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Short Communication

CD5 Expression in Thymic Carcinoma

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To determine the differences between the cellular characteristics of thymic carcinoma and thymoma, immunohistochemical analysis with lymphocyte markers (CD1a, 3, 4, 5, 8, 10, 20, 21, 25, 30, 57, and 72) was performed on 23 thymic epithelial tumors other than lymphocytic thymoma: overt thymic carcinoma (OC, n = 7), atypical thymoma (n = 5), and typical thymoma (epithelial or mixed thymoma, n = 11). Among the surface antigens examined, CD5, a type of receptor molecule that signals cell growth in T cells, was expressed in neoplastic epithelial cells of the thymus, in OC (seven of seven) and atypical thymoma (two of five), but not in typical thymoma. Double labeling immunofluorescence demonstrated expression of CD5 in cytokeratin-positive cells. The CD5 molecule extracted from an OC tumor showed the same molecular size as that in the spleen, but CD72, a ligand of CD5 on the surface of B cells, was not found in the epithelial cells of OC or atypical thymoma. Expression of CD5 was not observed in carcinomas of other organs, such as lung (n = 15), breast (n = 4), esophagus (n = 6), stomach (n = 6), colon (n = 9), and uterine cervix (n = 3). CD5 is closely related to morphological changes in thymic epithelial tumors and may play a role in the evolution of OC through receptor-ligand interaction. (Am J Pathol 1994, 145:268–275)

Thymic carcinomas and thymomas are anterior mediastinal tumors that originate from thymic epithelium. Thymic carcinoma and thymoma have been classified as distinct entities on the basis of morphology.[1–5] An advantage of this classification is that it removes most, though not all, malignant tumors from the undefined and broad category of thymoma. Thymoma now refers specifically to a thymic epithelial tumor, the malignant potential of which can not be predicted by morphology alone.[1] Thymic carcinoma consists of large cells with prominent nucleoli, a high nuclear/cytoplasmic ratio, and abundant mitoses and is frequently accompanied by multifocal or confluent necrosis.[2–6] On the other hand, thymoma has many organotypical features, such as perivascular space, epithelial palisading, medullary differentiation, and Hassall's corpuscles. However, it is occasionally difficult to distinguish between these two types of tumors, and the histological patterns in some cases change from thymoma to thymic carcinoma.[7]

To define both tumors more precisely on the basis of cytological and phenotypical characteristics, we studied thymic epithelial tumors by immunohistochemical analysis. Thymic epithelial tumors were tentatively divided into three groups based on the histological diagnoses of three pathologists: overt thymic carcinoma (OC), typical thymoma (TT), and atypical thymoma (AT), which we used in this study to describe tumors of intermediate morphology. The result of immunohistochemical analysis was compared among the three groups. The notable immunohistochemical feature was expression of CD5 on neoplastic epithelial cells of all OC tumors and some AT ones. CD5 is a monometric glycoprotein that is expressed by T cells during the various stages of T-cell differentiation in the thymus.[8] CD5 is a type of receptor molecule, one of the ligands for which is CD72 on the

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surface of B cells.[9,10] These molecules are believed to interact with each other, mutually stimulating proliferative activity. Because CD5 expression has never been demonstrated in the neoplastic epithelial component of any organ, CD5 may play a significant role in the morphogenesis of thymic carcinoma.

Materials and Methods

Twenty-three cases of thymic epithelial tumor were treated at Tokyo Metropolitan Komagome Hospital from 1983 to 1992. Specimens were obtained at surgery from primary tumors in 22 cases and from a metastatic lesion of the lung in one case. Clinical information regarding age, sex, associated disease, treatment, and outcome was extracted from the medical record of each patient. The clinical stage was determined from operative and histological findings, according to the criteria proposed by Masaoka et al.[11]

Histological Study

Formalin-fixed, paraffin-embedded sections of all cases were stained with hematoxylin and eosin and periodic acid-Schiff and alcian blue, pH 2.5. Each case was first classified into one of two major categories, thymic carcinoma or thymoma by three pathologists (TH, MF, and NF). A diagnosis of thymic carcinoma was based on the degree of architectural atypia (presence of necrosis, keratinization, broad fibrous stroma, and loss of lobular pattern, perivascular space, and epithelial palisading) and cytological atypia (large nuclear size, high nucleocytoplasmic ratio, conspicuous nucleoli, and abundant mitoses). Histological groups were defined after the independent evaluations; OC, when all three pathologists diagnosed the tumor as thymic carcinoma; TT, when all three pathologists diagnosed thymoma, and AT, or intermediate tumor, when the diagnoses were different or when it was difficult to determine whether the tumor was thymic carcinoma or thymoma.

Immunohistochemical Analysis

Tissues of all cases were fixed for ten hours with periodate-lysine-paraformaldehyde, embedded in OCT compound, frozen in dry ice-hexane, and stored at −80 C until they were processed for immunohistochemical study. Sections (5-μ-thick) were cut with a cryostat and stained by the avidin-biotin-peroxidase complex method for immunohistochemistry. Monoclonal antibodies used in this study were as follows: lymphocyte markers were CD1a (OKT6: Ortho Diagnostic Systems, Raritan, NJ), CD3 (Leu4), CD4 (Leu3a+3b), CD5 (Leu1 and UCHT2: Cymbus Bioscience, Southampton, UK), CD8 (Leu2a), CD10 (J5: Coulter Immunology, Hialeah, FL), CD20 (B1: Coulter Immunology), CD21 (CR2), CD25 (interleukin-2 receptor), CD30 (Ber-H2: DAKO, Glostrup, Denmark), CD57 (Leu7), CD72 (Immunotech S.A., Marseilles, France), and TdT (Life Science, St. Petersburg, FL). Monoclonal antibodies of the Leu series, CR2, and interleukin-2 receptor were purchased from Becton Dickinson (Mountain View, CA). Both of anti-CD5 monoclonal antibodies (MAbs) Leu1 and UCHT2 recognize two different CD5 epitopes.[12] Pan B-cell marker CD72, which appears at all stages of B-cell differentiation except in the plasma cell,[13] is one of the ligands for CD5.[9,10] Anti-cytokeratin (CAM5.2) antibody, which reacts with almost all epithelia, was obtained from Becton Dickinson.

Forty-three carcinomas originating from other organs that had been surgically resected from 1987 to 1991 and stored at −80 C (lung, $n = 15$; breast, $n = 4$; esophagus, $n = 6$; stomach, $n = 6$; colon, $n = 9$; uterus, $n = 3$), were also evaluated for CD5-expression by immunohistochemical analysis with Leu1 and UCHT2 MAb.

To prove that the CD5 is present on epithelial cells as well as the reactive T cells in thymic tumors, double labeling was performed using anti-cytokeratin (CAM5.2) antibody and anti-CD5 (Leu1) MAb. The periodate-lysine-paraformaldehyde-fixed sections as described above were incubated with anti-cytokeratine antibody for 60 minutes at room temperature, followed by Texas Red-conjugated horse anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Finally sections were incubated with the fluorescein isothiocyanate-conjugated Leu1 antibody (Becton Dickinson) for 120 minutes at room temperature. Between incubations, the slides were washed with phosphate-buffered saline. Visualization was achieved in a Nikon fluorescence microscope with separate filters.

Immunoaffinity Purification and Quantitation of CD5 from Tissues

All procedures were performed at 4 C. Membrane proteins prepared from spleen (3.5 g), TT (case 14, 5.8 g), and OT (case 5, 4.4 g), were resuspended in 10 ml of 10 mmol/L phosphate-buffered saline, pH 7.4/1 mmol/L ethylenediaminetetraacetic acid/1 mmol/L phenylmethysulfonyl fluoride/0.02% NaN_3, sonicated and dissolved by the addition of 250 μl of 20% Nonided P-40 with stirring for 30 minutes. The

samples were applied to Tresyl-activated Sepharose 4B (Pharmacia, Uppusala, Sweden) coupled with Leu1 (1 mg of antibody per 1 ml of gel), and recycled overnight. The column was washed with 30 ml of 10 mmol/L phosphate-buffered saline/0.5 mol/L NaCl/ 0.5% Nonided P-40 and bound proteins were then eluted with 0.2 mol/L glycine, pH 2.5/0.5% Nonided P-40. Fractions that contained CD5 were identified by immunoblot analysis. The samples were concentrated to 100 μl with Centricon 10 (Amicon), and the detergent was removed by methanol precipitation.[14] The samples were then dissolved in 50 μl of phosphate-buffered saline. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli.[15] The sample size was 20 μl. The gel was stained with silver (Silver stain kit, Wako, Osaka, Japan), and the amount of Leu1-immunoreactive protein was quantitated by the intensity of the staining.

Results

Histological Type and Clinicopathological Correlation

OC consisted of interconnected epithelial cords or small nests and a broad fibrous band, which occasionally showed hyalinization (Figure 1A). All of the OC tumors were classified as squamous cell carcinoma with moderate or poor differentiation. Tumor cells showed large, oval to polygonal nuclei with prominent nucleoli. Infiltrating lymphocytes were sparse in both epithelial cells and fibrous stroma.

AT tumors were heterogeneous, showing a mixture of the features of thymic carcinoma and thymoma to various degrees. In cases 8 and 9, interconnected cords of epithelial cells were accompanied by a diffuse infiltrate of lymphocytes with several lymphoid follicles (Figure 1B). Necrosis and mitoses were not prominent. In case 10, abundant mitoses were found in the tumor cells, but perivascular space with epithelial palisade was also observed, which is a landmark for typical thymoma. In cases 11 and 12, tumors showed well-developed keratinization with cytological atypia, but there were very few mitoses. The tumors grew in solid sheets with scanty fibrous stroma. Perivascular spaces were cystically dilatated or obliterated by hyaline fibrous tissue (Figure 1C).

TT consisted of predominantly epithelial thymomas ($n = 4$) (Figure 1D) and mixed epithelial-lymphocytic thymomas ($n = 7$). Organotypical structures such as perivascular space, epithelial palisading, and, medullary differentiation were apparent. Two patients with TT had myasthenia gravis. None of the patients with OC or AT exhibited myasthenia gravis, but one OC patient was associated with dermatomyositis. The tumor invaded adjacent structures in seven of seven OCs, five of five ATs, and three of 10 TTs. Pleural dissemination and hematogenous metastases were found in four OCs, two ACs, and one TT (Table 1). Seventeen patients were followed up for 5 months to 9 years. In OC, three patients died of the disease and two were alive and well without local recurrence or distant metastasis. In AT, one of four cases was alive with liver metastasis, and the others were alive and well. In the cases of TT, none of the patients died of the disease.

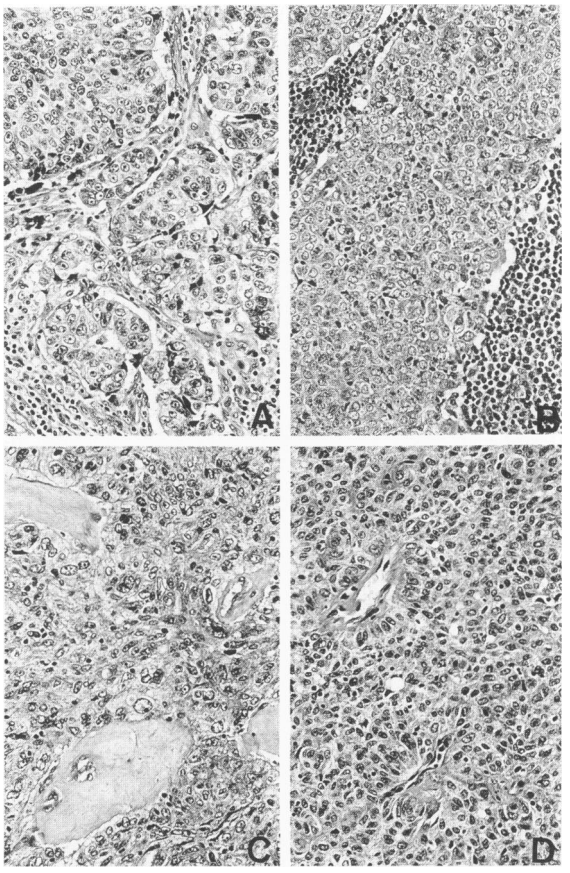

Figure 1. **A:** *Overt thymic carcinoma (case 6). The small nests, delineated by the fibrous tissue, are composed of large neoplastic cells with prominent nucleoli.* **B:** *Atypical thymoma (case 8). Tightly packed cells showing cytological atypia, which is intermediate between thymic carcinoma and thymoma.* **C:** *Atypical thymoma (case 11). Sheetlike arrangement of epithelial cells, which are markedly anisocytotic. However, obliterated perivascular spaces are apparent.* **D:** *Typical thymoma (epithelial) (case 16). Mild cytological atypia and epithelial palisading around a perivascular space are typical features.* **A** *to* **D**, *×240, H&E.*

Table 1. *Histological and Immunohistochemical Features in 23 Cases of Thymic Epithelial Tumor*

Case number	Histological subtype*	Stage	Immunohistochemistry of epithelial component†			Immunohistochemistry of lymphoid component‡	
			Leu 1	UCHT2	J5	TdT	OKT6
Overt thymic carcinoma							
1	Sq	IVb	+++	+++	−	−	−
2	Sq	II	+++	+++	−	−	−
3	Sq	III	+++	++	−	−	±
4	Sq	IVa	++	++	+++	−	−
5	Sq	III	+++	+++	−	−	−
6	Sq	IVa	++	+	+	−	−
7	Sq	IVb	++	±	−	−	−
Atypical thymoma							
8		II	++	++	±	−	−
9		II	+++	+++	+++	±	±
10		IVb	−	−	+++	−	±
11		IVa	−	−	−	±	±
12		II	−	−	−	−	−
Typical thymoma							
13	E	I	−	−	+++	−	−
14	E	I	−	−	−	±	−
15	E	IVa	−	−	−	±	±
16	E	III	−	−	−	±	−
17	Mx	I	−	−	−	++	+
18	Mx	I	−	−	−	++	++
19	Mx	NE	−	−	−	++	++
20	Mx	I	−	−	−	++	++
21	Mx	I	−	−	−	++	+
22	Mx	III	−	−	−	−	−
23	Mx	I	−	−	+++	++	++

* Sq: squamous cell carcinoma; E: predominantly epithelial thymoma; Mx: mixed epithelial-lymphocytic thymoma; NE: not examined.
† +++: diffuse staining in most cells; ++: many positive cells but not diffusely positive; +: considerable number of positive cells; ±: positivity or negativity difficult to evaluate; −: negative staining.
‡ ++: moderate number of positive cells; +: considerable number of positive cells; ±: small number of positive cells; −: negative staining.

Immunohistochemical Analysis

Epithelial Cells

Immunohistochemical results are summarized in Table 1. Neoplastic epithelial cells showed strong immunoreactivity with anti-cytokeratin antibody in seven of seven OCs (Figure 2A). Lymphocyte markers expressed in the epithelial cells of the thymus were CD10 (J5), CD57 (Leu7), and CD5 (Leu1 and UCHT2). CD10 (J5) was detected in epithelial cells in two of seven OCs, two of five ATs, and two of 11 TTs. The staining pattern was diffuse on the cell membrane, except in case 6, which showed CD10 (J5) in a small, confined area. Immunostaining with anti-CD 57 (Leu7) MAb showed intense positivity in only one case of TT (case 15). On the other hand, Leu1 or UCHT2, which define different CD5 epitopes, reacted with neoplastic epithelial cells of seven of seven OCs (Figure 2B) and two of five ATs, but none of the 11 TTs (Figure 2F). Positive staining appeared diffusely at the plasma membrane. In two of nine CD5-positive tumors, immunoreactivity for Leu1 was more intense than that for UCHT2. In two of the three cases (cases 11 and 12) of AT that did not show immunoreactivity for Leu1 and UCHT2, the structural pattern of the tumors differed from that of OC and of the positive cases of AT: ie, both had a sheetlike structure (Figure 1C). CD72, which is known to interact with CD5, was not found in the epithelial cells of any of the three groups.

Lymphocytes

Pan-T cell marker, CD3 (Leu4) was expressed in various numbers of lymphocytes in all 23 thymic epithelial tumors (Figure 2C). The phenotypical features of infiltrating lymphocytes in OC and TT agreed with those in previous reports.[16,17] CD1a (OKT6)- and/or TdT-positive lymphocytes are immature T lymphocytes that are attracted to the site by unknown factors produced by epithelial cells. These lymphocytes were absent in six of seven OCs, two of five ATs, and two of 11 TTs. In contrast to the epithelial cells, the infiltrating lymphocytes exhibited immunoreactivity for Leu1 and UCHT2 in all of the thymic epithelial tumors (Figure 2B) except one (case 8), thus serving as an intrinsic control for immunohistochemical analysis of CD5. CD72 was expressed in infiltrating lymphocytes in two cases (cases 8 and 9). These lympho-

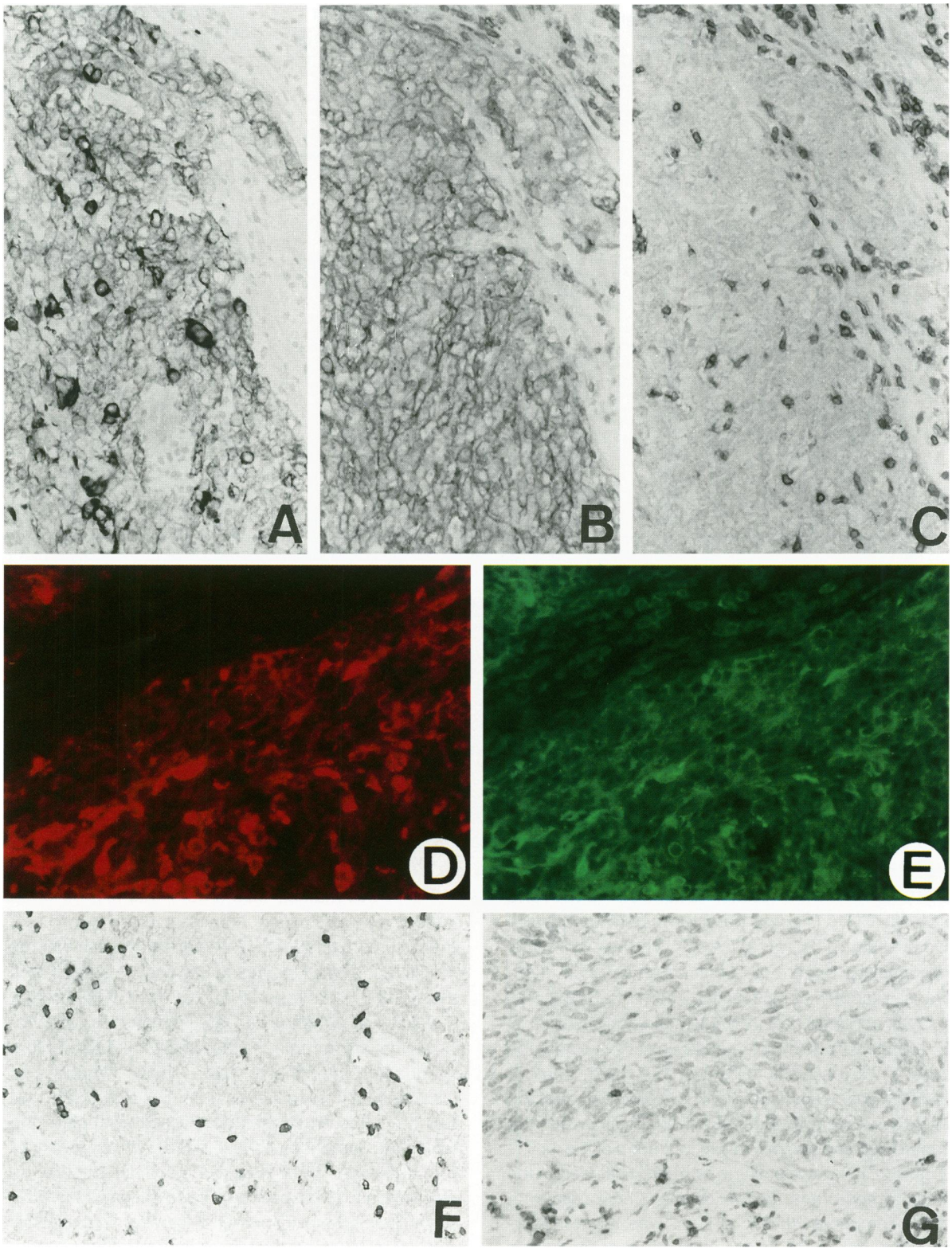
A
B
C
D
E
F
G

Table 2. *CD5 Expression in Carcinomas*

Organ	Number of cases	Number of positive cases	
		Leu1	UCHT2
Thymus*	7 (12)	7 (9)	6 (8)
Lung	15	0	0
Squamous cell carcinoma	10	0	0
Adenocarcinoma	3	0	0
Large cell carcinoma	2	0	0
Breast	4	0	0
Esophagus	6	0	0
Stomach	6	0	0
Colon	9	0	0
Uterine cervix	3	0	0

* Number of overt thymic carcinoma cases; (number of overt thymic carcinoma and atypical thymoma cases).

cytes were observed in the fibrous stroma and/or lymphoid follicles. However, there was no contact between CD72-positive lymphocytes and epithelial cells.

Immunofluorescence

Neoplastic epithelial cells, which were detectable with anti-cytokeratin antibody, were strongly labeled with anti-CD5 (Leu1) MAb, together with smaller numbers of the infiltrating lymphocytes in OC (Figure 2 D and E). These results confirmed those obtained with immunoperoxidase staining.

CD5 Expression in Carcinomas of Other Organs (Table 2)

The results of immunostaining with anti-Leu1 and UCHT2 MAb in carcinomas originating from various organs are presented in Table 2. None of the tumors that originated from other organs were positive for Leu1 or UCHT2 (Figure 2G).

CD5 in Thymic Carcinoma

Three different samples that were extracted from normal spleen and from each of the cases of TT and OC showed immunoreactive CD5 of the same molecular size at 67-kd (Figure 3A), which is the molecular weight of albumin. The possibility of contamination by albumin during the process of tissue extraction was

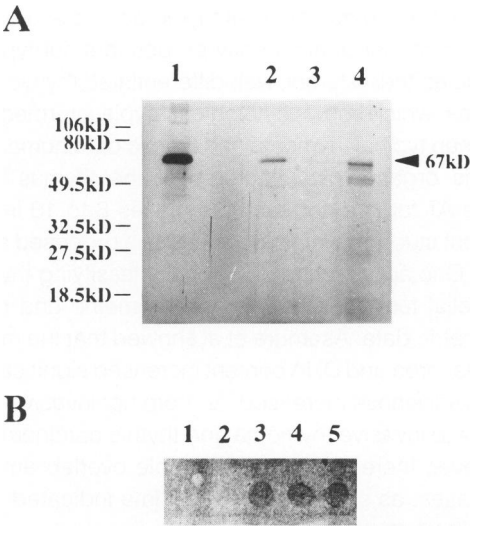

Figure 3. *CD5 in thymoma, thymic carcinoma and spleen.* (**A**) *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of CD5 purified by immunoaffinity; 12.5% acrylamide gel was subjected to silver staining.* **Lanes: 1,** *albumin (200 ng);* **2:** *OC (case 5);* **3:** *TT (case 14);* **4:** *spleen.* **B:** *Immunoblot analysis: the blots were incubated with Leu1 (1:2,000 dilution) overnight and the bindings were visualized using goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (1:2,000 dilution). Blots of 1 mg (blot 1) and 100 ng (blot 2) of human albumin served as controls.* **Blots 3** *to* **5** *represent fractions from immunoaffinity columns that had been loaded with tissue extraction from OC, TT, and spleen, respectively.*

eliminated by immunoblotting, which revealed that albumin was not reacted with Leu1 (Figure 3B). The total amounts of CD5 in the tissues, as estimated by silver staining, were 14 ng of CD5/g of tissue in the spleen, 23 ng/g in OC, and 0.34 ng/g in TT. Therefore, 70-fold increase of CD5 in OC relative to TT is apparently due to CD5 in epithelial cells of OC. It is notable that there were very few CD5-positive lymphocytes in both TT and OC, but only epithelial cells of OC showed diffuse immunostaining for CD5.

Discussion

Thymic epithelial tumors are histologically classified into two major categories, thymic carcinoma and thymoma. The distinction between the two has been based on cytoarchitectural atypism.[1–6] However, it is sometimes difficult to differentiate between thymic carcinoma and thymoma solely on the basis of histological features, as illustrated by the presence of the

Figure 2. **A** *to* **C** *Serial sections of OC (case 2) stained with anti-cytokeratin (CAM5.2) antibody* (**A**), *anti-CD5 (Leu1) MAb* (**B**), *and anti-CD3 (Leu4) MAb* (**C**). *The majority of the neoplastic epithelial cells are immunoreactive with both anti-cytokeratin (CK) antibody and anti-CD5 MAb. Some of the epithelial cells show strong cytoplasmic staining with anti-CK antibody. CD3- and CD5-positive T lymphocytes infiltrate in the stroma and between the epithelial cells.* **D** *and* **E:** *Double-labeling immunofluorescence of OC (case 2) with anti-CK antibody* (**D**) *and anti-CD5 (Leu1) MAb* (**E**). *Neoplastic epithelial cells are strongly double-labeled with anti-CK antibody (red) and anti-CD5 MAb (green). CD5-positive lymphocytes are also found in the stroma.* **F** *and* **G:** *Immunostaining with anti-CD5 (Leu1) MAb in TT* (**D**) *and lung cancer* (**E**). *CD5 was identified in a small number of infiltrating lymphocytes, but not in epithelial cells of TT (case 16) and lung cancer.* **A** *to* **C,** *×240, avidin-biotin complex immunoperoxidase staining.* **D** *and* **E,** *×200, indirect* (**D**) *and direct* (**E**) *immunofluorescence staining.* **F** *and* **G,** *×240, avidin-biotin complex immunoperoxidase staining.*

intermediate tumor, atypical thymoma, in the present study. Kirchner et al recently proposed a subtype of thymic epithelial tumor, well-differentiated thymic carcinoma, which shows cytological atypia intermediate between typical thymoma and thymic carcinoma and retains organotypical features of the thymus.[18–20] Some AT tumors, for example, cases 8 to 10 in the present study, may correspond to this proposed subtype. One approach to objectively classifying thymic epithelial tumors is to use cytoflowmetric and morphometric data. Asamura et al showed that the mean nuclear area and DNA content increased significantly as invasiveness increased: ie, from noninvasive thymoma to invasive thymoma and thymic carcinoma.[21] However, there was a considerable overlap among the cases, as our morphometric data indicated (unpublished observation).

Another approach is to demonstrate the differences in the cellular characteristics of thymic carcinoma and thymoma. Immunohistochemical analysis of lymphocyte markers demonstrates a unique characteristic of thymic epithelial tumors that is reflected in infiltrating lymphocytes: lymphocytes in squamous cell carcinoma show a CD1a (OKT6) (−) and TdT (−) mature T-cell phenotype, whereas there is an infiltration of CD1a (OKT6) (+) and TdT (+) immature T cells in thymoma.[16,17] The present study confirmed this finding. In addition, some lymphocyte-surface markers were found in neoplastic epithelial cells of the thymus. CD57 (Leu7) has been found in epithelial cells of the outer cortex of the thymus and cortical thymomas.[22] However, expression of CD57 was observed in only one case of epithelial thymoma in the present study. Expression of CD10, which has been found on a wide variety of normal and neoplastic cells,[23–25] was observed in six of 23 thymic epithelial tumors and such expression was not restricted to any particular tumor group.

CD5 expression on neoplastic epithelial cells has provided further insights into the cellular characteristics of thymic epithelial tumors. CD5 is expressed by thymocytes and mature T cells, and by a subpopulation of B cells at the mantle zone.[26] However, CD5 has not previously been found on normal or neoplastic epithelial cells. In the present study, CD5 was expressed on the surface of neoplastic epithelial cells of OC and AT, but not of TT. CD5 expression was observed in nine of 15 invasive tumors, but in none of the seven noninvasive tumors. Thus, CD5 may be closely associated with cellular characteristics of neoplastic epithelial cells of the thymus that lead to morphological atypia or local invasiveness.

CD5 is a type of receptor molecule on the lymphocyte surface, signals from which promote cellular proliferation in T cells.[27,28] Van de Velde and co-workers recently reported that one of the ligands for CD5 on T cells is CD72, which is expressed on the surface of B cells before final differentiation to plasma cells.[9,10] These molecules are thought to interact with each other on the cell surface, inducing activation and proliferation of their counterparts. In this context, CD5/CD72 interaction may contribute to the autoregulatory growth of CD5-positive B cells that express both CD5 and CD72, such as mouse B-cell lymphocytic lymphoma cells and human chronic lymphocytic lymphoma cells.[9,10] In the present study, however, CD72 was not expressed on CD5-positive thymic carcinoma cells. There may be another ligand for CD5 in neoplastic epithelial cells of the thymus that involves the growth of the tumor at the local site. Future studies to define the role of CD5 in thymic tumors, including identification of a possible ligand, are of great interest in terms of therapy for thymic carcinoma: the growth of a thymic carcinoma can perhaps be inhibited by blocking CD5 with monoclonal antibodies or analogs of the ligand.

Sometimes there were too few infiltrating lymphocytes to identify or characterize with lymphocyte markers, although CD1a (OKT6) and TdT expression in infiltrating lymphocytes is sometimes useful for developing a diagnosis. In this regard, CD5 immunohistochemistry can be an alternative diagnostic tool for classifying thymic epithelial tumors. Furthermore, because neoplastic epithelial cells originating from other organs did not express CD5, CD5 may also be valuable in differentiating between primary and metastatic carcinomas in the anterior mediastinum.

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