Immunological Characterization of a Human High Molecular Weight – Melanoma Associated Antigen Identified with Monoclonal Antibodies

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Summary. Sodium dodecyl sulfate polyacrylamide gel analysis of a high molecular weight (HMW) human melanoma associated antigen (MAA) defined by murine monoclonal antibodies revealed a number of distinct polypeptides ranging from 80,000 up to 280,000 daltons, in addition to an extremely heterogeneous group of components distributed over a wide range in apparent molecular weight (300,000–700,000 daltons). The 280,000 dalton and the larger heterogeneous molecular weight material are glycosylated since they are labeled with ³H-sugars. The HMW-MAA is readily solubilized in the absence of detergents and the entire series of polypeptides fractionates together in the void volume of a Sephadex G200 column. Peptide maps of the various polypeptides of the HMW-MAA, generated by Staphylococcus aureus V-8 protease, are essentially the same except that some of the proteolytic fragments derived from the lower molecular weight polypeptides (80,000 daltons) are present in greater amounts than are similar fragments derived from the larger molecular weight polypeptides; the latter finding suggests that the complexity in molecular weight of the MAA may reflect combinations of several base subunits. Proteolytic cleavage of the HMW-MAA generates a number of peptides ranging in molecular weight from 77,000 daltons to less than 12,000 daltons, which still react with monoclonal antibodies and can distinguish monoclonal antibodies specific for different antigenic determinants of this MAA.

Introduction

Serological analysis of human melanoma has identified various types of antigens not detectable on normal melanocytes and these antigens are referred to as melanoma-associated antigens (MAA). Clinical evidence suggests that these markers and the corresponding antibodies may play a role in the biology of the tumor, since the presence of antibodies in patients correlates with the stage of the disease [for review, see 18], and expression of some MAA is associated with a favorable clinical course [23]. Characterization of MAA was initially accomplished with antibodies from the sera of melanoma patients and from animals immunized with cultured melanoma cells or purified MAA. More recently, the hybridoma technique [15] has been applied to this problem and the efforts of several laboratories have resulted in the development of monoclonal antibodies to MAA [3, 4, 7, 9–11, 14, 17–19, 25, 28]. The availability of monoclonal antibodies has allowed for serological analysis at the antigenic determinant level and has facilitated the application of immunochemical approaches to define the structure of MAA. Several MAA have now been defined and their molecular profile characterized. These include MAA with single-chain structures [7, 10, 14, 18, 27, 28] as well as some composed of two or more polypeptide chains [10, 12, 14, 19, 25]. We have identified a multichain MAA of high molecular weight (HMW-MAA) which is expressed by melanomas, nevi, and some skin carcinomas but is undetectable on melanocytes and other normal and tumor tissues [19, 25]. In this paper we present an immunological analysis of this HMW-MAA, illustrating some of its unusual biochemical characteristics.

Materials and Methods

Radiolabeled Melanoma Cell Extracts. The melanoma cell lines M16, M21, and Colo38, maintained by serial passage in RPMI 1640 medium containing 10% calf serum, were surface labeled with ¹²⁵I (Amersham, Arlington Heights, IL, USA) using Iodogen as described by Salisbury and Graham [21]. Alternatively, cells were intrinsically labeled with ³H-sugars or with ³⁵SO₄ (both from Amersham, Arlington Heights, IL, USA) as described previously [24]. Labeled cells were washed three times with phosphate-buffered saline (PBS) pH 7.0 and then shaken for 30 min at 4 °C with 10 volumes of the appropriate extraction buffer (see Table 1) containing 10 μM of the fresh proteolytic inhibitor phenylmethylsulfonylfluoride. The extract was then cleared by centrifugation at 7,000 g and stored at −20 °C. Extraction buffers containing 3 M KCl or urea were dialyzed against PBS prior to reaction with antibody-linked Sepharose.

Monoclonal Antibodies (MoAb) to the HMW-MAA. The production, serological analysis, and immunochemical characterization of the three monoclonal antibodies 138.13, 225.28S, and 653.40S, which react with the same or close-ly-associated antigenic determinant [13], have been described previously [11, 19, 25, 27]. Briefly, MoAb 138.13 and 225.28S, both IgG2a, and MoAb 653.40, IgGl, react in binding assays with cultured melanoma cell lines but not with cultured carcinoma and lymphoid cells [19, 25, 27]. Indirect immunofluorescence analysis on cryostat thin sections of human biopsy material showed that these monoclonal antibodies react with melanoma, nevi, and skin carcinomas but fail to react with a large variety of normal and malignant human cells [19, 25, 27].
Table 1. Solubilization of the high molecular weight MAA detected by monoclonal antibody 225.28S

<table>
<thead>
<tr>
<th>Solubilization conditions</th>
<th>% Radioactivity solubilized from cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Radioactivity associated with HMW-MAA from solubilized extracts&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Total HMW-MAA solubilized&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>10 mM phosphate buffer pH 7.3</td>
<td>56</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>3 M KCl</td>
<td>51</td>
<td>0.9</td>
<td>24</td>
</tr>
<tr>
<td>0.1 M Urea/PBS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36</td>
<td>0.6</td>
<td>11</td>
</tr>
<tr>
<td>1.0 M Urea/PBS</td>
<td>43</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>1% NP40/10 mM phosphate</td>
<td>67</td>
<td>2.7</td>
<td>91</td>
</tr>
<tr>
<td>1% NP40/PBS</td>
<td>75</td>
<td>2.6</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were surface-labeled with <sup>125</sup>I by the iodogen procedure

<sup>b</sup> Determined by immunoprecipitation with MoAb 225.28S

<sup>c</sup> Determined relative to NP40-PBs

<sup>d</sup> PBS, phosphate-buffered saline pH 7.1

Another monoclonal antibody, 149.53, produced as described previously except that the immunogen was melanoma cells Colo38, reacts with the same structure as the other three antibodies but identifies a spatially distinct antigenic determinant since MoAb 149.53 does not block the binding of MoAb 225.28S to the HMW-MAA [26].

**Immunoadsorbent Purification of the HMW-MAA**
The monoclonal antibodies isolated from ascites fluids by precipitation with NH<sub>4</sub>SO<sub>4</sub> (33% of saturation) were either covalently attached to Sepharose 4B (Pharmacia, Inc., Piscataway, NJ, USA) by the CNBr method [6] or bound to Protein A Sepharose 4B (Pharmacia). In most experiments a ratio of 5 x 10<sup>6</sup> cpm was reacted per 10 μl packed immunoadsorbent diluted to approximately 0.5 ml with NET buffer [1]. After 2 h rotation at 4 °C, the immunoadsorbent was washed seven times with NET and three times with PBS to retain antigenic determinants from all components identified by the monoclonal antibodies. To obtain higher concentrations of 125I-labeled antigen needed in the peptide mapping experiments, large volumes of immunoadsorbents (1 ml) were eluted by boiling for 5 min with 0.2% SDS, concentrated by evaporation under vacuum, and then reconstituted to 10<sup>6</sup> cpm elution volume with 0.125 M Tris buffer pH 6.8.

**Gel Electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was performed in sodium dodecyl sulfate (SDS) using the buffer system described by Laemmli [16]. Gels were cast as 80 x 1.5 mm slabs and electrophoresed at 3 mV/gel constant current until the bromphenol blue tracking dye reached the bottom of the gel. The gels were stained with Coomassie blue in 40% methanol, 7.5% acetic acid, destained in 10% methanol, 10% acetic acid, and dried under vacuum. Kodak XAR-OMAT film was sandwiched between a dried gel and a Dupont Cronex High-plus intensifying screen for 1–6 days at -70°C.

Peptide mapping by partial proteolysis in SDS was performed using the Cleveland method [5] as modified for two-dimensional analysis by Bordier and Crettol-Harviner [2].

The first dimension was done in 1.5 mm thick slab gels and the second dimension in 2 mm thick slab gels. The only modification used here was that the *Staphylococcus aureus* V-8 protease (1 mg/ml) (Miles Laboratories, Inc. Elkhart, IN, USA) was mixed with bovine serum albumin (Fract V, Miles Laboratories, Inc. Elkhart, IN, USA) (1 mg/ml) to assure a similar enzyme/substrate ratio over the entire width of the gel.

One-dimensional SDS-PAGE analysis of antigenically active proteolytic fragments detectable with monoclonal antibodies was performed as follows: a hypotonic extract of 125I-labeled melanoma cells Colo38 was digested at 37° C, pH 7.4 with trypsin 10 mg/ml (Sigma Chemical Co., St Louis, MO, USA), chymotrypsin (Sigma Chemical Col, St Louis, MO, USA), Pronase (Calbiochem, Behring, La Jolla, CA, USA), and V-8 protease from *Staphylococcus aureus* (Miles Laboratories, Inc., Elkhart, IN, USA).

**Results**

**Solubilization of the HMW-MAA**
Surface-radiolabeled melanoma cells Colo38 were extracted with various agents and the extracts were analyzed for the total amount of radioactivity specifically bound by insolubilized MoAb 225.28S (Table 1). The nonionic detergent NP40 was three times more efficient than either high- or low-salt in solubilizing the HMW-MAA; the least efficient extraction buffer contained either 0.1 or 1.0 M urea. Using detergent solubilization, the MAA detected by MoAb 225.28S represented up to 2.5% of all the radioactivity released by the cells.

**Subunit Structure of the HMW-MAA**
An NP40 PBS extract of surface-radioiodinated melanoma cells was reacted with MoAb 138.13, 149.53, 225.28S, 653.40S insolubilized to Sepharose 4B and the bound antigens analyzed by SDS-PAGE. A representative gel pattern typical for all four monoclonal antibodies is shown in Fig. 1. Up to 12 distinct molecular-weight components can be seen when SDS-PAGE is performed in 7.5% polyacrylamide. The most typical size components are a very high molecular weight component at the top of the gel, a 280,000 dalton polypeptide, a 240,000 dalton polypeptide, a series of polypeptides of around 120,000 daltons, and three polypeptides around 80,000 daltons. Interestingly, the amount of 125I associated with the various components is greatest at the highest molecular weight and decreases proportionally with polypeptide size. The mobility of these various components in SDS-PAGE is not changed in the absence of a reducing agent (2-mercaptoethanol), indicating that none of the components identified by the monoclonal antibodies are bridged by disulfide bonds (data not shown). It should be noted that immunoprecipitation of these 125I-labeled extracts with monoclonal antibodies to HLA-A, B antigens or to human Ia antigens and SDS-PAGE analysis showed the typical two-chain structures without evidence of technical artifacts.

To increase the mobility of the labeled component at the top of the 7.5% acrylamide gel and therefore obtain an estimate of its apparent molecular weight, gels containing different mixtures of polyacrylamide (2%–5%) and agarose (0.5%–1%) were tested for their ability to separate high molecular weight standards. A gel containing 3% polyacrylamide and 0.75% agarose was chosen because it allowed the...