Flow cytometric analysis of lymphoid cells in thymic epithelial neoplasms

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Abstract

Objective: There have been conflicts concerning the criteria for diagnosing malignant epithelial neoplasms of thymic origin. To differentiate thymic carcinomas from thymomas, the maturation stage of T-lineage lymphoid cells infiltrating thymomas and thymic carcinomas was examined by flow cytometry to associate it with the degree of tumor malignancy. Methods: Multidimensional flow cytometric analysis was performed on the lymphoid cells extracted from 27 thymic epithelial neoplasms (14 encapsulated thymomas, ten invasive thymomas, and three thymic carcinomas) by using anti-CD3, -CD4, -CD8, -CD10, -CD20, -CD38, -CD45RA, and -CD45RO monoclonal antibodies. Results: CD4 and CD8 were co-expressed on 76.8% of the lymphoid cells in encapsulated thymoma (N = 14), 59.2% in invasive thymoma (N = 10), and 6.7% in thymic cancer (N = 3). The percentage of CD4+ or CD8+ single positive cells was 11.4% in encapsulated thymoma, 23.9% in invasive thymoma, and 77.7% in thymic cancer. The percentage of CD10-positive cells was 20.8% in encapsulated thymoma, 13.2% in invasive thymoma, and 6.0% in thymic cancer. The percentage of CD20-positive cells was 2.6% in encapsulated thymoma, 3.3% in invasive thymoma, and 31.6% in thymic cancer. There were significant statistical differences in the percentages of CD4/CD8 double positive cells, CD4- or CD8-single positive cells, CD10-positive cells and CD20-positive cells among the three groups. Two cases classified as invasive thymoma by pathohistological examination, however, showed the infiltration of mature lymphocytes like as thymic cancers. Conclusions: CD4+CD8+ or CD10+ T-lineage cells were the most reliable markers of the benignancy of thymic epithelial tumors. CD4- or CD8-single positive cells or CD20-positive cells were characteristic in thymic carcinoma. Flow cytometry on the maturity of lymphoid cells infiltrating thymic epithelial tumors was feasible for determining their degree of malignancy. Some invasive thymomas showed the intermediate characteristics with thymomatosus epithelia and mature lymphoid cells. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Thymoma; Thymus neoplasms; Flow cytometry; CD4-positive lymphocytes; CD8-positive lymphocytes; CD10

1. Introduction

Thymoma is defined as a cytologically bland epithelial tumor of the thymus [1]. It exhibits a diversity of degrees of malignancy, from benign neoplasm characterized by well-encapsulated tumors, to malignant one showing direct invasion of adjacent organs, dissemination in the pleural or pericardial cavity, and lymphogenous or hematogenous metastases. Both encapsulated and invasive thymomas include lymphoid cells in varying proportions. These lymphoid cells are mostly immature T-lineage cells characterized by coexpression of CD4 and CD8, similar to normal thymocytes [2,3]. Thymic carcinoma, which was first defined by Levine and Rosai, is a cytologically malignant thymic epithelial tumor showing poorer clinical prognosis. The thymic carcinoma does not contain immature lymphoid cells pathohistologically [4]. The cells of thymic carcinoma are very atypical and mitotic structures are often observed. Thus thymic carcinoma has been cytologically distinguished from those of invasive thymoma. However, some conflicts still exist in regard to distinguishing thymic carcinoma from invasive thymoma in borderline-malignant cases. Our study aimed to examine the maturation stage of lymphoid cells infiltrating thymic epithelial tumors by flow cytometry, and to associate pathohistological malignancy with the flow cytometric findings.

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2. Materials and methods

Thymoma or thymic carcinoma tissue had been harvested from patients who underwent extended thymectomy (i.e. en bloc resection of the thymoma, thymus, and the anterior mediastinal tissue around the thymus) between 1990 and 1999. For pathohistological studies, the resected specimens were fixed in 10% formaldehyde solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Thymomas were pathohistologically examined to determine the configuration of the neoplastic thymic epithelium (round-oval, mixed, or spindle cell type) and the degree of lymphocytic infiltration (predominantly, moderately, or scanty lymphocytic), as described by Levine and Rosai [4].

The thymomas were also subclassified according to Masaoka's clinicopathological staging classification [5]: stage I, completely encapsulated macroscopically and no capsular invasion microscopically; stage II, macroscopic invasion into surrounding fatty tissue or mediastinal pleura, or microscopic invasion of the capsule; stage III, macroscopic invasion into neighboring organs, i.e. pericardium, great vessels, or lung; stage IV, pleural or pericardial dissemination, or lymphogenous or hematogenous metastasis. Thymic carcinoma was distinguished from the thymoma based on the definition of Shimosato and Mukai [1], i.e. that thymic carcinoma is malignant thymoma type II by the Levine and Rosai classification [6] and several subtypes of thymic carcinoma exclusive of low grade well-differentiated thymic carcinoma as described by Kirchner and colleagues [7]. In this study, the tumors were divided into three groups: ‘encapsulated thymoma’, i.e. the tumors classified as stage I in Masaoka’s classification (group A); ‘invasive thymoma’, i.e. the tumors classified as stage II, stage III, or stage IV by Masaoka’s criteria, excluding thymic carcinoma (group B); and pathohistologically overt ‘thymic...
carcinoma’, characterized by diffuse growth of obviously atypical cells of an invasive nature (group C).

Flow cytometry of lymphoid cells in the thymoma and the thymic carcinoma was performed as described elsewhere [8]. Briefly, a neoplastic part of the resected tissues was minced in phosphate-buffered saline (PBS). The suspension of the cells and PBS was filtered through gauze to eliminate large particles, and the lymphoid cells in the tissue were retrieved by density-gradient centrifugation. The lymphoid cells were then stained with fluorescence-labeled monoclonal antibodies. Anti-CD3 (Leu-4, Becton Dickinson Co., CA, (BD)), anti-CD4 (Leu-3a, BD), anti-CD8 (Leu-2a, BD), anti-CD10 (CALLA, BD), anti-CD20 (Leu-16, BD), anti-CD38 (Leu-17, BD), anti-CD45RA (BD), and anti-CD45RO (BD) were used in this study to investigate T-cell differentiation. The FACScalibur system (BD) was used for three-color analysis of flow cytometry. Analysis of the two- or three-dimensional data was performed with CellQuest software (BD). Compensation adjustment for three-color analysis was performed before the assay with AutoCOMP software (BD). Ten thousand cells in each specimen were counted and examined by the FACScalibur system. Analysis of variance was performed to determine significant differences between mean values of percent specific positive staining, with a $P$ value $<0.05$ denoting statistical significance.

### 3. Results

Flow cytometric study was performed in 27 patients who underwent extended thymectomy between 1990 and 1998. Twenty tumors were diagnosed as thymoma. Fourteen of them were classified as stage I in Masaoka’s clinicopathological classification, and these cases belonged to group A in this study. Four tumors were diagnosed as stage II with minimal capsular invasion, five tumors as stage III, and one tumor as stage IV with lymph node metastasis. These ten tumors belonged to group B in this study. Three tumors were diagnosed as the thymic carcinoma belonged to group C in this study.

All of the patients in group A cases survived for 3 months–9 years postoperatively without tumor recurrence. Two of the group B patients died: one of perioperative acute myocardial infarction, and one of malignant lymphoma originating from the empyema cavity. One of the group C patients died of systemic metastasis of the tumor (Table 1).

Flow cytometric findings are shown in Fig. 1 and Table 2. The proportions of CD3+, CD4+, CD8+, CD10+, CD20+, CD45RA+, and CD45RO+ cells were shown in Table 2.

Two-dimensional flow cytometric analysis revealed that CD4 and CD8 were co-expressed on 76.8 ± 16.1% of the lymphoid cells in group A, 59.2 ± 33.7% in group B, and 6.7 ± 9.8% in group C. The percentage of CD4- or CD8-
single positive cells was 11.4 ± 5.9% in group A, 23.9 ± 21.9% in group B, and 77.7 ± 4.7% in group C. There were significant statistical differences in the percentages of CD10-positive cells, CD20-positive cells, CD4+CD8+ cells, CD4− or CD8-single positive cells among the three groups (Table 2). The percentage of CD10-positive cells was significantly higher in group A than in group C. The percentage of CD20-positive cells was significantly higher in group C than in groups A or B. The percentage of CD4+CD8+ double positive cells was significantly higher in group A than in group B ($P = 0.011$) and group C ($P = 0.00022$). The percentage of CD4- or CD8-single positive cells was significantly higher in group C than in group A ($P = 0.015$), and 50% of the CD4+CD8+ double positive fraction were also CD3-positive.

Two cases in group B, however, showed characteristics very similar to those in group C. Patho-histologically they were characterized by scant lymphocytic infiltration and mildly enlarged nucleoli without cellular atypia (Fig. 2), nonetheless, they showed macroscopically malignant characteristics.

4. Discussion

In this flow cytometric study, percentage of CD4- and CD8-double positive cell was higher in the encapsulated thymoma group (group A, 76.8%) than in invasive thymoma group (group B, 59.2%) and thymic cancer group (group C, 6.7%). The percentage of CD4- or CD8-single positive cells was higher in group C (77.7%) than in groups A (11.4%) and B (23.9%). The percentage of CD10-positive cells was higher in group A (20.8%) than in groups B (13.2%) and C (6.0%). The percentage of CD20-positive cell was higher in group C (31.6%) than in group A (2.6%) and group B (3.3%).

Lymphoid cells infiltrating thymoma have also been examined in an immunohistochemical study [2] and flow cytometric study [3]. The phenotypes of the lymphoid components in thymoma have been reported to be immature, similar to those of normal thymocytes, which mainly consist of immature T-lineage lymphoid cells. By contrast, the lymphoid cells infiltrating thymic carcinoma have been thought to be mature lymphocytes on the basis of immunohistochemical staining [1]. Recently we reported a quantitative analysis by flow cytometry of the immature lymphoid cells infiltrating thymomas. The report stated that the proportion of CD4+CD8+ double positive lymphoid cells in thymoma was similar to that in normal thymus tissue. CD10, which is expressed at a very low level on normal thymocytes, was expressed by 22 ± 10% of the lymphoid cells in the thymomas, suggesting that very immature subsets of T-lymphoid cells were even present in the thymoma tissue. Thymoma has also been reported to contain immature T-lymphoid subsets expressing CD10 on their surface along with CD4 and CD38, which indicates the existence of very immature pre-T fraction in thymomas [8]. In this study, the CD10-positive cells also expressed CD4, indicating that they were T-lineage cells.

Flow cytometric analysis of the lymphoid cells infiltrating thymic epithelial neoplasms in this study showed that the degree of malignancy correlated with the maturity of the lymphoid cells infiltrating in the tumor. That is, the proportions of CD4+CD8+ double-positive cells or CD10+ cells infiltrated thymic carcinoma less than invasive thymoma or encapsulated thymoma. The CD20+ fraction, representing B-lineage cells, were only observed in thymic carcinoma. The percentage of B-lineage cells in the thymic carcinoma tissue was similar to that in the peripheral blood. It also supports our finding that the degree of malignancy in thymic epithelial neoplasm is correlated with the maturity of lymphoid cells in the tumor.

Pathologists have struggled to diagnose the grade of malignancy of thymomas since the time of Bernatz, who

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Table 2

<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>Group A ($N = 14$)</th>
<th>Group B ($N = 10$)</th>
<th>Group C ($N = 3$)</th>
<th>$P$ value</th>
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<tr>
<td>CD3</td>
<td>39.4 ± 15.1</td>
<td>58.2 ± 25.3</td>
<td>39.1 ± 4.9</td>
<td>0.0729</td>
</tr>
<tr>
<td>CD4</td>
<td>83.4 ± 15.2</td>
<td>73.3 ± 27.0</td>
<td>45.9 ± 23.6</td>
<td>0.0191</td>
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<tr>
<td>CD8</td>
<td>80.5 ± 14.5</td>
<td>68.9 ± 26.7</td>
<td>30.4 ± 21.8</td>
<td>0.0039</td>
</tr>
<tr>
<td>CD10</td>
<td>20.8 ± 9.7</td>
<td>13.2 ± 10.8</td>
<td>6.0 ± 1.0</td>
<td>0.0446</td>
</tr>
<tr>
<td>CD20</td>
<td>2.6 ± 1.8</td>
<td>3.3 ± 5.2</td>
<td>31.6 ± 10.4</td>
<td>0.0010</td>
</tr>
<tr>
<td>CD38</td>
<td>95.7 ± 3.9</td>
<td>83.7 ± 23.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD45RA</td>
<td>3.8 ± 2.3</td>
<td>8.2 ± 7.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD45RO</td>
<td>89.9 ± 9.4</td>
<td>23.9 ± 21.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD4/8DP</td>
<td>76.8 ± 16.1</td>
<td>59.2 ± 33.7</td>
<td>6.7 ± 9.8</td>
<td>0.0006</td>
</tr>
<tr>
<td>CD4/8SP</td>
<td>11.4 ± 5.9</td>
<td>23.9 ± 21.9</td>
<td>77.7 ± 4.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Values are given as mean% ± SD.

* CD4/8DP, CD4 and CD8 double positive staining. CD4/8SP, CD4 or CD8 single positive staining.

* Group A, encapsulated thymoma.

* Group B, invasive thymoma.

* Group C, thymic carcinoma.
first demonstrated that thymoma is not a type of lymphoma, but a neoplasm of the thymic epithelium [9]. Rosai and Levine [4] classified thymomas by the cytological characteristics of neoplastic thymic epithelial cells among the paucity of lymphoid cells in the tumor. This classification was partly successful, because thymomas with scant lymphocytic infiltration tend to invade adjacent organs, and the postoperative outcome of thymoma patients is poorer when there is scant lymphocytic infiltration than when lymphocytic infiltration predominates [10]. However, the tumor classification by invasiveness of Masaoka was more successful in predicting the postoperative outcome of thymoma [5]. The definition of thymic carcinoma is still controversial. It is generally agreed that thymic carcinoma has the extremely malignant characteristic of thymic epithelial neoplasms with vast invasiveness and systemic metastases. Rosai and Levine revised the cytological classification of neoplastic thymic epithelium, highlighting cellular atypia, such as the configuration of the nuclei. As a result, some borderline-malignant thymic epithelial tumors have been classified as ‘type I thymic carcinoma’ [10], or as ‘well-differentiated thymic carcinoma of low-grade malignancy’ by Müller-Hermelink and colleagues [11], or, recently, as ‘cortical thymoma’ by Shimosato and Mukai [1]. Well-differentiated thymic carcinoma is a predominantly epithelial tumor in which the epithelial cells tend to be smaller than in cortical thymoma and possess round to oval or irregular grooved nuclei, inconspicuous nucleoli, and clear or oxyphilic cytoplasm with well-defined cell borders showing epidermoid features. Pallisading of epithelial cells toward the perivascular spaces and fibrous tissue is a characteristic feature.

Differential diagnosis of thymic carcinoma had been reported to be possible by immunohistochemical examination. Hishima and colleagues reported that they observed CD5 expression in the cytokeratin-positive fraction of thymic carcinoma tissue. CD5 was not expressed in carcinomas of other organs, such as lung, breast, esophagus, stomach, colon, or uterine cervix [12]. Fukai and colleagues stated that a kind of anti-cytokeratin monoclonal antibody was helpful in distinguishing carcinomas of thymic origin from primary lung cancer [13]. Gilhus and colleagues performed an immunohistochemical study on thymomas focusing on expression of oncogene proteins and proliferation antigens [14]. They reported that expression of EGF-R was increased, especially in the larger thymomas, but that neither p53 nor bcl-2 was detected in the neoplastic cells. The bcl-2 staining pattern in T-lymphocytes illustrates the...
broad spectrum of maturational stages in thymoma lymphocytes.

The flow cytometric analysis was very feasible, along with conventional pathohistological study, as a means of diagnosing thymic carcinoma. It was curious that some lesions classified as invasive thymomas by conventional pathohistological examination contained very mature T-lineage cells, like those in patent thymic carcinoma. This finding supported the ‘continuity theory’ of thymic epithelial neoplasms, i.e. that thymic carcinoma is an extremely malignant phenotype of thymoma with invasive characteristics.

References


Appendix A. Conference discussion

Dr C. Wright (Boston, MA, USA): This is a very interesting and provocative study that you have undertaken. I was trying to relate it to my clinical practice, and perhaps you could elucidate that for us. As you know, there is a new histologic grading system of thymomas which is in use throughout Europe but also throughout the world, the Müller-Hermelink system, medullary versus cortical versus well differentiated or poorly differentiated thymic carcinoma, and it appears to be a better prognostic instrument to use in selecting patients for adjuvant therapy and prognosis than the old staging system and the old histology. Could you correlate at all either old histology or this new Müller-Hermelink system with your CD4 and CD8 counts?

Dr Nakajima: From histopathological findings, I think that the maturational stage of the T-lineage lymphocytes was well correlated with the malignancy of the thymoma, and it is very curious that some invasive thymomas had a very similar characteristics of the T-lymphoid cells to thymic cancer. They were diagnosed as ‘thymoma’ by Shimosato’s classification, but they might be diagnosed as ‘well-differentiated thymic cancer’ by Müller-Hermelink’s criteria. Anyhow in this study, we did not analyze the correlation between the postsurgical prognosis and the expressions of CD4, CD8, and other T-cell markers. Actually two patients of the invasive thymoma died from extrathymic diseases, and one patient died of the thymic cancer.

Dr E. Rendina (Rome, Italy): As you know, there is evidence that especially if you use the Müller-Hermelink classification there might be areas in the same tumor with different histological patterns, and my question is, have you sampled the tumors extensively or are you just taking one sample for each tumor to make your investigation?

Dr Nakajima: I picked up the specimens from the tumors at least two or three different regions of the tumors, because we sometimes observed that the thymomas were made up of a hybrid combination of T-lineage cellular density. So I picked up several places and I mixed and analyzed by flow cytometry.

Dr T. Molnar (Pecs, Hungary): Can you see any implications for a possibility to use your results in the follow-up, in some sort of tumor markers after the resection?

Dr Nakajima: What kind of tumor markers?

Dr Molnar: Markers for the follow-up after the resection.

Dr Nakajima: Tumor markers, another type of tumor markers?

Dr Molnar: After you remove these thymic tumors, can you use these tumor markers for the follow-up after the resection?