molecules in L-132 became resistant to digestion with Endo H after a 60 min chase period. In contrast, about a half-portion of MHC class I molecules remained sensitive to Endo H digestion in H358 and A549, indicating that the MHC class I molecules did not transport past the *medial*-Golgi complex. Some fraction of the molecules, however, became resistant to Endo H treatment and it seems that they reached the late Golgi compartment and retained there since the FACS staining shown in Fig. 1 showed only a very little cell-surface expression of MHC class I molecules.

TAP1 and/or TAP2 Expression Is Decreased in Human LCC Lines

The peptides bound by the MHC class I heavy chains are supplied to the lumen of ER by TAP from the cytosol. Cells deficient in either TAP1 or TAP2 are markedly deficient in MHC class I surface expression and antigen processing [30]. Therefore, we also examined the expression of TAP in LCC lines. H358 and H522 showed no detectable TAP1 expression, and TAP1 signal was substantially decreased in the other LCC lines (Fig. 4, first panel). For TAP2, all except H889 lacked a detectable signal (Fig. 4, second panel). These results show that at least either TAP1 or TAP2 is missing in each LCC line

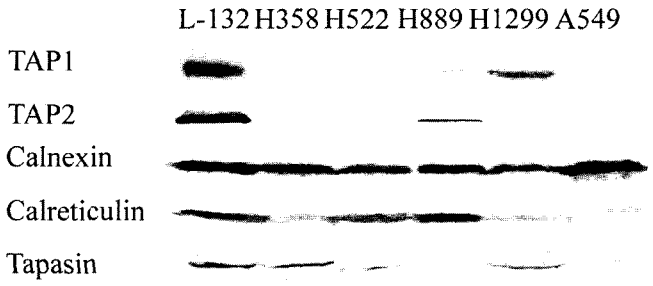

Fig. 4. Expression of MHC class I antigen processing components in the human LCC lines.
The cell extracts from human LCC lines were separated by SDS-PAGE, transferred to nitrocellulose membrane, probed for TAP1, TAP2, and chaperones with the appropriate antibodies, and detected by enhanced chemiluminescence.

analyzed. Defects in TAP raised the possibility of impaired transport of endogenous peptides as a primary reason responsible for downregulation of MHC class I molecules at the cell surface.

Some LCC Lines Are Deficient in the Expression of Chaperone Molecules

To reach the cell surface, MHC class I molecules must be properly assembled within the ER. In this organelle, there exists a network of molecular chaperones evolved to assist membrane and secretory proteins in the folding process [10]. It has been known that calnexin, calreticulin, and tapasin are involved in the process of MHC class I assembly. It prompted us to analyze whether chaperones are properly expressed in LCC lines. All of the LCC lines were positive for calnexin and calreticulin (Fig. 4, third and fourth panels, respectively). In contrast, tapasin, the chaperone mediating efficient peptide loading, was not detected in H889 and A549 and was normal in the other cell-lines (Fig. 4, last panel).

DISCUSSION

This study examined the MHC class I antigen processing machinery in human LCC lines. Deficiencies in translational levels were noted for at least one of MHC class I, TAP1, TAP2, and tapasin in each LCC line, and combined deficiencies of multiple components was common. Defects in TAP1, TAP2, and tapasin as well as MHC class I would result in the reduction of cell surface MHC class I expression. In contrast to the other components, β_2-m, calnexin, and calreticulin were expressed in all of the LCC lines, although expression levels appeared decreased in some cell lines.

The mechanisms by which deficiency occurs could be a genetic mutation or deletion in genes. A more detailed analysis at the DNA level is required to address this. Although a deletion in the MHC could explain the combined deficiencies of TAP1 and TAP2, as well as MHC class I, this mechanism cannot account for deficiency of tapasin which is encoded at a separate locus on a different chromosome. A more plausible mechanism is that the genes involved in antigen processing and presentation share common regulatory elements, which is supported by the fact that MHC class I, TAP1, and TAP2 genes are tightly clustered in the class II region of the MHC on human chromosome 6. When these regulatory elements are elucidated, new therapies developed for specific upregulation of antigen processing of these gene products may prove useful in tissue transplantation or in autoimmune diseases. Recently, it was shown that the proto-oncogene product PML induces expression of TAP1, TAP2, and proteasomal subunits, LMP2, LMP7 in an MHC class I negative, recurrent mouse tumor, leading to

the re-expression of cell-surface MHC in tumors and to rejection of the tumors [31]. It would be of interest to see whether the expression of the human homolog of PML can also restore the MHC class I antigen presentation machinery in human LCC lines.

Antitumor immune response may include multiple mechanisms of both cellular and humoral immunity and involve both innate and acquired immunity. The gene expression defects observed in these studies are predicted to disrupt MHC class I antigen presentation, which may help tumor cells to evade killing by CTLs. This could also be achieved by simply decreasing MHC class I expression, as H522 and H889 were such cases in our study [3, 7, 19]. However, decreased MHC class I expression may actually make tumor cells more susceptible to killing by NK cells [8, 21]. Thus, CTL and NK cytotoxicity may impose at least partially conflicting selection pressures on tumor cells, and the balance of these selection pressures may vary between different tumors. Alteration of antigen processing function may allow for a balance between sustained MHC class I expression to prevent NK susceptibility, while providing a blockade of tumor antigen processing and presentation to prevent CTL susceptibility.

The present studies do not address the question of whether defects in antigen processing machinery are actually associated with transformation or outgrowth of the tumor, or whether this poor processing is representative of the natural regulation of class I expression in the tissue of origin. However, those are plausible, noting the findings that a significant proportion of tumor tissues (particularly those that are more malignant) show reduced expression of cell-surface MHC class I owing to multiple antigen-presentation defects [17, 29]. Whatever the case may be, it is expected that the LCC lines analyzed in this study are incompetent to present antigen. The therapeutic implications of these findings are that poor class I expression and poor antigen processing capability must be overcome if LCC and other possible tumor histologies sharing these characteristics are to be made susceptible to CTL-based immunotherapy. Experiments are in progress to test whether genetically manipulated LCC lines are more capable to functionally present endogenous peptides to tumor-reactive CTLs. Further understanding of the mechanisms whereby tumor cells may evade immune responses is essential to proper design of an effective strategy for immunotherapy for cancer.

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