Expression of Class II Major Histocompatibility Complex Molecules Correlates with Human Colon Tumor Vaccine Efficacy

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ABSTRACT

Vaccination of colon cancer patients with X-irradiated autologous tumor cells and Bacillus Calmette-Guérin results in a significant reduction in tumor recurrence. A study was undertaken to determine whether the expression of tumor-associated antigens, expression of major histocompatibility complex molecules, or the cellular composition of the vaccine cells correlates with vaccine efficacy. A significant increase in the percentage of histocompatibility leukocyte antigen (HLA) class II molecule-expressing tumor cells was the only marker with a positive correlation. Because HLA class II molecule expression is not a prognostic marker in control patients, it was hypothesized that HLA class II molecules are involved in the induction of tumor immunity in patients treated with the autologous colon tumor vaccine. Enhancement of HLA class II molecule-expressing cells could be induced in X-irradiated colon tumor cells injected into the skin of mice when the cells were mixed with γ-interferon. Therefore, addition of γ-interferon to the colon tumor vaccine, resulting in increased numbers of HLA class II molecule-expressing cells, could potentiate the generation of tumor immunity.

INTRODUCTION

Active specific immunotherapy of colon cancer has been achieved by administering a vaccine composed of enzymatically dissociated, X-irradiated, autologous tumor cells admixed with BCG² to patients (1, 2). In a 65-month median follow-up of a prospectively randomized phase II clinical trial designed to evaluate the autologous colon tumor vaccine, Hoover and Hanna (3) reported a significant increase in time to tumor recurrence. They observed a 21% rate of recurrence in the immunotherapy group versus 52% in the surgery-only controls. Although not statistically significant, they reported 12.5% mortality after immunotherapy and 30% after surgery alone. Tumor immunity developed, as demonstrated by the delayed cutaneous hypersensitivity response to autologous tumor cells (4), which appeared to correlate with clinical outcome (3). In this paper, an immunohistochemical analysis of the vaccine cells was performed to determine whether any cell-associated markers correlate with vaccine efficacy, such that the possible reasons why not all immunized patients were cured can be elucidated.

Expression of three different classes of markers was considered for evaluation: (a) phenotypic markers indicative of the vaccine’s cellular composition, (b) tumor-associated antigens, and (c) major histocompatibility complex molecules. It was hypothesized that vaccine efficacy could result simply from the total cellular composition of the vaccine, i.e., vaccines that were ineffective could have contained more normal cells and leukocytes than immunizing tumor cells. To discriminate among the colon tumor cells, normal fibroblasts, and leukocytes in the dissociated tumor cell suspension, two sets of phenotypic markers were chosen. Because colon carcinomas are of epithelial origin, the malignant cells present in the vaccine should express cytokeratin 18. The normal fibroblasts of mesenchymal origin express the intermediate filament protein vimentin. Leukocytes also express vimentin. However, because leukocytes are morphologically distinguishable from the tumor cells and normal fibroblasts, vimentin-positive leukocytes were not enumerated when the presence of normal mesenchymal and malignant epithelial cells in the vaccines was quantitated. It is possible that, although irradiated, these metabolically active leukocytes may play a role in host immune recognition, by secreting bioactive lymphokines.

A number of colon tumor-associated antigens have been identified and characterized using human and mouse monoclonal antibodies. If the expression of one or more of these antigens on the vaccine cells correlates with a positive clinical outcome, this antigen could possibly be used as the primary vaccine or as an adjuvant to the cellular vaccination. Haspel et al. (5, 6) have immortalized and cloned cells producing colon tumor-reactive human monoclonal antibodies, from patients undergoing active specific immunotherapy. These antibodies were selected because they have specificity for tumor-associated antigens, they recognize cell surface and cytoplasmic determinants, and some have been shown to localize to tumor in human colon carcinoma patients (7–11). Moreover, because these antibodies are the products of the human immune response to an autologous tumor, our supposition is that their respective antigens would be more relevant to tumor immunity than those isolated after immunization of mice. Carcinoembryonic antigen and tumor associated antigen-72 (TAG-72) are well characterized antigens associated with colon carcinoma and recognized by murine monoclonal antibodies, which have also been used successfully for imaging of colon tumors (12, 13). HER2 is a proto-oncogene that has been reported to have prognostic significance in breast cancer (14) and also has been shown to be expressed by colon carcinomas (15), but whose prognostic significance for colon carcinoma is not known. Monoclonal antibodies to monomorphic determinants of HLA-ABC (class I) and HLA-DR, -DP, and -DQ (class II) molecules were also used to characterize the vaccine tumor cells. HLA class I and class II molecules of the MHC are the cell surface molecular structures that present antigens to cytotoxic T lymphocytes and T helper/inducer lymphocytes, respectively (16–18). MHC molecules are expressed on a variety of neoplasms, with apparent wide variation in expression and differences in prognostic implication, depending upon the type of malignancy (19–22). However, reports have indicated that the MHC has a role in tumor resistance (23, 24). We sought to determine whether MHC molecule expression played a role in conferring tumor immunity.

MATERIALS AND METHODS

Colon Tumor Vaccines. Tumors were obtained at the time of surgery and, with the assistance of a surgical pathologist, non-neurotic tumor tissue was dissected, minced with a scalpel, and dissociated into a
single-cell suspension with collagenase and DNase (25). Cells were cryopreserved and maintained in the vapor phase of a liquid nitrogen cylinder until prepared for either patient vaccination or marker characterization. Cells used for characterization were surplus dissociated cells obtained from the tumors of patients in the reported phase II trial (1, 2). Due to the limited clinical material, marker analysis was performed once for each patient.

Monoclonal Antibodies. The monoclonal antibodies described in Table 1 were quantitated expression of the three classes of markers used for vaccine characterization. All of the human monoclonal antibodies were provided by M. V. Haspel (Organon Teknika/Biotechnology Research Institute, Rockville, MD). The specificity and biochemical characteristics of their cognate antigens have been described (7-11). SC20 was provided by B. T. Butman (Organon Teknika/Biotechnology Research Institute, Bethesda, MD). M736 and M704 were from DAKO (Santa Barbara, CA). mAB1 was from Triton Biosciences (Alameda, CA). The remaining antibodies were from Becton Dickinson Corp. (Hialeah, FL). The concentrations of monoclonal antibodies to intermediate filament proteins were from Behring Diagnostics (Somerville, NJ).

Table 2 Comparison of cell marker expression with clinical course in patients vaccinated with the autologous colon tumor vaccine

<table>
<thead>
<tr>
<th>Marker</th>
<th>NED</th>
<th>REC</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue markers</td>
<td>Cytokeratin 18</td>
<td>55 ± 18</td>
<td>55 ± 18</td>
</tr>
<tr>
<td>Vimentin</td>
<td>41 ± 13</td>
<td>37 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>CD4</td>
<td>23 ± 14</td>
<td>23 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>CD8</td>
<td>11 ± 7</td>
<td>10 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>CD20</td>
<td>21 ± 10</td>
<td>14 ± 15</td>
<td>NS</td>
</tr>
<tr>
<td>CD11C</td>
<td>40 ± 9</td>
<td>32 ± 15</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 81AV78</td>
<td>38 ± 18</td>
<td>37 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 79AV829</td>
<td>42 ± 18</td>
<td>42 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 104MV148</td>
<td>22 ± 20</td>
<td>12 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 88BV59</td>
<td>27 ± 12</td>
<td>29 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 97AV1017</td>
<td>3 ± 6</td>
<td>8 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>CTAA 81AV117</td>
<td>11 ± 18</td>
<td>14 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>TAG-72</td>
<td>14 ± 12</td>
<td>11 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>24 ± 15</td>
<td>24 ± 23</td>
<td>NS</td>
</tr>
<tr>
<td>CEA</td>
<td>57 ± 28</td>
<td>67 ± 19</td>
<td>NS</td>
</tr>
<tr>
<td>HLA molecules</td>
<td>HLA-ABC</td>
<td>87 ± 21</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>48 ± 24</td>
<td>24 ± 12</td>
<td>0.02</td>
</tr>
<tr>
<td>HLA-DP</td>
<td>45 ± 22</td>
<td>23 ± 21</td>
<td>0.04</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>27 ± 15</td>
<td>18 ± 16</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NED, no evidence of disease; REC, tumor recurrence. Values are mean ± SD.

* Probability by Student's t test (two-tailed), performed on the transformed percentages using the arcsin of the square root of the percentage (32), that a statistically significant difference exists between the groups with no evidence of disease and those with tumor recurrence. The P values reported are not corrected for between-group comparisons.

NS, not significant at P = 0.05.
to this were that the human IgG and IgA monoclonal antibodies used were conjugated directly to biotin, to minimize background staining, and binding was visualized with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), using 3-amino-9-ethylcarbazole as the substrate.

In a blind coded fashion, at least 200 cells were enumerated for positive or negative reactivity, by microscopic examination. Counting 200 cells was found to yield a difference in enumeration of identically stained slides of <7%. The intensity of a positive staining reaction was dramatic with all of the antibodies used in this study and, therefore, discrimination between positive and negative staining was clear. Only nonleukocytic cells were enumerated for expression of tumor-associated antigens, intermediate filament proteins, and major histocompatibility complex molecules. The leukocyte antigen expression represents the percentage of positive cells in the leukocyte population alone.

Up-regulation of MHC Molecules. γ-Interferon was used to determine whether MHC molecule expression could be up-regulated in an in vivo model of the colon carcinoma vaccine. γ-Interferon dose-response studies were initially performed in vitro. One million tumor cells were plated in 10 ml of RPMI 1640, 10% fetal bovine serum, 50 μg/ml Gentamicin, in 100-mm tissue culture dishes. Of the four tumor cell lines used, two were the established colon tumor lines HT-29 and WiDr (obtained from the American Type Culture Collection, Rockville, MD), and the other two were early-passage colon tumor cell lines, CW and HAR (obtained after 10 population doublings), established in our laboratory from dissociated primary colon tumors. After a 24-h incubation at 37°C in a 95% air-5% CO2 incubator, γ-interferon, in increasing concentrations from 0 to 0.7 μg/ml, was added and an additional 48-h incubation was performed. γ-Interferon (specific activity, 1.5 × 10^7 units/mg) was obtained from Genentech, Inc. (South San Francisco, CA). The cells were harvested after brief trypsinization, and 5 × 10^5 cells were incubated with 100 μl of 1 μg/ml anti-HLA-ABC, anti-HLA-DR, or IgG control monoclonal antibodies (as described in Table 1), for 1 h on ice. After washing with HBSS, 0.01% sodium azide, 1% bovine serum albumin, the cells were incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (diluted 1:50; KPL, Gaithersburg, MD), on ice. After washing, the cells were subjected to flow cytometric analysis, using an Epics C flow cytometer (Coulter, Inc., Hialeah, FL). The results are expressed as percentage of fluorescent cells and, when increases in marker expression were analyzed, as increase in mean positive channel number (determined by subtracting the mean channel number of positively staining cells of the untreated control from the mean positive channel number of the treated cells).

In the in vivo MHC up-regulation experiments, cells were prepared similarly to preparation of the autologous human colon tumor vaccine (1). The same four cell lines described above were harvested from culture. The cells were suspended in 10 ml of HBSS and X-irradiated, for a total dose of 20,000 rad, in a Nordion Gamma-cell Elite irradiator (Ontario, Canada). After centrifugation, 10^7 cells were resuspended in 0.1 ml of HBSS containing 10^7 colony-forming units of BCG and graded doses of γ-interferon and were injected intradermally into the skins of SCID mice. SCID mice were chosen to eliminate participation of host immunological factors. After 2 days, the injection site was excised and immediately frozen in optimal cutting temperature compound (OCT) suspended in liquid nitrogen. Serial sections were cut on a cryostat, and immunohistochemical staining for expression of MHC molecules was performed as described above. At least four microscopic fields (×400 magnification) were enumerated for positively staining cells (approximately 1000 cells).

RESULTS AND DISCUSSION

Immunohistochemical analysis of marker expression was performed on vaccine cell cytospins from patients who had under-
gone active specific immunotherapy. The data were sorted into two groups. The first group contained data from patients who remained disease free after at least a 5-year follow-up from the time of vaccination. The second group was from patients whose tumor recurred after vaccination. We were looking for a difference in markers among the two groups that would be indicative of a possible role in the induction of tumor immunity. As can be seen in Table 2, the colon tumor vaccines were composed of colon carcinoma cells, fibroblasts, and leukocytes that had infiltrated the tumor. About one half of the nonleukocytic cells in the vaccine expressed the intermediate filament cytokeratin 18. This suggests that only half of the nonleukocytic cells in the vaccine are carcinoma cells that are of epithelial origin. The number of infiltrating leukocytes ranged from 36 to 67% of the total cells in the vaccine (data not shown). The vaccine contained macrophages, CD4+ and CD8+ T cells, and B cells. There were no significant differences in the cellular composition of the vaccine in the two groups of vaccinated patients, regardless of their clinical outcome. This suggests that the difference in vaccine efficacy was not due simply to the total numbers of tumor and other cell types.

There were also no significant differences in the expression of any of the tumor-associated antigens between the two groups (Table 2). Therefore, no indication of the importance of a tumor antigen for induction of tumor immunity was evident. There were, however, significant differences in expression of HLA molecules between the two groups (Table 2). Vaccine tumor cells from both groups had high levels of expression of HLA-ABC markers. Vaccine tumor cells in patients with no evidence of disease had statistically significantly higher levels of expression of both the HLA-DR and HLA-DP molecules than found on the tumor cells from patients whose tumors recurred after vaccination. There was a somewhat lesser expression of HLA-DQ molecules on the tumor cells, with no significant differences between the two groups. HLA class II molecules (HLA-DR and DP) present antigen to T helper cells. Even though the study groups were not large, because the differences were statistically significant it is compelling to speculate that increased expression of these two HLA class II molecules in the vaccines of patients who were rendered disease free suggests that the HLA class II molecules on the tumor cells in the vaccine may play an active role in the development of tumor immunity. Hanna et al. (26) have reported that an inadequate number of tumor cells in the vaccine renders the vaccine ineffective. Therefore, having the appropriate numbers of tumor cells expressing HLA-DR and -DP molecules in the vaccines of patients who had no evidence of disease may be sufficient to drive the tumor immune response adequately. Once T cell immunity is established, the residual tumor, which has nearly 100% of cells expressing HLA class I molecules, as demonstrated by the data in Table 2, possesses the appropriate target structures for recognition and elimination by cytotoxic T cells.

We next asked the question: do increased numbers of HLA-DR and -DP molecule-expressing cells correlate with vaccine efficacy, or are these markers just generally prognostic? The same set of HLA molecules, tumor markers, and tissue markers were compared on dissociated cells from colon tumors of control patients who received surgery without vaccination and had appropriate clinical follow-up. There were no statistically significant differences in MHC molecule expression in the two groups of control patients, who either had no evidence of disease after surgery or had recurring tumors (data not shown). This is consistent with the findings of Möller et al. (27, 28), who reported a lack of correlation between expression of HLA class I and class II molecules and survival of colon cancer patients treated by surgical resection. Although HLA class II molecule expression has also not been prognostic in other tumors, a number of investigators have reported correlations between molecule expression on tumor cells and the effectiveness of immunotherapy. Cohen et al. (29) reported an association of HLA-DR expression and response to interleukin 2 immunotherapy of melanoma. In follow-up studies, Rubin et al. (23) also found that pretreatment HLA-DR antigen expression was not predictive of clinical response but that regressing metastases had increased HLA-DR expression, compared with lesions that did not respond. Mitchell (30) has also reported that certain HLA subclass-specific antigens on an allogenic melanoma vaccine correlate with response rates. Furthermore, Prescott et al. (24) reported that HLA-DR molecules were expressed on regressing bladder tumors only after immunotherapy with BCG. In our study, there is no prognostic correlation with expression of HLA class II molecules by colon tumors, but there is an association showing elevated numbers of tumor cells expressing HLA class II molecules in the vaccines of patients who were cured; this suggests that expression of these molecules is important to confer tumor immunity.

Another interesting observation found when comparing marker expression with clinical course in control patients is that the expression of the tumor marker recognized by the

![Graph A](image1.png)

**Fig. 2.** In vivo dose-response studies of γ-interferon up-regulation of MHC molecules. Cells, CW (●), HAR (○), WIDR (▲), and HT-29 (■), were X-irradiated with 20,000 rads, mixed with 10⁶ colony-forming units of BCG and varying doses of γ-interferon, and injected intradermally into the skins of SCID mice. After 2 days, the injection site was excised, frozen, sectioned, and stained for HLA-ABC (4) and HLA-DR (8) expression.
Fig. 3. Photomicrographs of the stained frozen sections of the intradermal inoculation site of human tumor cells treated with γ-interferon, demonstrating the up-regulation of HLA-DR expression in vivo. CW colon tumor cells (10^7) were X-irradiated with 20,000 rads, admixed with 10^7 colony-forming units of BCG, and suspended in either HBSS (top 4 panels) or HBSS with 0.3 μg of γ-interferon (lower 4 panels), and injected intradermally in 0.1 ml into the dorsal flank of SCID mice. After 2 days, the inoculation site was excised, frozen, sectioned, and stained indirectly for expression of HLA-ABC or directly with a biotinylated antibody to HLA-DR or with the appropriate IgG control antibodies, as described in Table 1. The appearance of the red 3-amino-9-ethylcarbazole substrate in a section is indicative of positive expression of a particular marker.
HUMAN COLON TUMOR VACCINE CHARACTERIZATION

81AV78 human monoclonal antibody was elevated in control patient's tumors that did not recur after surgery (61 ± 19% positive for patients with no evidence of disease, compared to 43 ± 16% positive for patients whose tumors recurred; \( P = 0.046 \)). This human monoclonal antibody recognizes a lipid antigen, and these data suggest that this may have some prognostic implications. This warrants further study. There was no association with elevated 81AV78 antigen expression in the vaccinated patients, suggesting that this antigen is not critical for induction of tumor immunity in the vaccine setting.

If critical numbers of cells expressing HLA class II molecules are required for effective vaccination, then we may be able to improve the vaccine by augmenting the proportion of HLA-DR-expressing cells. The next set of experiments were designed to determine whether we could up-regulate the expression of the HLA molecules on a colon tumor vaccine after inoculation into the skin of SCID mice. γ-Interferon has the ability to up-regulate HLA class I and class II molecules on a variety of cell types. In preliminary dose-response experiments performed in vitro, four cell lines were selected. As can be seen in Fig. 1A, none of the four cell lines expressed HLA-DR before γ-interferon treatment, but both the percentage of positive cells and the fluorescent intensity per cell were maximally increased after exposure to as little as 0.007 μg/ml γ-interferon (Fig. 1, A and B). These four cell lines had relatively high percentages of HLA-ABC-positive cells. γ-Interferon did not augment the percentage of HLA-ABC-positive cells but did increase the fluorescent intensity per cell (Fig. 1, A and B). The data in Figs. 2 and 3 demonstrate that coinjection of irradiated colon tumor cells with γ-interferon results in the up-regulation of HLA-DR molecules in vivo. As was seen in vitro, the cells did not express HLA-DR when γ-interferon was not included in the inoculum (Figs. 2B and 3). The maximal expression in all four cell lines was achieved at a dose of 2.0 μg. All four cell lines were induced to the same extent in vitro; however, their in vivo responses and minimal dose-response varied (Fig. 2B).

The percentages of HLA-ABC-positive cells (used as a positive control for staining) were not as high as those in vitro and were not as greatly affected by γ-interferon. Although these cells were lethally irradiated, they were still sufficiently metabolically active to respond to γ-interferon, via a mechanism believed to involve the induction of proteins that bind to the promoter region of the MHC gene. Because the number of tumor cells in a vaccine is critical to confer tumor immunity (26, 31), the increase in percentages of HLA-DR-expressing tumor cells in the vaccine, which are capable of presenting tumor-associated antigens to the immune system, may potentiate the development of tumor immunity.

In summary, enhanced expression of HLA class II molecules on the tumor vaccine cells of colon carcinoma patients correlates with therapeutic effectiveness. This marker, however, is not indicative of a good prognosis in patients not receiving immunotherapy. No other tumor-associated antigen showed this correlation and, therefore, could be identified as critical for vaccination. Augmentation of HLA class II molecule expression can be achieved by coadministration of the vaccine with γ-interferon, thereby possibly providing a means of enhancing the effectiveness of colon cancer active specific immunotherapy.

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REFERENCES


3465


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