Expression of HLA Class I and II Antigens in Bronchogenic Carcinomas: Its Relationship to Cellular DNA Content and Clinical-Pathological Parameters

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ABSTRACT

We studied the presence of HLA class I antigens in 115 samples of bronchogenic carcinomas (66 frozen and 49 formalin-fixed and paraffin-embedded specimens) by the immunoperoxidase alkaline and immunoperoxidase methods with antibodies against major histocompatibility complex antigens. We also studied HLA class II antigens on the 66 frozen tumor samples. Nonneoplastic lung tissue was also analyzed for purposes of comparison.

Pneumocytes and epithelial respiratory cells expressed HLA class I and II antigens. The expression of class I antigens was totally lost in 29 tumors (25%). The defect in HLA gene expression affected both heavy chain and \( \beta_2 \)-microglobulin, as demonstrated by the null reactivity with specific antibodies. In 2 cases of 66 studied in cryostatic section, the selective loss of A locus was observed, and in three cases selective loss of B locus was detected.

The expression of class I antigens was compared with clinical-pathological parameters such as histological type, degree of differentiation, and tumor stage, as well as tumoral ploidy. The absence of expression of HLA class I molecules was significantly associated with poorly differentiated and undifferentiated tumors (\( P < 0.0001 \)) and with aneuploid tumors (\( P < 0.001 \)), suggesting that some lung tumors may escape immune surveillance and become biologically more aggressive.

Class II antigens were expressed in 13 cases of 66 studied (18%) in frozen specimens, and a clear relationship was observed with well-differentiated tumors (\( P < 0.05 \)).

INTRODUCTION

The prognosis in patients with lung cancer is determined by several factors, the most important being the specific histological diagnosis, tumor stage, and host performance status.

DNA content in human solid tumors is usually analyzed by flow cytometry, which has recently been used to help determine the prognosis in several types of solid tumors, including non-SCLC. In SCLC aneuploidy is frequent, but the relationship between ploidy status and prognosis has not been well established. The presence of class II antigens and a high degree of differentiation and the absence of aneuploidy may have a decisive influence on the immune response to the tumor and on the tumor's metastatic capacity.

The loss of HLA class I expression is a relatively frequent event in human tumors, such loss being associated with the degree of differentiation and clinical aggressiveness in colon, breast, and laryngeal carcinomas.

HLA-DR expression is observed in two different situations: in advanced stages of malignant melanoma with an unfavorable prognosis and in highly differentiated colon adenocarcinoma and laryngeal carcinoma. These findings suggest that in human tumors, histocompatibility antigens may directly influence the type of immune response and possible escape mechanisms.

Few studies have dealt with the relationship between HLA and lung cancer. We used immunohistochemical techniques to examine HLA class I antigens in 115 bronchogenic carcinomas. Our results demonstrate a significant relationship between the loss of HLA class I antigens and the markers of biological aggressiveness: a poor degree of differentiation and the presence of aneuploidy.

When HLA class II antigens were examined in the 66 frozen tumor specimens, a direct association was found between the presence of class II antigens and a high degree of differentiation. HLA class II expression was observed in epidermoid carcinomas and adenocarcinomas but not in any of the SCLC tumor specimens studied.

MATERIALS AND METHODS

Subjects. One hundred fifteen bronchogenic carcinoma specimens from patients undergoing surgical treatment at the Thoracic Surgery Units of the Virgen de las Nieves Hospital in Granada and the Marques de Valdecilla Hospital in Santander (Spain) were studied. The patients had not received radiotherapy or chemotherapy prior to surgery.

Pathological Examination. Gross examinations were carried out under the supervision of two of us (A. Co. and R. O.). Representative blocks from the tumors were fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and independently classified according to the WHO system.

Sections of nonrepresentative tissues were not used for counting purposes and not selected for DNA analysis.

Antibodies. The following antibodies were used: W6/32 against a common HLA class I determinant (24); GRH1 against \( \beta_2 \)-microglobulin (25); anti-\( \beta_2 \)-microglobulin (DAKO, Copenhagen, Denmark); HC10 anti-HLA heavy chain, kindly provided by Dr. H. Ploegh (26); \( \alpha \)-HLA-A (27) and JOAN I (anti-HLA-A and anti-HLA-B, respectively, both kindly provided by Dr. J. Vives); \( \alpha \)-HLA-B (YT-76, anti-HLA-B molecules), kindly provided by Dr. C. Milstein (28); GRB1 against DR antigen (29); B7/21 against DP (30); Tu22 against DQ (31); and GRT2, a monoclonal antibody that reacts with the common leukocyte antigen (CD45), to measure leukocyte infiltration of the tumor (32).

Alkaline Immunophosphatase and Immunoperoxidase Techniques. Sections measuring 5–8 \( \mu \)m in thickness were cut from 66 frozen specimens and 49 paraffin-embedded specimens.

For tissues embedded in paraffin, the sections were routinely deparaffinized with xylene and progressively rehydrated in decreasing concentrations of ethanol. The frozen specimens were fixed in cold acetone for 10 min, air-dried at room temperature (2–24 h), and rehydrated with phosphate-buffered saline.

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1 To whom requests for reprints should be addressed.

2 The abbreviation used is: SCLC, small cell lung cancer.
Binding of the antibody to sections was assayed by the alkaline immunophosphatase and immunoperoxidase techniques according to previously published methods (33, 34). The sections were incubated first with the specific antibody, then with rabbit anti-mouse immunoglobulin (DAKO) at 1:20, and finally with the alkaline phosphatase anti-alkaline phosphatase complex (DAKO) at 1:50. The sections were washed between incubations for 5 min in Tris-buffered saline. The binding sites were revealed with a fresh solution of naphthol AS-Mx phosphatase, dimethylformamide, Tris buffer (pH 8.2), and fast red substrate. Levamisole was added to inactivate the endogenous alkaline phosphatase. A final rinsing with water was followed by counterstaining with hematoxylin.

The immunoperoxidase technique was similar to the previous essay, except for the third step with peroxidase anti-alkaline phosphatase complex (DAKO) and the use of chromogen diaminobenzidine (Sigma Chemical Co., St. Louis, MO) plus H2O2. When rabbit anti-human HLA class I antigens were used as the first antibody, the second antibody was swine anti-rabbit immunoglobulin, and the third step used peroxidase anti-peroxidase rabbit (rabbit immunoglobulin to peroxidase).

Controls. The intrinsic positive controls were the stromal cells in each case (endothelium, infiltrating lymphocytes, and fibroblasts). In addition, we used the GRT2 monoclonal antibody which recognizes CD45 antigens. As a negative control in each experiment, the primary antibody was replaced in one section with Tris-buffered saline.

Sections of tumors and histologically normal lung tissue from the same patient were studied at the same time.

Interpretation of Immunohistochcmical Results. Microscopic observations were carried out by two observers. We classified a tumor as HLA-negative when less than 10% of its cells were stained and HLA-positive when more than 75% of its cells were stained. When the percentage of stained cells was between 10% and 75%, the tumor was classified as having heterogeneous expression, although these cases were not frequent in our study.

Preparation of Nuclear Suspensions. The preparation of a single cell suspension was similar to that described by Hedley et al. (35) for tissues embedded in paraffin. Three 30-μm sections from the paraffin blocks of the primary tumor were obtained. The tissue was deparaffinized with xylene and then progressively rehydrated in decreasing concentrations of ethanol. The specimen was washed with distilled water and incubated in a 0.5% pepsin solution (Sigma). Following incubation, the specimen was vortexed, filtered, and centrifuged. The remaining pellet was resuspended in phosphate-buffered saline and incubated with a solution containing 0.1% RNase (Sigma) and 5% Triton X-100 (Sigma).

Cellular DNA Measurement. Propidium iodide solution (Sigma) in RPMI (Gibco Laboratories, Grand Island, NY) at a final concentration of 50 μg/ml was added to the single cell preparation as a DNA stain. The DNA content was measured by flow cytometry (fluorescence-activated cell sorter; Becton Dickinson). A minimum of 10,000 cells from each specimen were analyzed. We classified the samples according to the method of Hiddeman (36).

Statistical Analysis. The χ2 and Fisher tests were used to compare clinical-pathological data and immunohistological results.

RESULTS

In all, 115 specimens of primary tumors were studied; 66 were frozen (Table 1) and 49 were paraffin embedded (Table 2). Most of the tumors (n = 76) were epidermoid carcinomas; the remainder were adenocarcinomas (n = 33), SCLC (n = 4), and large cell carcinomas (n = 2). There were 24 well-differentiated, 56 moderately differentiated, 29 poorly differentiated, and 6 undifferentiated tumors. For practical purposes we considered undifferentiated tumors as those with little squamous or glandular differentiation.

The postsurgical tumoral stage was only available for evaluation in 58 cases (patients from the Virgen de las Nieves Hospital). According to the new International System for Stag-
**Table 2** HLA class I expression in bronchogenic carcinomas (paraffin-embedded specimens)

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* HLA class I expression was evaluated by the HC10 monoclonal and anti-β2-microglobulin mAb (DAKO). (+/n), positive cases/total number of cases; SQ, squamous cell carcinoma; AC, adenocarcinoma; LC, large cell carcinoma. I, well-differentiated tumors; II, moderately differentiated tumors; III, poorly differentiated tumors; IV, undifferentiated tumors.

Expression of HLA Class I and II Antigens in Nontumor Lung Tissue

We studied the expression of HLA class I and II antigens on pneumocytes, epithelial airway cells, endothelial cells, lymphocytes, and myocytes from the same lung as the original tumor, but from a place that was distant from the tumor and which was considered normal by the pathologist. We observed expression of class I antigens (Fig. 1) in all cases, except on myocytes. The intensity of expression of class II antigens in each case was variable in the bronchiolar epithelium and constant on pneumocytes and alveolar macrophages.

Expression of HLA Class I and II Antigens in Bronchogenic Carcinomas

Expression of HLA Class I Antigens. Of the 115 cases studied, 86 (75%) showed positive expression to heavy chain and β2-microglobulin, as confirmed by the reactivity with the antibodies anti-β2-microglobulin, W6/32, and HC10 (Tables 1 and 2). Twenty-nine (25%) patients were class I-negative (Fig. 2). No discordant results were obtained between anti-β2-microglobulin and anti-heavy chain antibodies.

Selective loss of A locus was detected in two cases and loss of B locus in three cases, in tumors that were previously classified as HLA-ABC-positive (Table 1).

The absence of expression of HLA class I antigens was significantly associated (P < 0.001) with the presence of aneuploidy (Fig. 3). In this study, adequate tumor samples were available from 100 patients; 69 tumors were diploid and 31 were aneuploid.

When we compared HLA class I antigens and the degree of differentiation a statistically significant association was found between the loss of these antigens and the two lowest degrees of differentiation (P < 0.001). Seventy-one of 86 tumors with positive expression of class I molecules were well or moderately differentiated, whereas 20 of 29 tumors which did not express class I antigens were poorly differentiated and undifferentiated tumors (Fig. 2).

No significant relationship was found between the expression of HLA class I antigens and other clinicopathological parameters such as tumoral stage or histological type (Fig. 4).

Because of the short period of follow-up (overall range, 3–36 months; for most patients, 3–12 months) we were not able to establish an accurate picture of the usefulness of HLA phenotype for predicting survival.

Expression of HLA Class II Antigens. HLA class II antigens were detected in 13 of the 66 tumors studied in cryostatic section (Fig. 5). DR-positive cells were found more frequently than DP- and DQ-positive cells (Table 1).

A statistically significant relationship was found when we compared the presence of HLA class II antigens on well-differentiated tumors with the other histologic grades (P < 0.05) (Fig. 5). Thus, 6 of 13 tumors with positive expression were well differentiated, 4 were moderately differentiated, 3 were poorly differentiated, and none of the undifferentiated carcinomas were positive for HLA class II expression (Table 1).

DISCUSSION

It has been postulated that tumor cells which have lost class I antigens have the advantage of being able to escape lysis by...
HLA CLASS I EXPRESSION IN BRONCHOGENIC CARCINOMAS

Fig. 2. Immunophosphatase alkaline technique. There is no reaction with W6/32 in a poorly differentiated epidermoid carcinoma.

We detected two types of alterations in immunohistological studies: total loss of HLA expression and selective losses of HLA-A and HLA-B antigen expression. These alterations were restricted to neoplastic cells.

In our study we detected total loss of HLA class I expression in 25% of lung carcinomas. We found no discrepancies between β2-microglobulin and heavy chain expression (Table 1). These results are similar to those of previous studies of lung cancer (20) but contrast with the discrepancies obtained in colon carcinomas (41).

We observed 2 selective losses for A locus and 3 for B locus expression; such uncoordinated expression of HLA-A and HLA-B antigens has been reported in other tumors (42, 43).

The mechanisms for HLA selective losses are not yet well known, although locus-specific regulation of HLA gene subsets has been documented (44, 45). In addition, locus-specific DNA binding factors have been found for the regulation of HLA class I genes in colon cancer (46).

The loss of class I antigen expression was significantly associated with poorly differentiated and undifferentiated tumors (P < 0.0001), as has also been reported in colon (13) and laryngeal carcinomas (15). However, in other studies of colon carcinoma (18, 47, 48) and gastric carcinoma (49), this correlation was not found.

Doyle et al. (20) reported a loss of class I antigens in special histological types of tumors such as SCLC, which invades or metastasizes rapidly. In our study, 2 of 4 SCLCs were positive for class I expression (Fig. 4). However, we found no statistically significant differences between expression in this tumor and expression in other histological types. In contrast, when we compared the group of tumors without squamous or glandular differentiation (SCLC and large cell carcinomas) with the group of epidermoid carcinomas and adenocarcinomas, a statistically significant difference was found (P < 0.039), indicating that in lung cancer, HLA class I expression is related to the degree of differentiation in certain histological types. A similar correlation between the grade of differentiation and HLA-A,B,C expression was also described in a recent study of lung cancer (22).

Chromosomal aberrations and changes in DNA content are distinctive features of human tumors. DNA content and proliferative activity are two parameters obtained by flow cytometry with important prognostic implications in many types of solid tumors (50). In lung carcinoma, a strong association between non-SCLC and abnormal DNA content has been described (1, 2), in contrast with results obtained by others (51).

The 31% rate of aneuploidy found in our study is lower than previously published figures (1, 2, 52). In addition, as we observed in breast carcinomas (14), there was a strong association between the absence of class I antigen expression and the presence of aneuploidy (P < 0.001) in lung carcinomas. Thus, these cases may have a worse prognosis (1, 2).
Our findings fail to demonstrate rearrangements of HLA class I genes in tumors with total or partial HLA losses of these antigens (15, 53). However, regulatory mechanisms of the expression of HLA genes can be affected in aneuploid tumors, and in this sense, although aberrant DNA content apparently does not cause structural gene damage, it may be responsible for altered gene expression, leading to the surface alterations frequently seen on cancer cells.

Major histocompatibility complex class II genes are immune response genes that control the production of specific T-cells in response to a particular antigen (54). We found uncoordinated expression of HLA-D region products (DR, DP, and DQ) in our tumors (Table 1), as has been detected in the hematopoietic system and other tissues (48, 55, 56). This suggests the presence of different regulatory mechanisms for each of three molecules.

Our results demonstrate an evident relationship between the expression of HLA class II molecules and the degree of differentiation ($P < 0.05$) as reported in breast carcinomas (57) and laryngeal carcinomas (19). In the latter, positive class II expression was associated with an excellent prognosis.

This association suggests that HLA class II expression in bronchogenic carcinomas may be constitutive (recalling the expression in normal epithelium and pneumocytes). In fact, the distinct mechanisms responsible for the differential control of class II gene expression during cell differentiation have been documented (58). Alternatively, HLA class II expression may be modulated by an exogenous stimulus such as IFN-$\gamma$, al-
though we found no significant differences between HLA class I antigen expression in bronchogenic carcinomas is related to infiltrate, which in most cases was scarce.

Based on our findings, we suggest that the absence of HLA class I antigen expression in bronchogenic carcinomas is related to aggressive phenotypes and that HLA class II expression may reflect variations in the degree of differentiation. Extensive follow-up studies will be necessary to document the prognostic significance of the lack of HLA-ABC antigen expression and HLA class II expression in lung carcinomas.

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REFERENCES

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