Altered HLA Class I Expression in Non-Small Cell Lung Cancer Is Independent of c-myc Activation

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ABSTRACT

We studied the expression of major histocompatibility complex class I antigens in 59 bronchogenic carcinomas, as well as in pneumocytes and epithelial respiratory cells distant from the tumor. We observed in all cases that normal lung tissue expressed major histocompatibility complex class I antigens, while this expression was completely lost in 16 tumors (27%). The defect in HLA gene expression affected both heavy chain and β2-microglobulin, as demonstrated by the null reactivity with the monoclonal antibodies GRH1, W6/32, and HC10. Selective underexpression was detected in 1 tumor for HLA-A locus antigens and in 3 tumors for HLA-B locus antigens.

Southern blot analyses of major histocompatibility complex class I genes were performed in 20 tumor tissue specimens and 6 cell lines. No class I gene rearrangements were detected using HLA coding and locus specific noncoding probes.

We also used the Southern blot method to investigate the possible relationship between c-myc amplification and HLA class I antigens in non-small cell lung cancers and detected no apparent amplification in 20 tumor tissue specimens (5 negative for HLA class I antigens) and 6 cell lines (3 with decreased expression).

Northern blot analysis revealed no relationship between c-myc mRNA levels and specific mRNA for HLA-A and HLA-B antigens in cell lines with imbalanced HLA-A or HLA-B expression.

INTRODUCTION

Carcinoma of the lung is the most common cancer in males. The subdivision of bronchogenic carcinoma into different clinicopathological entities such as small cell carcinoma of aggressive behavior and non-small cell carcinoma of slower evolution is well established and of practical use.

The evidence available to date suggests that all the major subtypes of bronchogenic carcinoma arise in the basal cells of the bronchial epithelium, which is of endodermal origin. During the early phases of neoplastic development the malignant cells tend to differentiate as squamous cells, glandular cells, large undifferentiated epithelial cells, or small cells (1, 2).

The widely distributed HLA class I antigens (3, 4) are involved in the process of recognition of tumor antigens by cytotoxic T-lymphocytes (5). It has been postulated that both tumor growth and invasiveness are modified not only by the absence of expression (6–8) but also by imbalance in the expression of the different class I molecules (9) and even by increased expression (10), this latter case reflecting a decrease in sensitivity to natural killer cells.

The loss of HLA class I expression is a relatively frequent event in human neoplasias and has been correlated with the degree of differentiation in laryngeal carcinomas (11), histological type in carcinoma of the lung (12), degree of tumor progression in melanoma (13, 14), and also with the malignancy of B-cell lymphomas (15).

In this paper we analyze the expression of class I antigens in 59 bronchogenic carcinomas and 6 NSCLC cell lines. Our results lead to the following conclusions: (a) lung cancer exhibits major alterations in HLA antigens compared with other tumors (11, 16); (b) alterations in HLA gene expression are found in both heavy chain and β2-microglobulin; (c) mRNA levels of MHC class I seem to be independent of the existence of c-myc amplification or high levels of c-myc mRNA.

MATERIALS AND METHODS

Tissue Specimens. Tissue fragments were obtained from 59 patients at the time of tumor resection. The patients had not received radiotherapy or chemotherapy prior to surgery. We studied 40 epidermoid, 15 adenocarcinomas, and 4 oat cell carcinomas.

Fresh tumor tissue and normal tissue samples were taken from surgical specimens immediately after removal. The samples were immersed in cooled isopentane and snap-frozen in liquid nitrogen.

Paraffin sections of all tumors were routinely stained with hematoxylin and eosin and were independently classified according to the WHO system (17).

Monoclonal Antibodies. The following mAbs were used: W6/32 against a common HLA class I determinants (18) kindly provided by Dr. W. Bodmer; GRH1 against β2-microglobulin (19); HC10 anti-HLA heavy chain (20); α-HLA-A and JOAN 1 (anti-HLA-A and anti-HLA-B, respectively) and α-HLA-B (anti-HLA-B molecules) (21); and GRT2, a mAb which reacts with the common leukocyte antigen (CD45), to measure tumor leukocyte infiltration (22).

Immunohistochemical Staining. Binding of MAbs to frozen sections was assayed by the alkaline immunoperoxidase and immunoperoxidase techniques according to presumably published methods (23, 24). Briefly, the sections were incubated first with the specific antibody, then with rabbit anti-mouse immunoglobulin (DAKO, Copenhagen, Denmark) at 1/20, and finally with the alkaline phosphatase-monoclonal mouse antibody anti-alkaline phosphatase complex (DAKO) at 1/50. The binding sites were revealed with Fast Red TR salt and levamisole was added to inactivate the endogenous alkaline phosphatase. A final washing with water was followed by counterstaining with hematoxylin.

The immunoperoxidase technique was similar to the previous assay except for the third antibody (swine anti-rabbit immunoglobulin), which was conjugated with peroxidase, and for the use of chromogen diaminobenzidine plus H2O2.

Interpretation of Immunohistochemical Results. Sections were examined with an Olympus BH2 microscope and photographed at low magnification. The distribution of HLA expression was examined by two observers. A minimum of 10 microscopic fields were counted independently. Areas of obvious tissue necrosis were avoided for counting purposes. The results were scored as negative HLA expression when <5% of the cells were positively stained.

Immunofluorescent Staining and Flow Cytometry. Immunofluorescence studies were performed in NSCLC lines according to previously published methods (25). Analysis of fluorescence was performed on a FACScan (Becton Dickinson, Mountain View, CA). Data analyses were performed with LYSIS software.
HLA CLASS I EXPRESSION AND c-myc ACTIVATION IN NSCLC

based on readings of 10,000 cells/sample. The background number of fluorescent cells (no relevant MAbs) was generally adjusted to 5–10% by using the photomultiplier gain setting. The relative increase in percentage mean fluorescence (MF) was calculated with the formula

$$\text{MF}_{\text{IFN}} - \text{MF}_{\text{control}} \times 100$$

Cell Lines and Interferon Induction of HLA Class I Antigens. The human NSCLC lines were grown in our laboratory in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO₂ atmosphere. The cell lines used in this study included A549 (adenocarcinoma), A427 (epidermoid), CALU1 (epidermoid), CALU6 (large cell carcinoma), SK-LU-1 (adenocarcinoma), and SK-MES-1 (adenocarcinoma). All cell lines were obtained from the American Type Tissue Collection.

All cell lines were incubated during 3 days with human recombinant γ-interferon, supplied by Dr. Adolf of the Boehringer Institute, at a titer of $4.6 \times 10^7$ units/ml and a specific activity of $2 \times 10^7$ reference units/mg of protein. Interferon induction of class I antigens was determined by FACScan.

DNA Isolation and Southern Blot Analysis. High-molecular-weight DNA was isolated from tumors, normal lung tissues, and cell lines (26). Aliquots (10 μg) of DNA were digested with 50 units of EcoRI for 4 h according to the supplier’s specifications (Amersham). DNA fragments were separated by electrophoresis in 0.8% agarose gels for 16 h and transferred to a nylon filter (Zeta-Probe membranes; Bio-Rad, Richmond, CA). We used two HLA-A and -B locus-specific probes (pHLA 2a.1, a 400-base pair probe, and pHLA-1.1, a 358-base pair probe) (27); and the 1.3-kilobase fragment Clal-EcoRI, cloned in the plasmid

Table 1 Expression of HLA class I antigens in normal tissue and bronchogenic carcinomas

<table>
<thead>
<tr>
<th>Positive cases/no. of cases tested</th>
<th>W6/32</th>
<th>GRH1</th>
<th>HC10</th>
<th>HLA-A</th>
<th>HLA-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>43/59</td>
<td>43/59</td>
<td>43/59</td>
<td>42/59</td>
<td>40/59</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>59/59</td>
<td>59/59</td>
<td>59/59</td>
<td>59/59</td>
<td>59/59</td>
</tr>
</tbody>
</table>

Fig. 1. Photomicrographs showing a poorly differentiated epidermoid carcinoma. (A) Immunoperoxidase alkaline technique showing no reaction in tumor cells with GRH1 (anti-β2-microglobulin). (B) Immunoperoxidase technique showing negative pattern in tumor cells with HC10 (anti-heavy chain).
vector pBR322 (28), was used as a probe for c-myc protooncogene.

For hybridization the membranes were treated with random priming
32P-labeled probes (29) in a hybridization solution composed of 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate, and 1 M sodium chloride, during 48 h at 42°C. Membranes were washed first in 2 changes of 5 min with 2 × SSC at room temperature, then for 30 min at 65°C in 2 changes of 2 × SSC/1% sodium dodecyl sulfate, and finally for 30 min at room temperature in 0.5 × SSC followed by a final wash in 0.5 × SSC for 1 min. The membranes were then dried and autoradiographed at −70°C until the signals became visible.

Northern Blot Analysis. Total cellular RNA from cell lines was
prepared by the guanidine isothiocyanate method (26). RNA (20 µg total) was electrophoresed in 1% agarose gel containing formaldehyde and transferred to nylon membranes (Gene Screen Plus). Hybridization and washing were performed as for Southern blotting, except that the second washing was at 60°C. The probes were the same as for Southern blotting, plus β-actin as a DRβ complementary DNA (30) and a β-actin probe (31).

RESULTS

Expression of HLA Class I Antigens on Pneumocytes and
Epithelial Respiratory Cells

We studied the expression of these antigens on pneumocytes and epithelial respiratory cells from the same lung as the origin of the original tumor but distant from the tumor and considered normal by the anatomopathologist. We observed the expression of HLA-ABC antigens in all cases.

Expression of HLA Class I Antigens in Brongogenic
Carcinomas

Tissue Specimens. We studied 59 tumors and observed a total loss of expression of HLA class I antigens in 16 (27%), selective loss of A locus in 1 case, and B locus loss in 3 cases. These class I alterations were due to simultaneous loss of heavy chain and β2-microglobulin, as confirmed by the null reactivity of monoclonal antibodies GRH1, W6/32, and HC10 (Fig. 1; Table 1).

HLA class I expression was absent in 2 of 4 oat cell carcinomas (50%), 10 of 40 epidemoid carcinomas (25%), and 4 of 15 adenocarcinomas (26.6%).

No significant differences were found in the degree of differentiation, although only 1 case of 9 well-differentiated tumors was negative for class I expression.

Cell Lines. NSCLC lines variably expressed surface HLA antigens. While three lines (SK-LU-1, CALU1, and SK-MES-1) presented a high expression of class I antigens, three other lines (CALU6, A427, and A549) showed a reduced level of expression. However, imbalanced expression of HLA-A and HLA-B antigens was observed in the six lines (Fig. 2A). After IFN-γ treatment (Fig. 2B) HLA-B antigen was preferentially modulated.

Imbalances in HLA-A or HLA-B Gene Expression Are Not
Due to Rearrangements of Structure Genes

We investigated HLA gene in tumor cell lines (Fig. 3) and solid tumors (data not shown) with different HLA-A or HLA-B gene expression. Southern blot analysis using locus specific probes showed a similar pattern of bands when compared with normal tissue samples (peripheral blood leukocytes and autologous normal tissue). In addition, no apparent defects or genetic rearrangements were revealed with nonspecific coding probes (data not shown).

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Fig. 2. Analysis of fluorescence performed by FACScan in six NSCLC lines and an Epstein-Barr virus (EBV) B cell line as control, with anti-HLA-A and -B monoclonal antibodies. (A) Mean fluorescence. (B) Relative increase of fluorescence after stimulation with IFN-γ, calculated as described in “Materials and Methods.” • HLA-A; ®, HLA-B.

Fig. 3. DNA blot hybridization analyses of genomic DNA from six NSCLC lines and peripheral blood leukocytes (PBLs) as control. DNA (20 µg) was digested, electrophoresed, and probed with pHLAI.1 (HLA-B locus specific). kb, kilobase.
Expression of Class I MHC Antigens and c-myc Genes in NSCLC Carcinomas and Cell Lines

HLA class I negative expression has been related to c-myc amplification in SCLC. To investigate this possibility, we used Southern blot analysis to compare DNA from 20 cases of NSCLC, including 5 cases of HLA negative tumors and 15 cases of HLA positive tumors (Fig. 4A). We observed no apparent differences in c-myc gene copy numbers between the two groups. Southern blot showed a 12.5-kilobase band, where the HL60 cell line was used as a c-myc amplified control. In tumor cell lines (Fig. 4B), no differences in c-myc copy number were observed.

Imbalances in HLA-A and HLA-B expression on the cell membrane surface corresponded to differences in mRNA levels. We investigated whether the expression of c-myc mRNA was related to the level of HLA expression on the cell surface or to mRNA steady state. As reflected in Fig. 5, no consistent relationship was found between c-myc mRNA levels and specific mRNA for HLA-A and HLA-B antigens. For example, A427 and CALU6, which both showed low HLA mRNA levels, differed significantly in c-myc mRNA steady state. In contrast, SK-LU-1 and SK-MES-1, which clearly differed in HLA-B mRNA levels, show similar c-myc mRNA transcripts. After 72 h of IFN-γ treatment, c-myc mRNA levels rose in CALU1, SK-MES-1, and CALU6 and declined in SK-LU-1, A549, and A427 (Fig. 5).
DISCUSSION

The mechanism of HLA class I antigen underexpression in human tumors is not well known. In cases of laryngeal carcinomas nonexpression of HLA class I has been correlated with the degree of differentiation (11), in contrast with other tumors (32, 33). However, the malignant cellular transformation may itself alter the major histocompatibility complex phenotype (34).

It has been postulated that selective down-regulation of β2-microglobulin is responsible for the lack of HLA expression in HLA negative colon tumors (35). However, this mechanism does not seem to operate in lung cancer. In 59 lung carcinomas, we found total loss of expression of HLA in 16 cases (27%) (Table 1). In addition, we observed a coordinated underexpression of β2-microglobulin and heavy chain (Table 1).

Differential expression of HLA-A and HLA-B antigens was also evident at the protein and RNA levels. A close relation between the level of expression on the membrane and mRNA was not observed in all cases, making it possible that posttranscriptional control may be involved in the differential expression of HLA-ABC products. Moreover, IFN-γ induced variable expression of HLA-A and HLA-B antigens. This effect has been observed in variants of the MOLT-4 cell line (21, 36).

Differential expression of HLA products does not reflect different gene copy numbers or damage to gene structure (Fig. 3) (11, 32). In fact, IFN-γ preferentially modulates HLA-B expression. RNA analyses indicated that IFN-γ established a difference between HLA-A or HLA-B molecules, probably corresponding to differences in interferon consensus sequences (37).

On the other hand, some oncogene products have been suggested to act as cellular factors that may modulate class I gene expression. An inverse correlation has been described between c-myc mRNA levels and class I gene expression in melanoma cell lines (38) and SCLC tumors and cell lines (12).

Amplification of c-myc DNA appears frequently in the variant of SCLC tumors and cell lines (39). However, in 20 cases of NSCLC tested, we detected no amplification (Fig. 4). Therefore, at least in NSCLC lines and tumors, c-myc amplification is not related to HLA phenotype. Furthermore, we could find no relationship between c-myc and HLA mRNA levels, indicating that an inverse correlation between HLA class I and c-myc expression may not be a constant finding (40); hence this c-myc product may act through other tissue specific nuclear regulatory factors for class I gene expression.

Our results show that HLA class I alterations occur preferentially in lung carcinomas and may be manifested as simultaneous underexpression of β2-microglobulin and heavy chain (27%) and selective HLA-A or HLA-B loss (1.7 and 5%, respectively), probably through the mediation of nonspecific or locus specific regulatory mechanisms. Furthermore, expression of class I histocompatibility antigens in NSCLC tumors and cell lines is independent of c-myc expression.

ACKNOWLEDGMENTS

We would like to thank Dr. H. Ploegh for HC10 mAb, Dr. J. Vives and Dr. C. Milstein for HLA-Locus specific mAbs, Dr. H. Orr for HLA locus specific probes, and Dr. A. Cueto (Servicio de Cirugía Torácica, Hospital Virgen de las Nieves, Granada, Spain) for providing specimens.

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