Detection of Antibodies to Human Melanoma Cell in Vitiligo by Western Blot Analysis

Seung-Kyung Hann and Jung-Bin Kim

Vitiligo is a disease in which melanocytes are selectively destroyed. The disease is thought to be an autoimmune process being there are antibodies to pigment cells in the sera of patients and animals with vitiligo. In the present study, sera from vitiligo patients were examined for reactivity with the human melanoma cell line, SK-Mel-28, by Western blot analysis of solubilized membrane antigens of these cells to identify the pigment cell antigens defined by antibodies in the patients with vitiligo.

Antibody reactivity to human melanoma cells (SK-Mel-28) was investigated in 14 patients with vitiligo, and 16 with normal control individuals. Antibodies to the 116-113, 60, 40 KD antigens were associated with vitiligo being present in 79%, 86%, and 43% respectively of the patients with vitiligo, but in only 6%, 38% and 6% of the normal controls. In contrast, antibodies to the 160-155, 78 and 64 KD antigens were equally common in vitiligo and in normal individuals.

The results suggest that autoreactivity to pigment cells occurs more commonly in patients with vitiligo than in the normal control and high autoreactivity to pigment cells in the vitiligo sera might be an impertinent epiphemenon to destroyed pigment cell.

Key Words: Vitiligo, antibodies, pigment cell

Vitiligo is a disease in which melanocytes are selectively destroyed. The disease is an autoimmune process on the ground of the following findings that there are antibodies to pigment cells in the sera of patients and animals with vitiligo (Lamont et al. 1982; Naughton et al. 1983a; Naughton et al. 1983b; Bystryn and Naughton, 1985; Naughton et al. 1986a), that there is a correlation between the presence and level of these antibodies to the extent (Naughton et al. 1986b) and activity of vitiligo (Harning et al. 1991) and that these antibodies can selectively kill human melanocytes in vitro (Norris et al. 1988). However, it is not known whether these antibodies actually injure melanocytes in vivo or if they are simply an interesting epiphenomena.

In the present study, sera from patients with vitiligo were examined for reactivity with the human melanoma cell line, SK-Mel-28, by Western blot analysis of solubilized membrane antigens of these cells to identify the pigment cell antigens defined by antibodies in the patients with vitiligo.

MATERIALS AND METHODS

Sera

Sera were obtained from 14 patients with active vitiligo and 16 normal individuals who did not have any autoimmune diseases. The activity of vitiligo was determined by a questionnaire on the history of the illness taken during each patient's visit to our clinic. Active disease was defined as the appearance
of new lesions or the extension of existing lesions within 3 months prior to the collection of sera.

Cells

Pigment cell, M-28 Human melanoma cell line (SK-Mel-28), was obtained from American Tissue Culture Collection (Rockville, MD, USA) and was maintained in culture in RPMI 1640 medium supplemented with 10% fetal calf serum.

SK-Mel-28 membrane preparation

Cells were harvested using 1mM EDTA in Hank’s balanced salt solution and homogenized in sucrose buffer (0.25 M sucrose, 0.5 mM MgCl₂, 50 mM Tris, 1 mM PMSF, 1μM leupeptin, pH 7.4) at 4°C in a glass homogenizer. The homogenate was centrifuged at 2,000 × g for 10 min, and the supernatants were pooled and centrifuged at 105,000 × g for 1 h. The resulting pellet was washed in 0.15M NaCl, 50 mM Tris, 1 mM EDTA buffer, pH 8.0, re-suspended in 0.5% NP-40 to a final concentration of 2mg/ml, and stored at −70°C until use.

SDS-PAGE and Western blotting procedures

Cell lysates contained 200mg protein were run on SDS-8% PAGE under reducing conditions (2-mercaptoethanol and boiling) and electroblotted onto polyvinylidene difluoride microporous membranes. The membranes were blocked with 5% non-fat milk in PBS (pH 7.4) for 2 h at 25°C and reacted with the individual sera, diluted 1:100 in blocking buffer, overnight at 4°C. The membranes were then incubated with biotinylated goat anti-human IgG or IgM monospecific antisera (Organon Technika, Westchester, PA, USA) diluted 1:100 for 2 h and then avidin-peroxidase (Organon Technika, Westchester, PA, USA), diluted 1:100, for 1 h and developed with 4-chloro-l-napthol with intervening washes in PBS-Tween.

Measurement of level of antibodies

The level of antibody to individual antigens was estimated by quantitative densitometry using an image 1 system gel scanning system (Universal Imaging Corporation, Media, PA, USA) to measure band intensity. The level of individual antibodies was calculated by subtracting background density from band density. Total level of all antibodies in a serum was calculated from the sum of individual antibody level in that serum.

RESULTS

Sera of 14 patients with active vitiligo and 16 normal individuals were tested for antibodies to pigment cell antigens by Western blot analysis. The sera of patients and normal control showed antibody responses to multiple antigens on Western blot analysis (Fig. 1). We selected several significant molecular weight bands including 160-155, 116-113, 78, 64, 60, 40 KD and analyzed the incidence of positivity and density of the bands. The results are shown in Table 1, 2. Antibodies to the 116-113, 60, 40 KD antigens were associated with vitiligo being present in 79%, 86%, and 43% respectively of patients with vitiligo, but in only 6%, 38%, and 6% of the normal controls. By contrast, antibodies to the 160-155, 78 and 64 KD antigens were almost equally common in vitiligo and normal individuals. Antibody levels were estimated from the density of the bands on Western blot, as quantitated by computer-assisted densitometry. The analysis was restricted to patients who were antibody positive. The average level of antibody to the major antigens 116-113, 64, 60, 40 KD in patients with vitiligo was higher than in the normal controls.

DISCUSSION

In the present study, using a Western blotting assay that measures antibodies to melanoma membrane antigens, we have found that patients with vitiligo have antibodies to antigens with MWs of 160-155, 116-113, 78, 64, 60, and 40 KDs. But antibodies to the 116-113, 60
Fig. 1. Immunoblot analysis of SK-Mel-28 antigens defined by vitiligo and control sera. Reduced 0.5% NP-40 extractable proteins derived from SK-Mel-28 membrane preparation were separated by SDS-8% PAGE and immunoblotted using vitiligo, control sera diluted 1:100 and then incubated with goat anti-human IgG antisera. Each lane contained 10 μg protein.

Table 1. Incidence of antibodies to melanoma 28 membrane antigen in vitiligo

<table>
<thead>
<tr>
<th>Antigen molecular weight</th>
<th>Patients with antibodies</th>
<th>Vitiligo (n=14)</th>
<th>Normal control (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160-155 KD</td>
<td></td>
<td>15 (94%)</td>
<td></td>
</tr>
<tr>
<td>116~113 KD</td>
<td></td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>78 KD</td>
<td></td>
<td>16 (100%)</td>
<td></td>
</tr>
<tr>
<td>64 KD</td>
<td></td>
<td>13 (81%)</td>
<td></td>
</tr>
<tr>
<td>60 KD</td>
<td></td>
<td>6 (38%)</td>
<td></td>
</tr>
<tr>
<td>40 KD</td>
<td></td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>any antigen</td>
<td></td>
<td>13 (81%)</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05 by Fisher’s Exact Test

Table 2. Level of antibodies to melanoma 28 membrane antigen in vitiligo

<table>
<thead>
<tr>
<th>Antigen molecular weight</th>
<th>Antibodies Level*</th>
<th>Vitiligo (n=14)</th>
<th>Normal control (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160-155 KD</td>
<td>31.28±8.24</td>
<td>25.39±7.48</td>
<td></td>
</tr>
<tr>
<td>116~113 KD</td>
<td>39.07±7.71</td>
<td>3.42</td>
<td></td>
</tr>
<tr>
<td>78 KD</td>
<td>130.21±24.25</td>
<td>121.46±19.25</td>
<td></td>
</tr>
<tr>
<td>64 KD</td>
<td>92.46±13.70</td>
<td>62.06±12.90</td>
<td></td>
</tr>
<tr>
<td>60 KD</td>
<td>72.30±12.68</td>
<td>31.66±10.67</td>
<td></td>
</tr>
<tr>
<td>40 KD</td>
<td>46.61±14.26</td>
<td>2.43</td>
<td></td>
</tr>
</tbody>
</table>

* Values are mean±SD

* p<0.05 by t-test
and 40 KD antibodies were associated with vitiligo, as antibodies to the 160-155, 78 and 64 KD antigens were almost equally common in vitiligo and normal individuals.

The antibodies detected by immunoprecipitation in patients with vitiligo in another study (Cui et al. 1992) reacted with antigens of 35, 40-45, 75, 90, 150 KD. These detected antigens were present mostly on the surfaces of both human melanocytes and melanoma cells. The antigens detected in another study (Cui et al. 1992) were iodinated by lactoperoxidase technique which labels preferentially the cell surface antigen suggesting that they may be expressed on the surface of pigment cell. We assumed that some differences in the pigment cell antigen between ours and Cui et al’s study may come from the assay method and cell preparation used. The nature of the SK-Mel-28 antigens recognized mostly by the sera of patients with vitiligo in this experiment is not definitely known. We think that membrane preparation of SK-Mel-28 could have the solubilized proteins of mostly cell membrane and some several cytoplasmic components including melanosome, mitochondria, lysosome, etc. Therefore the antibodies detected by Western blot analysis in our patients with vitiligo might be directed to multiple antigens on mostly the membrane portion of the pigment cell. The multitude of pigment cell antigen found in patients with vitiligo in this study differs from a recent report by Song et al. (1994) who, using a similar Western blot technique, reported that patients with vitiligo had antibodies to only two pigment cell antigens- a 69 KD and a 60 KD protein (Song et al. 1994). These differences may reflect differences in the cell lines used as the antigen source for antibody assays (SK-mel-28 in our study compared to HTB-70 human melanoma cells in Song's studies), or in the methods used to extract the antigens (from a membrane fraction in our study compared to whole cell lysates in Song's studies).

In another study, there was an increased prevalence of reactivation to melanoma antigen of 53 KD in vitiligo sera (Galbraith et al. 1988). It is suggested from these findings that autoreactivity to pigment cell occurs in the patients with vitiligo. The disparity between the results obtained in the current and another report (Song et al. 1994) in the reactivity of vitiligo could be related with the human melanoma cell line used (SK-Mel-28 in our study compared to M14 in Galbraith's studies) or in the different antigen extraction method.

In summary, vitiligo sera showed higher incidence and level of autoreactivities to pigment cells. But the autoreactivities to pigment cells in the normal control were also high. These findings may suggest that the high incidence and level of autoreactivities to pigment cells in vitiligo sera could be an impertinent epiphenomenon to destroyed pigment cells such as melanocyte.

REFERENCES

Naughton GK, Reggiard D, Bystryn JC: Correlation between vitiligo antibodies and extent of depig-
Antibodies of Vitiligo

Norris DA, Kissinger RM, Naughton GK, Bystryn
JC: Evidence for immunologic mechanisms in
human vitiligo: patients' sera induce damage to
human melanocytes in vitro by complement-
mediated damage and antibody dependent cel-
lular cytotoxicity (ADCC). J Invest Dermatol 90:
783-789, 1988
Song YH, Connor E, Li Y, Zorovich B, Balducci P,
Maclaren N: The role of tyrosinase in autoim-