Intratumoral T-Cell Infiltrates and MHC Class I Expression in Patients with Stage IV Melanoma

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Abstract
The infiltration of tumors by T cells has been shown to correlate with prolonged patients’ survival. However, it remains unclear why only some tumors are infiltrated with T cells. This study was designed to investigate possible correlations between intratumoral T-cell infiltrates and the expression of cancer-associated antigens and MHC class I and II molecules in patients with melanoma. Fresh frozen samples from 124 stage IV melanoma patients were analyzed by immunohistochemistry for the expression of Melan-A/MART-1, tyrosinase, gp100, NY-ESO-1, and MHC class I and II. Intratumoral T-cell and B-cell infiltrates were detected by staining with anti-CD4, anti-CD8, anti-CD3, and L26 antibodies. The NY-ESO-1 serum antibody status was assessed by Western blot analysis. Intratumoral CD8+ and CD4+ T cells were detected in 63.9% and 71.3% of patients, respectively. We observed a significant heterogeneity of the expression of the melanocyte differentiation antigens, NY-ESO-1, and MHC class I and II molecules. The only significant correlation was found between the expression of MHC class I and the presence of CD4+ and CD8+ T cells (P < 0.0001). There was a strong association between these two variables with respect to the density and distribution of infiltrating T cells and the pattern of MHC class I expression (focal versus homogenous). Intratumoral T-cell infiltration is closely correlated with the MHC class I expression but not with the expression of differentiation antigens, cancer-associated antigens, or MHC class II molecules. These results may have implications for the definition of prognostic variables and for the identification of patients who may benefit from antigen-specific cancer immunotherapy.

Introduction
The infiltration of tumors by T cells has been shown to correlate with the clinical outcome of patients with several types of cancer, including melanoma, renal cell, prostate, breast, colorectal, and esophageal cancers (1–6). Most recently, a clear association between the presence of intratumoral T cells and prolonged patients’ progression-free and overall survival in ovarian cancer was established (7). However, the pattern of T-cell infiltration of tumors is heterogeneous and it remains unclear why only some tumors are infiltrated with T cells. The present study was designed to investigate this issue by evaluating the expression of cancer-associated antigens, MHC class I and II molecules, and intratumoral T and B cells in samples of advanced melanoma.

Materials and Methods
Patients and samples. We evaluated fresh frozen samples from 124 stage IV melanoma patients between January 2001 and December 2002. All patients had metastatic disease and had failed at least one palliative chemotherapy regimen. All tumor samples were obtained from metastatic lesions. Informed consent was obtained from all patients.

Immunohistochemistry. Cryosections (5 μm thickness) were fixed in acetone and stained with H&E or immunostained by indirect immunoperoxidase method (Dako, Glostrup, Denmark) as recommended by the manufacturer. Monoclonal antibodies against the T-cell markers CD3, CD4, and CD8, against B-cell marker (L26), against the MHC class I complex (W6/32; ref. 8), and against the HLA-DR of MHC class II complex (L243), the melanocyte differentiation antigens Melan-A/MART-1 (A103; ref. 9), tyrosinase (T311; ref. 10), and gp100 (HM565; ref. 11), and the cancer testis antigen NY-ESO-1 (E978; ref. 12) were used. The sections were examined by a pathologist and two independent additional investigators trained in the histopathology of melanoma. Tumor-infiltrating T cells were defined as T cells detected within the tumor cell nests (intratumoral T cells) and were evaluated by semiquantitative scoring on a scale of 1+ to 3+ (scattered or mild 1+, moderate 2+, and strong 3+). Tumor-associated antigens and MHC molecules were graded as 1+, 2+, or 3+ (24%, 25-50%, or >50% positive tumor cells, respectively). The overall distribution of stained cells was graded as focal (one or few tumor cell nests were positive or infiltrated by T cells) or diffuse. Heterogeneous staining (1+ to 3+) was considered as diffuse and graded according to the area of the highest level of antigen expression.

Tumor typing for NY-ESO-1 mRNA. Expression of NY-ESO-1 mRNA in tumor specimens was assessed by reverse transcription-PCR (RT-PCR) using primers described previously (13).

Detection of NY-ESO-1 serum antibody. NY-ESO-1 serum antibodies were assayed by Western blot analysis using NY-ESO-1 recombinant protein purified from Escherichia coli (14) as described.

Results
Intratumoral T-cell infiltration. Intratumoral CD8+ and CD4+ T cells were detected in 78 of 122 (63.9%) and 87 of 122 (71.3%) patients, respectively. In most cases, the observed T-cell infiltration was mild to moderate (Table 1). The results for CD8+ and CD4+ cells were closely correlated. In the majority of cases (109 of 122), intratumoral CD8+ and CD4+ cells were either both detectable or both absent. Total intratumoral (CD3+) lymphocytes were evaluated in a smaller patient population and found to be positive in 43 of 62 (67.7%) patients, correlating well with the results of intratumoral CD8+ and CD4+ cells. Positive immunoreactivity of the B-cell marker L26 was detected in 90 of 116 (77.6%) cases.
NY-ESO-1 expression and NY-ESO-1 antibody. Tumors from 48 of 124 (38.7%) patients with stage IV melanoma expressed NY-ESO-1 as detected by either immunohistochemistry (28 of 60; 46.6%) or RT-PCR (20 of 64; 31.3%). The staining pattern for NY-ESO-1 was heterogeneous. In most cases, 5% to 50% of the tumor cells were positive, corresponding to 1+ to 2+ in our grading system (Table 1). No positive correlation between NY-ESO-1 expression and the presence of intratumoral CD4+ or CD8+ T cells could be found (P = 0.21 and 0.11, respectively, Fisher's exact test). There were no correlations between both variables with respect to the intensity or the overall distribution of immunoreactivity in the tumor. Eight of 45 (17.8%) patients had detectable NY-ESO-1 serum antibody, and in seven of eight patients, tumor samples expressed NY-ESO-1 as detected by RT-PCR.

Expression of the melanocyte differentiation antigens. The expression proportions are shown in Table 2. In the cases shown here, the number of positive cells was comparatively high (3+ in 38% of patients), staining was more homogenous, and focal expression was less frequent. No statistically significant correlations between the expression of any antigen and the T-cell infiltration were found.

MHC class I and II expression. Eighty-nine of 123 (72.4%) samples were positive for MHC class I (W6/32) and 53 of 55 (96.4%) samples tested were positive for MHC class II (HB55). The staining pattern for MHC class I was more heterogeneous compared with MHC class II. Focal expression was observed in 17 of 89 (19.2%) cases (Table 1).

Relation between MHC class I expression and T-cell infiltration. Positive W6/32 immunoreactivity was strongly associated with the presence of CD8+ and CD4+ T cells. In addition, there was a clear correlation between W6/32 immunoreactivity and CD8+/CD4+ infiltration with respect to the number and distribution of positive cells and the overall pattern of MHC class I expression (focal versus homogenous). For instance, focal MHC class I staining was observed in 17 cases, 13 of which exhibited focal CD8+ lymphocyte infiltration (Fig. 1A and B). Only one of these cases showed a homogenous but weak (+1) infiltration, and the remaining three were negative. Conversely, negative staining with W6/32 was found in 34 cases, 30 of which were also negative for CD8+ T cells (Fig. 1E and F), 3 showed a weak positive infiltration, corresponding to 1+ in our grading system, and 1 was not evaluable for CD8+ T-cell infiltration. Tables 2, 3, and 4 show the strong statistical associations between the intensity and distribution of T-cell infiltrates and MHC class I expression.

Discussion

Growing evidence has supported the view that the immune system may be able to interact with determinants of tumor cells in vivo (15, 16). Mainly the presence of tumor-infiltrating T cells has been shown to correlate with improved patients' clinical outcome (1–7). In some cases, the specificity of infiltrating CD8+ T cells was directed against cancer-associated antigens expressed by the tumor sample. However, it remains unclear why only some tumors are infiltrated with T cells, and little is known about the relation between tumor-infiltrating T cells and the expression of tumor-associated antigens or MHC molecules.

Previous studies on limited patient populations described a decrease of expression of the melanocyte differentiation antigens along with more advanced clinical stages (Table 5; refs. 11, 17, 18). The expression of MHC molecules has also been shown to be reduced in advanced tumor stages (19–21).

Table 1. Intratumoral T-cell and B-cell infiltrates and the expression of cancer-associated antigens and MHC class I and II molecules

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Patients</th>
<th>Positive, n (%)</th>
<th>Scoring</th>
<th>Focal n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>122</td>
<td>87 (71.3)</td>
<td>1+ 50</td>
<td>15 (12.3)</td>
</tr>
<tr>
<td>CD8</td>
<td>122</td>
<td>78 (63.9)</td>
<td>2+ 42</td>
<td>14 (11.5)</td>
</tr>
<tr>
<td>CD3</td>
<td>62</td>
<td>42 (67.7)</td>
<td>3+ 8</td>
<td>3 (0.71)</td>
</tr>
<tr>
<td>L26</td>
<td>116</td>
<td>90 (77.6)</td>
<td>4+ 42</td>
<td>10 (8.71)</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>124</td>
<td>48 (38.7)*</td>
<td>5+ 16</td>
<td>4 (3.4)</td>
</tr>
<tr>
<td>Melan-A/MART-1</td>
<td>121</td>
<td>106 (87.6)</td>
<td>6+ 20</td>
<td>7 (6.7)</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>121</td>
<td>103 (85.1)</td>
<td>7+ 36</td>
<td>5 (4.8)</td>
</tr>
<tr>
<td>Gp100</td>
<td>121</td>
<td>95 (78.5)</td>
<td>8+ 32</td>
<td>13 (13.7)</td>
</tr>
<tr>
<td>MHC I</td>
<td>123</td>
<td>89 (72.4)</td>
<td>9+ 39</td>
<td>17 (19.2)</td>
</tr>
<tr>
<td>MHC II</td>
<td>55</td>
<td>53 (96.4)</td>
<td>10+ 12</td>
<td>7 (13.2)</td>
</tr>
</tbody>
</table>

a As detected by either immunohistochemistry (28 of 60; 46.6%) or RT-PCR (20 of 64; 31.3%).

Table 2. Correlation between MHC class I expression and CD8+ T-cell infiltration with respect to the variables: positive/negative

<table>
<thead>
<tr>
<th></th>
<th>CD8+ cells present</th>
<th>CD8+ cells absent</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6/32 negative (n = 33)</td>
<td>3</td>
<td>30</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td>W6/32 positive (n = 89)</td>
<td>75</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

*Number of samples evaluable for W6/32 and CD8+ T cells.
† Fisher's exact test.
a loss of expression of MHC class I molecules was observed in single growing tumors of patients with a mixed response to peptide vaccination therapy, representing a potential mechanism of immune escape from antigen-specific T-cell recognition (22–24). Our results confirm these observations, showing that in most cases of advanced melanoma MHC class I molecules were expressed in <50% of the tumor cells or were absent. Interestingly, MHC class II molecules were regularly expressed in the majority of samples evaluated.

We compared in our samples the presence of tumor-infiltrating T cells with the expression status for MHC molecules and melanocyte differentiation antigens and found that the immunohistochemical detection of MHC class I molecules by positive staining with W6/32 was significantly associated with tumor-infiltrating CD4+ and CD8+ T cells. On the other hand, no significant correlations were found between detectable tumor-infiltrating T cells and the expression of melanocyte differentiation antigens or MHC class II molecules. The presence of tumor-infiltrating T cells did not correlate either with the expression of the highly immunogenic cancer testis antigen NY-ESO-1 (13, 14) or with the status of NY-ESO-1 serum antibody.

Both the loss of tumor-associated antigens and the down-regulation or alteration of MHC class I and II molecules have been considered to be potential means of tumor escape from immune recognition (25–28). Our analysis of tumor samples of patients

<table>
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<th>Table 3. Correlation between MHC class I expression and CD8+ T-cell infiltration with respect to the variables: focal/homogenous</th>
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<tbody>
<tr>
<td>n = 75*</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>W6/32 focal expression (n = 14)</td>
</tr>
<tr>
<td>W6/32 homogenous expression (n = 61)</td>
</tr>
</tbody>
</table>

*Number of samples evaluable for W6/32 and CD8+ T cells.
†Fisher’s exact test.
with metastatic melanoma by immunohistochemistry showed a significant heterogeneity of the expression of the melanocyte differentiation antigens Melan-A/MART-1, tyrosinase, and gp100, the cancer testis antigen NY-ESO-1, and MHC class I and II molecules and the levels of tumor-infiltrating CD4+ and CD8+ T cells. However, tumor-infiltrating CD4+ and CD8+ T cells were restricted to tumors that were MHC class I positive. In a recent study, clonally expanded tumor-infiltrating lymphocytes in melanoma lesions did not correlate with the heterogeneous expression of melanocyte differentiation antigens, whereas MHC class I molecules were not evaluated (29). A positive correlation between tumor-infiltrating lymphocytes and MHC class I molecules was found in a series of 18 head and neck squamous cell carcinoma biopsies (30). Our results add to these observations and indicate that MHC class I–expressing tumor cells may trigger intratumoral T-cell infiltrates in vivo. Low levels of MHC expression may lead to reduced antigen presentation, which might prevent sufficient immunologic recognition and subsequently the intratumoral accumulation of CD4+ and CD8+ T cells. The expression of MHC class I molecules in metastatic disease may represent a major prognostic factor for the clinical benefit resulting from spontaneous and specific vaccine-induced antitumor immunity. The targeting of MHC class II–restricted epitopes, the administration of immunotherapy in adjuvant settings or in early stages of cancer, the use of drugs that enhance the expression of MHC class I molecules, or the selection of patients with tumors that homogeneously express MHC class I molecules for clinical vaccine trials may represent effective strategies for making antigen-specific cancer vaccines more effective.

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