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% more recurrence in the immunotherapy group versus 52% in the surgery-only controls. They observed a 21% rate of recurrence in the immunotherapy group versus 52% in the surgery-only controls. However, 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, 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
TABLE 1 Characteristics of monoclonal antibodies used for colon carcinoma vaccine characterization

<table>
<thead>
<tr>
<th>Antibody characteristics</th>
<th>Species</th>
<th>Isotype</th>
<th>Identification</th>
<th>Character</th>
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<tbody>
<tr>
<td>Tissue markers</td>
<td>Mouse</td>
<td>IgG</td>
<td>Cytokeratin 18</td>
<td>Intermediate filament of epithelial cells</td>
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<tr>
<td></td>
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<td>IgG</td>
<td>Vimentin</td>
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<td>SK3/SK4</td>
<td>Mouse</td>
<td>IgG</td>
<td>CD4</td>
<td>Helper T cells</td>
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<td></td>
<td>MOUSE</td>
<td>IgG</td>
<td>CD8</td>
<td>Cytotoxic suppressor T cells</td>
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<tr>
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<td>Mouse</td>
<td>IgG</td>
<td>CD20</td>
<td>B cells</td>
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<td>CD11C</td>
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<td>Tumor antigens</td>
<td>Human</td>
<td>IgM</td>
<td>CTAA 81AV78</td>
<td>Cell surface phospholipid</td>
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<td></td>
<td>Human</td>
<td>IgM</td>
<td>CTAA 79AV829</td>
<td>Cell surface protein</td>
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<td>CTAA 104MV148</td>
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<td>Human</td>
<td>IgG</td>
<td>CTAA 1688</td>
<td>Altered cytokeratins</td>
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<td>IgA</td>
<td>CTAA 79AV1017</td>
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<td>IgA</td>
<td>CTAA 81AV117</td>
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<td>TAG-72</td>
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<td>IgG</td>
<td>HER-2/new</td>
<td>Proto-oncogene epidermal growth factor receptor-like</td>
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<td>Mouse</td>
<td>IgG</td>
<td>CEA</td>
<td>Carcinembryonic antigen</td>
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<td>IgG</td>
<td>HLA-ABC</td>
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<td>IgG</td>
<td>HLA-DR</td>
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<td>Mouse</td>
<td>IgG</td>
<td></td>
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<tr>
<td>M704</td>
<td>Mouse</td>
<td>IgG</td>
<td></td>
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<tr>
<td>B7/21</td>
<td>Mouse</td>
<td>IgG</td>
<td></td>
<td></td>
</tr>
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<td>HLA-DQ</td>
<td>Class II molecules; T helper cell induction</td>
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Monoclonal Antibodies. The monoclonal antibodies described in Table 1 were used to quantitate 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). B7/23 was a gift from J. Schliom (National Cancer Institute, Bethesda, MD). M736 and M704 were from DAKO (Santa Barbara, CA). mAb1 was from Triton Biosciences (Alameda, CA). The antibodies to intermediate filament proteins were from Behring Diagnostics (Somerville, NJ). The remaining antibodies were from Becton Dickinson Corp. (Hialeah, FL). The concentrations of monoclonal antibodies ranged from 1 to 10 μg/ml and were selected based on optimal reactivity, with no nonspecific binding. Appropriate class-matched control antibodies were used, and no background reactivity was observed.

Immunohistochemical Analysis. Twenty thousand cells from collagenase/DNase-dissociated, cryopreserved, colon tumors were thawed in HBSS with 10% fetal bovine serum, cryocentrifuged onto slides, air dried, and frozen at −70°C. The cells were stained by incubating the slide in 25 μl of an appropriate concentration of monoclonal antibody for 2 h at room temperature, washing with PBS, incubating for 1 h with 25 μl of an appropriate concentration of peroxidase-conjugated goat anti-human IgM or goat anti-mouse IgG (KPL Laboratories, Gaithersburg, MD), washing with PBS, incubating for 40 min with 3-amino-9-ethylcarbazole substrate (Sigma, St. Louis, MO), washing with PBS, and counterstaining for 5 min with hematoxylin. The exceptions...
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. Interferon was used to determine whether MHC molecule expression could be up-regulated in an in vivo model of the colon carcinoma vaccine. 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, interferon, in increasing concentrations from 0 to 0.7 µg/ml, was added and an additional 48-h incubation was performed. 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 × 10^6 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 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 differ-
eence 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 con-
tained 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, regard-
less 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
classes 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 expres-
sion 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 expres-
sion 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,

We next asked the question: do increased numbers of 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.

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

![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 109 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.](cancerres.aacrjournals.org)
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 (10⁷) were X-irradiated with 20,000 rads, admixed with 10⁷ 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. \( \gamma \)-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 \textit{in vitro}, four cell lines were selected. As can be seen in Fig. 1A, none of the four cell lines expressed HLA-DR before \( \gamma \)-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 \( \mu \text{g} \/ \text{ml} \) \( \gamma \)-interferon (Fig. 1A and B). These four cell lines had relatively high percentages of HLA-ABC-positive cells. \( \gamma \)-Interferon did not augment the percentage of HLA-ABC-positive cells but did increase the fluorescent intensity per cell (Fig. 1A and B). The data in Figs. 2 and 3 demonstrate that coinjection of irradiated colon tumor cells with \( \gamma \)-interferon results in the up-regulation of HLA-DR molecules \textit{in vivo}. As was seen \textit{in vitro}, the cells did not express HLA-DR when \( \gamma \)-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 \( \mu \text{g} \). All four cell lines were induced to the same extent \textit{in vitro}; however, their \textit{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 \textit{in vitro} and were not as greatly affected by \( \gamma \)-interferon. Although these cells were lethally irradiated, they were still sufficiently metabolically active to respond to \( \gamma \)-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 \( \gamma \)-interferon, thereby possibly providing a means of enhancing the effectiveness of colon cancer active specific immunotherapy.

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REFERENCES


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