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Determination of Carrier Status for the Wiskott-Aldrich Syndrome by Flow Cytometric Analysis of Wiskott-Aldrich Syndrome Protein Expression in Peripheral Blood Mononuclear Cells

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The Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by severe recurrent infections due to immunodeficiency, tendency to bleeding due to thrombocytopenia with small platelets, and severe eczema (1, 2). The responsible WAS protein (WASP) gene was recently identified (3), and since then a lot of mutations in this gene have been reported with defective or reduced expression of WASP (4, 5). WASP is preferentially expressed in hemopoietic cells, such as lymphocytes, monocytes, and platelets (6). Although the definite WASP function remains to be determined, WASP seems to play roles in signal transduction pathways for cell growth (7) and in cytoskeletal organization in response to activation (8).

In a previous study, we have established the technique of flow cytometric analysis of intracellular WASP expression (FCM-WASP analysis) in lymphocytes (9) and demonstrated its clinical usefulness for the diagnosis of WAS patients. However, the results suggested that application of this technique for the carrier diagnosis is not possible because all the three carriers studied had only the WASPbright population of lymphocytes.

In this study, we used monocytes for FCM-WASP analysis, and found that all the nine carriers studied had both the WASPbright and WASPdim population in varying degree. None of control individuals possessed the WASPdim population. In contrast, lymphocytes from all the carriers except two lacked the WASPdim population. The difference of the WASPdim population in monocytes and lymphocytes observed in WAS carriers suggests that WASP plays a more critical role in the development of lymphocytes than in that of monocytes. The present studies suggest that a skewed X-chromosomal inactivation pattern observed in WAS carrier peripheral blood cells is not fixed at the hemopoietic stem cell level but progresses after the lineage commitment. The Journal of Immunology, 2000, 165: 1119–1122.

Materials and Methods

Carriers studied

Nine WAS carrier mothers from different families and a WAS patient, who had received bone marrow transplantation (BMT) from his carrier mother, were included in this study. One noncarrier mother of a WAS patient was also studied. The results of mutations in the WASP gene belonging to each family, some of which were reported elsewhere (10, 11), are shown in Table I. Carrier diagnosis was performed by molecular methods as described previously (10). Carrier mothers in families 1 and 3 are the mothers of patients 1 and 2 in the previous study (9). Carrier mothers in families 3 and 4 are unrelated but had the same mutated allele. BMT was performed in the patient in family 3 from his mother 3 years previously. The severity of WAS-associated symptoms of patients in all the families was scored from 1 to 5, based on the criteria by Zhu et al. (12).

FCM-WASP analysis

FCM-WASP analysis was performed as previously described (9) with minor modification. In brief, PBMC washed in PBS containing 1% FBS were treated with Cytofix/Cytperm solution from CytoStain kit (PharMingen, San Diego, CA) at 4°C for 30 min. They were then reacted with FITC-labeled goat anti-mouse IgG1 Ab (Southern Biotechnology Associates, Birmingham, AL). Samples, thus processed, were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Gating of lymphocytes or monocytes was

Abbreviations used in this manuscript: WAS, Wiskott-Aldrich syndrome; WASP, WAS protein; FCM-WASP, flow cytometric analysis of WASP expression; BMT, bone marrow transplantation.
made from a distribution pattern in forward and side scatter. A total of 20,000 events of each cell were studied.

**Results**

**Significant difference of WASP expression in monocytes between normal individuals and WAS patients**

As we have shown the significant difference of WASP expression in lymphocytes previously (9), the same difference of WASP expression in monocytes was observed between normal individuals and WAS patients. An example of a normal individual (A, WASP bright) and a WAS patient from family 3 (B, WASP dim) was shown (Fig. 1).

**WAS carriers possessed the WASP dim population in monocytes**

In all the carriers, the WASP dim population was detected in monocytes in variable degree with the major population of WASP bright. The pattern of the WASP dim population in WAS carriers was largely divided into three groups according to the proportion of WASP dim cells: skewed (A, <10%), moderately skewed (B, >10%, <30%), and random (C, >30%) groups (Fig. 2, A–C). Carriers in families 1 and 2 belong to group A, carriers in families 3, 4, 5, 6, and 10, and a patient in family 5 after BMT to group B, and carriers in families 7 and 8 to group C. The proportion of the WASP dim population ranged from 3.5% to 50.7% (Table I). None of control individuals including a noncarrier mother in family 9 possessed the WASP dim population (Fig. 2 D).

**The WASP dim population in lymphocytes was not detected in most of WAS carriers**

In contrast to FCM-WASP analysis in monocytes, most of carriers (seven of nine) were shown to have only the WASP bright population of lymphocytes (Fig. 3 and Table I). The WASP dim population of lymphocytes was detected only in carriers in families 7 and 8 (the data of a carrier in family 8 is shown in Fig. 3 C).

**Discussion**

In this study, we report that FCM-WASP analysis in monocytes could be a useful tool for WAS carrier diagnosis. All the definitely diagnosed WAS carriers possessed the WASP dim population of monocytes together with the WASP bright one. The proportion of the WASP dim population ranged from 3.5% to 50.7% and could be detected clearly.

In the previous study, we have established the technique of FCM-WASP analysis in lymphocytes (9) and demonstrated its clinical usefulness for the diagnosis of WAS patients. However, the application of this technique for the carrier diagnosis seemed not to be possible because all the three carriers studied had only the WASP bright population of lymphocytes. We could not find any difference between WAS carriers and control females in these studies. This finding is not surprising, because a skewed X-chromosomal inactivation pattern is observed in female carriers of some X-linked genetic disorders including WAS in constitutional or in some specific lineage cells. The cause of this phenomenon is attributable to negative selection of cells, which have the growth disadvantage due to the mutant gene on the active X chromosome.
Wengler et al. further reported that a skewed X-chromosomal inactivation pattern in WAS-obligate carriers occurs early during the hemopoietic differentiation, speculating that hemopoietic progenitors are affected by WASP mutations (14). In contrast, a random X-chromosomal inactivation pattern was reported in some carriers, although these reports are limited to carriers of X-linked thrombocytopenia or mild WAS with missense mutations (15).

In the present study, we performed FCM-WASP analysis in PBMC to study whether a skewed X-chromosomal inactivation pattern is universally observed in all hematological cells of WAS carriers. The results showed that all the WAS carriers have variable proportion of WASP dim population of monocytes, together with the WASP bright one. We then quantitatively evaluated the X-chromosomal inactivation pattern in monocytes from each carrier. The WASP dim monocyte population detected in carriers in families 1 and 2, whose WASP gene mutation seemed to result in nearly null function of WASP, was 5.4% and 3.5%, respectively, whereas carriers in families 7 and 8 who have the missense mutations were revealed to have the WASP dim population of 30.1% and 50.7%, respectively. These results may suggest the proportion of the WASP dim monocyte population correlates with their genotypes. However, a carrier in family 5 who was supposed to have the most severe mutation, and a carrier in family 6 with missense mutation, both belong to the same B group of moderately skewed pattern (the WASP dim population of 17.3% and 11.7%, respectively). Thus, genetic, stochastic, or other unknown factors might contribute to the skewed pattern in monocytes as well as types of WASP gene mutations.

In contrast to the observation in monocytes, we could not detect the WASP dim population of lymphocytes in seven of nine carriers. Only two carriers with random X-inactivation pattern in monocytes were shown to have the WASP dim population of lymphocytes. Even in the two carriers, the WASP dim population of lymphocytes was smaller than those of monocytes were. Thus, WASP seemed to be more critical for the development of lymphocytes than that of monocytes. Alternatively, accelerated cell death in WASP dim lymphocytes by spontaneous apoptosis (16) might be responsible for the lack of WASP dim lymphocytes in most of WAS carriers. We speculate that the mutant WASP in these two families still remains to have a residual function that makes the carriers’ WASP dim lymphocytes survive in their peripheral blood.

Although FCM-WASP analysis in monocytes is much simpler and faster than molecular methods for WAS carrier diagnosis, it is possible that some carriers will have undetectable percentages of WASP dim monocytes. Therefore, we should carefully rule out the possibility of carriers when the WASP dim population was not detected. And as was discussed for the diagnosis of WAS in the previous study (9), this method of carrier detection should be also limited to families where the affected boys have a WASP dim population, although a normal amount of WASP expression has not been reported in WAS patients.

We had a chance to study the WAS patient (patient in family 5) who had received BMT from his carrier mother (carrier in family 5) 3 years previously. He was shown to have almost the same

<table>
<thead>
<tr>
<th>Family No.</th>
<th>Mutation in the WASP Gene</th>
<th>Clinical Score of the Patient</th>
<th>Findings in the Carrier Mother</th>
<th>Proportion of the WASPdim population in</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arg211→stop (exon 7)</td>
<td>3 (BMT at 1 year)</td>
<td></td>
<td>5.4 (b)</td>
<td>38 (c)</td>
</tr>
<tr>
<td>2</td>
<td>Deletion including exons 3–7</td>
<td>4 (died at 3 years)</td>
<td></td>
<td>3.5</td>
<td>32 (c)</td>
</tr>
<tr>
<td>3</td>
<td>Glu31→Lys (exon 1)</td>
<td>2 (BMT at 1 year)</td>
<td></td>
<td>14.6</td>
<td>34 (c)</td>
</tr>
<tr>
<td>4</td>
<td>Glu31→Lys (exon 1)</td>
<td>3 (BMT at 1 year)</td>
<td></td>
<td>25.1</td>
<td>28 (c)</td>
</tr>
<tr>
<td>5</td>
<td>G deletion after Gly31 (exon 1)</td>
<td>4 (BMT at 4 years)</td>
<td></td>
<td>17.3 (18.5)</td>
<td>27 (c)</td>
</tr>
<tr>
<td>6</td>
<td>Tyr107→Cys (exon 3)</td>
<td>2→3 (7 years)→4 (11 years)→5 (25 years)</td>
<td></td>
<td>11.7</td>
<td>55 (c)</td>
</tr>
<tr>
<td>7</td>
<td>Pro69→Ser (exon 11)</td>
<td>3</td>
<td></td>
<td>30.1</td>
<td>34 (c)</td>
</tr>
<tr>
<td>8</td>
<td>Ile65→Thr (exon 2)</td>
<td>3</td>
<td></td>
<td>50.7</td>
<td>41 (c)</td>
</tr>
<tr>
<td>9</td>
<td>G deletion at exon 11/intron 11</td>
<td>3</td>
<td></td>
<td>–</td>
<td>39 (c)</td>
</tr>
<tr>
<td>10</td>
<td>C deletion after Asp59 (exon 9)</td>
<td>3</td>
<td></td>
<td>11.9</td>
<td>30 (c)</td>
</tr>
</tbody>
</table>

a The mother in family 9 was not a carrier.

b The WASP dim population was not detected.

c Observed in a patient of family 5 after BMT.
proportion of the WASPdim population of monocytes as his mother (18.5% vs 17.3%). Also similar to his mother, he did not have WASPdim lymphocytes, either. Because the karyotype of the patient had completely changed to 46, XX after BMT, both lymphocytes and monocytes analyzed were originated from the hematological progenitor cells of the carrier mother. The results indicate that transplantable hematopoietic progenitor cells in the carrier mother was not severely skewed, and the proportion of the reconstructed WASPdim monocyte population was reproducible in vivo.

We previously demonstrated that most of the WAS carriers possessed lymphocytes and granulocytes expressing the mutant WASP message by allele-specific RT-PCR methods (17). These results were partly inconsistent with the present results, because seven of nine carriers did not have the WASPdim lymphocyte population in this study. In the previous study, however, we did not separate monocytes from the lymphocyte fraction. Therefore, it was possibly monocytes that predominantly expressed the mutant WASP message because “lymphocytes” as described in the previous report included lymphocytes and monocytes. If it is the case, the previous results obtained by RT-PCR methods mostly agree with those by flow cytometric analysis in this study.

In conclusion, this study demonstrated the usefulness of FCM-WASP analysis in monocytes for the carrier diagnosis of WAS. The difference of the WASPdim population between in monocytes and lymphocytes from WAS patients and their familial carriers suggests that a skewed X-chromosomal inactivation pattern observed in WAS carriers’ blood cells is not fixed at the hematopoietic stem cell level but progresses after the lineage commitment.

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References