Identification of Melanoma Antigens That Are Immunogenic in Humans and Expressed In Vivo

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**Background:** In the development of an antimelanoma vaccine, a critical factor is the identification of antigens that induce a strong immune response in humans and that are expressed by melanoma cells in vivo. The aim of this study was to identify candidate antigens for such vaccine. **Methods:** Sixty-nine patients with surgically resected melanomas (American Joint Commission on Cancer [AJCC] stage III) were immunized with a polyvalent vaccine containing multiple melanoma antigens. Antimelanoma antibodies generated in the patients’ sera were used as probes to identify the melanoma antigens that are immunogenic in humans and that are expressed on the tumor tissue in vivo. Such responses were determined by an immunoblotting assay that employed an antigen source prepared from membrane fractions of freshly excised melanoma tissue. **Results and Conclusions:** Vaccine treatment stimulated antibody responses in 35 (51%; 95% confidence interval [CI] = 39%–63%) of 69 sequentially enrolled patients. The antibodies were directed to one or more antigens with molecular masses of 45, 59, 68, 79, 89, 95, and/or 110 kd. The most immunogenic antigens were p110 and p68, which induced responses in 33% (95% CI = 22%–44%) and 25% (95% CI = 15%–35%) of patients, respectively. Both antigens were commonly expressed on different melanomas, but they were absent on autologous normal tissue and on an unrelated allogeneic tumor. All the above antigens are attractive candidates for vaccine construction. [J Natl Cancer Inst 1998;90: 146–9]

A major challenge in the design of effective vaccines for melanoma is the identification of candidate melanoma antigens for vaccine development. The minimal essential requirements for such antigens are that they be immunogenic in humans and be expressed by melanoma cells in vivo. Two major approaches are currently being used to identify candidate antigens for vaccines; neither of these approaches is completely satisfactory. The first approach involves identifying antigens expressed on surgically resected melanoma tissue in vivo that are reactive with ‘‘natural’’ melanoma-specific T cells or melanoma-specific antibodies present in patients with this cancer (2–5). Unfortunately, while this approach identifies molecules that are antigenic (i.e., able to react with an immune cell or antibody), it does not provide direct evidence of immunogenicity (i.e., the ability to stimulate an immune response in humans). The second approach is to directly identify antigens that are immunogenic in humans, as evidenced by their ability to stimulate an immune response in patients immunized to the antigen (6–10). With this approach, however, the source of antigen for immune assays is usually the cells or antigen extract that was used to prepare the vaccine; therefore, it is difficult to exclude the possibility that the induced responses are directed against artifacts in the vaccine preparation. Neither approach identifies antigens that can stimulate tumor-protective immune response, which can be evaluated only by subsequent analysis of the effects of active immunization to the antigen on tumor progression.

This report describes the identification of multiple melanoma antigens that are both immunogenic in humans and expressed in vivo. The strategy used to achieve that goal was to employ vaccine-induced antibodies as probes to identify the immunogenic proteins in melanoma tissues. Extracts prepared from fresh, surgically resected melanoma tissue were used as a source of target antigens expressed in vivo and to avoid detection of antibodies to antigens that may be artifacts of the vaccine construction proteins. A polyvalent vaccine that contains a broad range of potential immunogens was used to detect and to compare the immunogenic potency of different antigens.

**Materials and Methods**

**Melanoma Vaccine**

A soluble, partially purified, polyvalent melanoma antigen vaccine was prepared from the material shed into culture by four human melanoma cell lines (SF-SKMel128, SF-M14, SF-M20, and SF-HM54) that were adapted to long-term growth in serum-free medium, as previously described (11,12). Briefly, these cells were incubated at 2 x 10^6/mL in serum-free RPMI-1640 medium for 3 hours at 37°C. The spent culture medium from each cell line was collected, centrifuged at 100,000g for 90 minutes at 4°C to remove particulate material, and concentrated, and equal protein amounts of the concentrated shed material were collected from the four cell lines pooled. The non-ionic detergent Nonidet P-40 was added to a final concentration of 0.5% to the pooled shed material, which was then ultracentrifuged at 100,000g for 90 minutes at 4°C to remove insoluble material, dialyzed against normal saline, and sterilized by filtration through a 0.22-μm filter. The protein concentration of the final product was adjusted to 0.2 mg/mL and dispensed into pyrogen-free glass vials. For use, the vaccine was usually admixed with alum as an adjuvant. The biochemical and antigenic properties of the vaccine have been published (12).

**Patients and Immunizations**

Sixty-nine sequentially registered patients (43 men and 26 women between the ages of 18 and 75 years) with surgically resected melanoma (American Joint Commission on Cancer [AJCC] stage III) were enrolled in this study, which was conducted at the New York University Medical Center from 1992 to 1995. Other criteria for patient selection were as follows: no evidence of distant metastatic disease, no history of other cancers or other serious systemic disease, positive skin test response to recall antigens or ability to be sensitized to dinitrochlorobenzene, and no prior therapy for melanoma other than surgery or local radiotherapy. All patients signed informed consent. The patients were immunized with 20–40 μg of vaccine protein administered intradermally every 3 weeks four times and then monthly three times and at longer intervals thereafter. No other therapy for melanoma or immunosuppressive agents were given to the patients while they were being treated with the vaccine. Sera were collected from the patients before the first immunization and 1 week following six to eight immunizations. The sera were stored at −80°C until used.

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See “Notes” following “References.”

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Preparation of Antigen Extracts for Immunoblotting

Two lots of melanoma antigen extracts were prepared; each was from equal volumes of surgically resected tumor tissue obtained from two separate patients. Lot 1 was prepared from hepatic metastases resected from one patient and from splenic metastases resected from a second patient; lot 2 was prepared from metastases resected from the chest wall of one patient and from the large intestine in a second patient. To prepare tumor tissue extracts, we separated the melanoma tissue from surrounding normal tissue and removed necrotic material. The tumor tissue was finely diced and homogenized, and nuclear and other cellular debris was removed by three cycles of centrifugation at 10000 rpm for 10 minutes at 4 °C. The supernatants were ultracentrifuged at 105 000 rpm for 1 hour at 4 °C, and the pellets were resuspended in Tris–EDTA buffer (pH 8.0) and ultracentrifuged again at 105 000 rpm for 20 minutes at 4 °C. The resulting pellets were solubilized in 6 M urea and stored at −80 °C. A normal autologous tissue extract was prepared similarly from normal uninvolved liver and spleen obtained from the patient used to prepare melanoma antigen extract lot 1. A similar procedure was used to prepare antigen extracts from a control human parotid mixed tumor. Normal tissue from the patient from whom lot 2 was prepared was not available. Total protein concentration in all lots of antigens was measured by use of a Bio-Rad kit (Bio-Rad Laboratories, Paris, France) and normalized to 240 μg/g.

Assay of Melanoma Antibodies

Antibodies to melanoma and the identity of antigens to which they were directed were determined by immunoblotting. Antigen extracts (240 μg of protein) were admixed with Laemmli’s buffer (13) and 2-mercaptoethanol, boiled for 5 minutes, and centrifuged at 6400 rpm for 2 minutes at 4 °C. The entire aliquot was run on sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (13). Proteins were transferred onto the PVDF (Immobilon-P; Millipore Corp., Bedford, MA) membrane in 0.192 M glycine and 0.025 M Tris (pH 8.3) without methanol, blocked in 5% low-fat milk, and washed three times in 0.05% Tween 20 in phosphate-buffered saline (PBS). The membrane was then cut into equal strips (each strip containing 10 μg of protein) and incubated overnight in a 1:50 dilution of the patient’s serum (unabsorbed). The strips were washed seven times with 0.05% Tween 20 in PBS, incubated for 4 hours in a 1:100 dilution of horseradish peroxidase-conjugated anti-human antibody [F(ab′)2 fragment; Sigma Chemical Co., St. Louis, MO] in 0.3% Tween 20 in PBS, washed seven times with 0.05% Tween in PBS, and incubated with a substrate containing 0.01% hydrogen peroxide and 0.5 mg/mL 4-chloro-1-naphthol. Vaccine-induced antibodies were evidenced by bands that were present in serum from the patient after vaccine treatment but absent or present in lesser density (<50% decrease) in baseline serum obtained from the same patient before vaccine treatment. Approximate 95% confidence intervals (CIs) were generated by use of the formula

\[ p \pm 1.96 \sqrt{\frac{p \times q}{n}} \]

where \( p \) and \( q \) are the proportions with and without antibody responses, respectively, and \( n \) is the total number of patients, i.e., 69.

Results

The ability of vaccine treatment to stimulate antibodies to melanoma antigens expressed in vivo was investigated by a comparison of the pattern and level of melanoma antibodies in sera collected from 69 patients with surgically resected AJCC stage III malignant melanoma prior to vaccine treatment and 1 week after six to eight immunizations. The same melanoma antigen extract (lot 1), pooled from metastases resected from two patients, was used for all assays. The results are illustrated in Fig. 1 and summarized in Table 1. Vaccine treatment induced a new antibody response, or augmented a pre-existing response, to one or more antigens expressed in surgically resected melanoma tissue in 35 (51% [95% CI = 39%–63%]) of 69 patients. The antibodies were directed to antigens of molecular masses of 45, 59, 68, 79, 89, 95, and/or 110 kD. Vaccine-induced immune responses were directed most commonly to the p110 antigen and to the p68 antigen (in 33% [95% CI = 22%–44%] and 25% [95% CI = 15%–35%] of patients, respectively) and least commonly to the p89 antigen and to the p45 antigen (in 10% [95% CI = 3%–17%] and 15% [95% CI = 7%–23%] of patients, respectively).

The specificity of the antigens defined by vaccine-induced antibodies was investigated by the measurement of their expression in extracts of surgically resected normal tissue and non-melanoma tumor, by use of a post-vaccine treatment serum with high levels of antibodies to the target antigens as a probe. The tissues tested included normal autologous tissues obtained from sites (liver in one patient and spleen in the other) adjacent to the metastases used to prepare melanoma antigen extract lot 1, another melanoma antigen extract (lot 2) prepared from metastases resected from two other patients, and an unrelated tumor (a parotid mixed tumor). All extracts were prepared identically and tested at identical protein concentrations. The results are illustrated in Fig. 2 and summarized in Table 2. None of the melanoma antigens targeted by these antibodies were detected in the autologous normal tissue. With the exception of antigens p68 and p89, none were detected in extracts of the parotid mixed tumor. The antigens were commonly expressed in melanoma, inasmuch as all were detected in a second lot of melanoma antigen extract (lot 2) prepared from two additional patients.

Discussion

This study shows that immunization to a polyvalent melanoma vaccine prepared from antigens shed from melanoma cells cultured in vitro induces antibody responses to multiple melanoma antigens of molecular masses 45, 59, 68, 79, 89, 95, and/or 110 kD that are expressed in vivo in fresh melanoma tissue. This observation demonstrates that the vaccine contains multiple antigens that are immunogenic in humans and that the antimalanoma antibody responses that these antigens induce are not directed to artificial antigens; moreover, it identifies these antigens as candidates for construction of melanoma vaccines.

There is presently considerable interest in the development of vaccines to treat, and possibly to prevent, some cancers. To be effective, such vaccines must be able to stimulate antitumor immune responses directed to antigens that are expressed in vivo by the tumor. This requires that the vaccine contains tumor antigens that are both immunogenic in humans and expressed in vivo (1). In the
case of malignant melanoma, the identity of such antigens remains to be fully defined.

Few melanoma antigens satisfy the criteria of being both immunogenic in humans and expressed in vivo by melanoma. These include the GM2 and GD2 gangliosides (8,14). These gangliosides are weakly immunogenic, requiring conjugation to a carrier protein and administration with potent adjuvants to induce long-lasting antibody responses in patients (15). Other melanoma antigens present in vaccines are immunogenic in humans, as determined by the induction of antibodies, but their expression in vivo is unknown. These include a number of antigens ranging in molecular masses from 34- to 100-kd antibodies that were induced by immunization in molecular masses from 34- to 100-kd antibodies that were induced by immunization to a melanoma vaccinia viral oncolysate (6); a 31-kd glycoprotein antigen to which an antibody was induced by a vaccinia virus melanoma oncolysate (9); and cell surface antigens of 38-43, 75, 110, 150, and 210 kd induced by the polyvalent shed antigen vaccine used in the current study (7). However, in all these cases, the antigen source used to detect vaccine-induced antibodies was derived from cultured cells, making it difficult to exclude the possibility that the antibodies were induced and directed to artifacts of tissue culture rather than being expressed in vivo. Other melanoma antigens are known to be expressed in vivo and to react with human T cells obtained from patients with melanoma, but their immunogenicity in vivo is unknown. These include the MAGE-1, MAGE-3, and MART-1 peptides (3,4), gp100 (16), and tyrosinase (17), all of which are recognized by human leukocyte antigen (HLA)-restricted cytotoxic lymphocytes obtained from patients with melanoma. However, although these studies show that these molecules are antigenic in vitro, they do not establish with certainty whether they are immunogenic in vivo because the lymphocytes could have been sensitized by different or cross-reacting antigens.

In this study, our strategy to identify antigens that are both immunogenic in humans and expressed in vivo has been to use as probes antibodies induced by vaccine immunization. The detection of several antigens that satisfy this definition was made feasible by immunization of patients to a polyvalent melanoma vaccine that contains a broad range of potential immunogens. A large number of patients was studied (n = 69) to permit the evaluation of relative immunogenicity of the antigens and the identification of

### Table 1. Generation of vaccine-induced antibodies that react with antigens present in fresh melanoma tissue

<table>
<thead>
<tr>
<th>Melanoma antigen, kd</th>
<th>Vaccine-induced responses, No. (%)‡</th>
<th>Vaccine-enhanced responses, No. (%)§</th>
<th>Any antibody response to immunization,[¶] No. (%)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>9 (13)</td>
<td>14 (20)</td>
<td>23 (33; 22–44)</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>10 (15)</td>
<td>5 (7)</td>
<td>15 (22; 12–32)</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>2 (3)</td>
<td>7 (10; 3–17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>9 (13)</td>
<td>2 (3)</td>
<td>11 (16; 7–25)</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>10 (15)</td>
<td>7 (10)</td>
<td>17 (25; 15–35)</td>
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<tr>
<td>59</td>
<td>9 (13)</td>
<td>6 (9)</td>
<td>15 (22; 12–32)</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>8 (12)</td>
<td>2 (3)</td>
<td>10 (15; 7–23)</td>
<td></td>
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<tr>
<td>Any antigen</td>
<td>23 (33)</td>
<td>24 (35)</td>
<td>35 (51; 39–63)</td>
<td></td>
</tr>
</tbody>
</table>

*Total number of patients = 69.
†Measured by immunoblot analysis, with the use of a membrane fraction of fresh melanoma tissue as the antigen source.
‡Antibody band present in post-treatment but not pretreatment serum in same patient.
§Density of antibody band greater in post-treatment serum than in pretreatment serum.
¶Approximate 95% confidence intervals were generated by use of the formula

\[
(p ± 1.96 \sqrt{\frac{pq}{n}})
\]

where \( p \) and \( q \) are proportions with and without antibody responses, respectively, and \( n = 69 \), i.e., the total number of patients.

### Table 2. Tissue distribution of melanoma antigens defined by antibodies induced by vaccine immunization

<table>
<thead>
<tr>
<th>Antigen expression*</th>
<th>Melanoma antigen lot 1†</th>
<th>Melanoma antigen lot 2‡</th>
<th>Normal autologous tissue§</th>
<th>Unrelated tumor¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>+</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>89</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>+</td>
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<td>79</td>
<td>++</td>
<td>+</td>
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<td>–</td>
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<td>68</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>59</td>
<td>++</td>
<td>++</td>
<td>Not tested</td>
<td>–</td>
</tr>
<tr>
<td>51</td>
<td>+</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>45</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*The number of + signs are indicative of relative band density on immunoblots. + = the lowest; +++ = the highest; – = absence of the band(s).
†Prepared from metastatic nodules obtained from the liver and spleen of two different patients.
‡Prepared from metastatic nodules obtained from the chest wall and bowel of two different patients.
§Normal tissue obtained from autologous liver and spleen from patients utilized for the preparation of melanoma pool #1.
¶Parotid mixed tumor.
weakly immunogenic antigens that induce responses in only a small proportion of patients. The latter antigens are still potentially valuable for vaccine development because the immunogenicity of even weakly immunogenic entities can be markedly increased by appropriate immunizing strategies (15).

Our results indicate that a large number of antigens expressed by melanoma cells in vivo can be immunogenic in humans. These include proteins with molecular masses of 45, 59, 68, 79, 89, 95, and 110 kd. Vaccine-induced antibody responses to one or more of these antigens were detected in approximately half of 69 sequentially registered patients with surgically resected stage III melanoma. The immunodominant antigens (p110 and p68) were those of molecular masses of 110 kd and 68 kd and stimulated antibody responses in 33% and 25% of patients, respectively. All of these antigens appear to be melanoma associated, inasmuch as none could be detected in normal tissue. They appear to be common melanoma antigens because all could be detected in different melanomas. The 89-kd and 68-kd antigens were weakly expressed on an unrelated cancer. The relation of these antigens to previously described melanoma antigens remains to be defined. However, all but p45 are unrelated to HLA antigens on the basis of their molecular size. If these antigens are shown to differ from currently known melanoma antigens, the sera used to identify them could be used for cloning them.

In summary, we have demonstrated that a polyvalent melanoma vaccine prepared from antigens shed from cultured melanoma cell lines can stimulate immune responses to multiple melanoma antigens expressed in vivo. These antigens are attractive candidates for the development of melanoma vaccines because they possess the dual property of being immunogenic in humans and selectively expressed by melanoma cells in vivo.

References


Notes

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