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Expression of a lymphocyte adhesion molecule (CD44) in malignant lymphomas: relevance to primary site, histological subtype and clinical stage.

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Abstract

Lymphocyte adhesion molecules defined by anti-CD44 antibody (Hermes-3) may be involved in lymphocyte binding to high endothelial venules at sites where lymphocytes exist the blood. CD44 expression was immunohistochemically examined in 167 well characterized cases of malignant lymphomas (MLs). None of 12 nodal follicular lymphomas (FLs) were CD44+, whereas 3 of 4 extranodal ones showed distinct CD44 expression. In contrast to nodal FLs, 28 of the 38 (74%) nodal diffuse B-cell lymphomas were CD44+ ($p < 0.0001$). T-cell lymphomas showed a significantly higher expression of CD44 antigen than diffuse B-cell lymphomas in the nodal cases ($p < 0.04$), but not in the extranodal ones. In nodal diffuse lymphomas, 3 of 5 stage I lymphomas (60%) were CD44+ in contrast to 53 of 63 stage II-IV lymphomas (84%), but the difference was not statistically significant. Of 14 Hodgkin's diseases, 9 cases were CD44+ with no significant correlation with clinical stage. The data of flow cytometric analysis confirmed the results of immunohistochemical analysis. In conclusion, CD44 expression is relevant to primary sites of distinctive MLs originating in the mucosal regions (MALToma) and some histological subtypes, but the relation with clinical stage was not defined. Some other adhesion molecules or different mechanisms must also be taken into account concerning the genesis and the expansion of MLs.

KEYWORDS: malignant lymphomas, adhesion molecules, CD44, clinical staging, histological classification

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Expression of a Lymphocyte Adhesion Molecule (CD44) in Malignant Lymphomas: Relevance to Primary Site, Histological Subtype and Clinical Stage

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Lymphocyte adhesion molecules defined by anti-CD44 antibody (Hermes-3) may be involved in lymphocyte binding to high endothelial venules at sites where lymphocytes exit the blood. CD44 expression was immunohistochemically examined in 167 well characterized cases of malignant lymphomas (MLs). None of 12 nodal follicular lymphomas (FLs) were $CD44^+$, whereas 3 of 4 extranodal ones showed distinct CD44 expression. In contrast to nodal FLs, 28 of the 38 (74%) nodal diffuse B-cell lymphomas were $CD44^+$ ($p < 0.0001$). T-cell lymphomas showed a significantly higher expression of CD44 antigen than diffuse B-cell lymphomas in the nodal cases ($p < 0.04$), but not in the extranodal ones. In nodal diffuse lymphomas, 3 of 5 stage I lymphomas (60%) were $CD44^+$ in contrast to 53 of 63 stage II-IV lymphomas (84%), but the difference was not statistically significant. Of 14 Hodgkin's diseases, 9 cases were $CD44^+$ with no significant correlation with clinical stage. The data of flow cytometric analysis confirmed the results of immunohistochemical analysis. In conclusion, CD44 expression is relevant to primary sites of distinctive MLs originating in the mucosal regions (MALToma) and some histological subtypes, but the relation with clinical stage was not defined. Some other adhesion molecules or different mechanisms must also be taken into account concerning the genesis and the expansion of MLs.

Key words : malignant lymphomas, adhesion molecules, CD44, clinical staging, histological classification

Recently, adhesion molecules have been suggested to play a very important role in the accumulation and the localization of leukocytes in inflammatory tissues (1). Lymphocytes move to specific organs such as the peripheral lymph node, mucosal lymphoid tissue, skin, and synovial tissue. This lymphocyte homing is exquisitely controlled by sequential expression of adhesion molecules (2-11).

Butcher recently proposed the multi-step theory, which is now widely accepted (12). The first step of lymphocyte homing is the transient and reversible lymphocyte adhesion to specialized high endothelial venules (HEV) mediated by the lymphocyte adhesion receptors and the ligands of HEV. After this initial adhesion, lymphocytes are further activated by specific chemoattractant or cell contact-mediated signals (the second step), which activates or upregulates the activation-dependent adhesion receptors and induces tighter adhesion (the third step).

Most of the adhesion molecules belong to the integrin family, the selectin family or the immunoglobulin supergene family (13). CD44 is a unique adhesion molecule composed of a C-terminal cytoplasmic tail, a hydrophobic transmembrane domain of 23 amino acids, and on N-terminal domain of 248 amino acids (14). This molecule is expressed not only on lymphocytes but also on various nonhematolymphoid cells in diverse normal human tissues, including many types of epithelium, mesenchymal elements such as fibroblasts and smooth muscle, and a subset of glia in the central nervous system (15-17).

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CD44 seems to participate in steps 2 and 3 of the above adhesion pathway on the lymphocyte (12). CD44 is rather an accessory molecule to lymphocyte-endothelial cell recognition than a tissue-specific homing receptor.

Malignant lymphomas (MLs) are thought to be malignant counterparts of lymphocytes, and most of them express various kinds of adhesion molecules (1-3). Interestingly, some cell lines derived from lymphoid malignancies selectively bind to peripheral and/or mucosal lymphoid HEVs (11, 14, 18-20). Some recent studies on non-Hodgkin's lymphomas (NHLs) suggested that a high level of CD44 expression correlates with wide-spread blood-born dissemination and poor prognosis of NHLs (21-25).

CD44 expression has not been analyzed in terms of nodal versus extranodal MLs in the previous studies. Herein, we examined the expression of CD44 in nodal and expranodal MLs, correlating them with the histological classification and clinical stages of lymphoma, and evaluated the significance of the biological behavior of CD44.

Materials and Methods

Cases and classification of malignant lymphoma. A total of 167 cases of MLs (153 NHLs and 14 cases of Hodgkin's disease) selected from the surgical pathology files of the 2nd Department of Pathology, Okayama University Medical School from 1989 to 1992 were examined. All of these cases were well characterized immunohistologically and immunogenotypically. These cases were staged according to the Ann Arbor classification (26). Of 153 NHLs, 80 were nodal, and 73 were extranodal (Table 1). B-cell lymphomas were classified according to the International Working Formulation (27), and T-cell lymphomas according to the Updated Kiel Classification (28).

Histology and immunohistochemical staining. Surgically obtained tissues were fixed in 10% buffered-formalin solution for 3h and embedded in paraffin, or were frozen in liquid nitrogen. Tissue sections were routinely stained with hematoxylin and eosin and examined immunohistochemically. Lymphomas were immunophenotyped on both paraffin sections and acetone-fixed frozen sections with a panel of antibodies against lymphoid cell type-related antigens.

The anti-CD44 monoclonal antibody (MoAb) used in this study was Hermes-3 (kindly donated by E. C. Butcher). Hermes-3 (11, 18), one of the anti-CD44 antibodies; produced by immunization with Hermes-1 antigen isolated from a mucosal HEV-specific cell line, selectively blocked lymphocyte binding to mucosal HEV, which suggests that CD44 has some function in lymphocyte recognition of mucosal HEV (11). Furthermore, CD44 binds cell surface hyaluronate (18, 29). We preliminarily tested the reactivity of Hermes-3 on both frozen and paraffin sections and found no difference in reactivity. For the diagnosis of lymphomas, MoAbs: Anti-CD15 (LeuM1), anti-CD20 (PanB), anti-CD43 (MT-1), anti-CD45 (LCA), anti-CD45R (MB-1), anti-CD45RO (UCHL-1, OPD4), anti-CDw75 (LN-1), and anti-epithelial membrane antigen (EMA) were used for paraffin sections, and MoAbs: Anti-CD3 (Leu4), anti-CD4 (Leu3a), anti-CD5 (Leu1), aoti-CD8 (Leu2a), anti-CD10 (CALLA), anti-CD14 (LeuM3), anti-CD19 (Leu12), anti-CD20 (Leu16), and anti-CD22 (Leu14) were used for frozen sections. Anti-immunoglobulin kappa (Ig κ) and lambda (Ig λ) light chain MoAbs were used for both frozen and paraffin secitons. UCHL-1, OPD4, and EMA were purchased from Dakopatts (Copenhagen, Denmark), MT-1 and MB-1 were from Bio-Science Product AG (Emmenbrücke, Switzerland), anti-Ig κ and anti-Ig λ were from Tago. Inc. (Burlingame, CA, USA), PanB was from Kyowa Medex Co. (Tokyo, Japan), LN-1 was from Techsiclone International Co. (Santa Ana, CA, USA), and the others were from Becton-Dickinson (San Jose, CA, USA). The antibodies were diluted with phosphate-buffered saline supplemanted with bovine serum albumin and NaN_3 to a dilution which gave specific staining with minimum nonspecific background. Hermes-3 (supernatant of hybridoma) was used at a dilution of 1:10. For immunostaining, the avidin-biotin-peroxidase complex (ABC) method was used as described previously (30). Biotinylated goat anti-mouse IgG and peroxidase-conjugated streptavidin were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). Immunostaining of paraffin sections was preceded by bleaching and destruction of endogenous peroxidase using methanol-H_2O_2. Sections were counterstained with hematoxylin or methyl green.

The immunostaining was evaluated independently by the two observers (K. F and T. Y) without other information. The results were identical in more than 90% of the cases, and the other cases in disagreement were excluded from the evaluation. CD44 reactivity with Hermes-3 was semiquantitatively scored on a scale of Grade 0 to Grade 4 (0, negative; 1, faintly positive, but equivocal; 2, weakly positive; 3, moderately positive; 4, positive) with Grades 0 and 1 being considered negative and Grades 2 to 4 being considered positive (Fig. 1). The expression of the CD44

Table 1 Site of extranodal lymphomas

B-cell Lymphomas	57	T-cell Lymphomas	16
Gastrointestine	39	Gastrointestine	2
Tonsil	4	Skin (CTL[a])	6
Orbit	9	Tonsil	2
Liver	1	Nasal cavity	3
Breast	1	Thymus	1
Subcutis	1	Thoracic cavity	1
Oral palate	1	Skeltal muscle	1
Skin	1		

a: Cutaneous T-cell lymphoma.

antigen in Hodgkin's disease was judged by reactivity of Reed-Sternberg cells. CD44 was expressed in not only lymphoma cells but also non-neoplastic lymphocytes, the latter being strongly positive except for germinal center B cells. Some large cell type B-cell lymphomas contain many non-neoplastic, mature T cells, which are known as T-cell rich B-cell lymphoma. In angioimmunoblastic T-cell lymphomas, lymphoma cells are frequently intermingled with non-neoplastic tissue components including reactive lymphocytes. In most of these cases, lymphoma cells were easily defined monrphologically, but were sometimes very difficult to

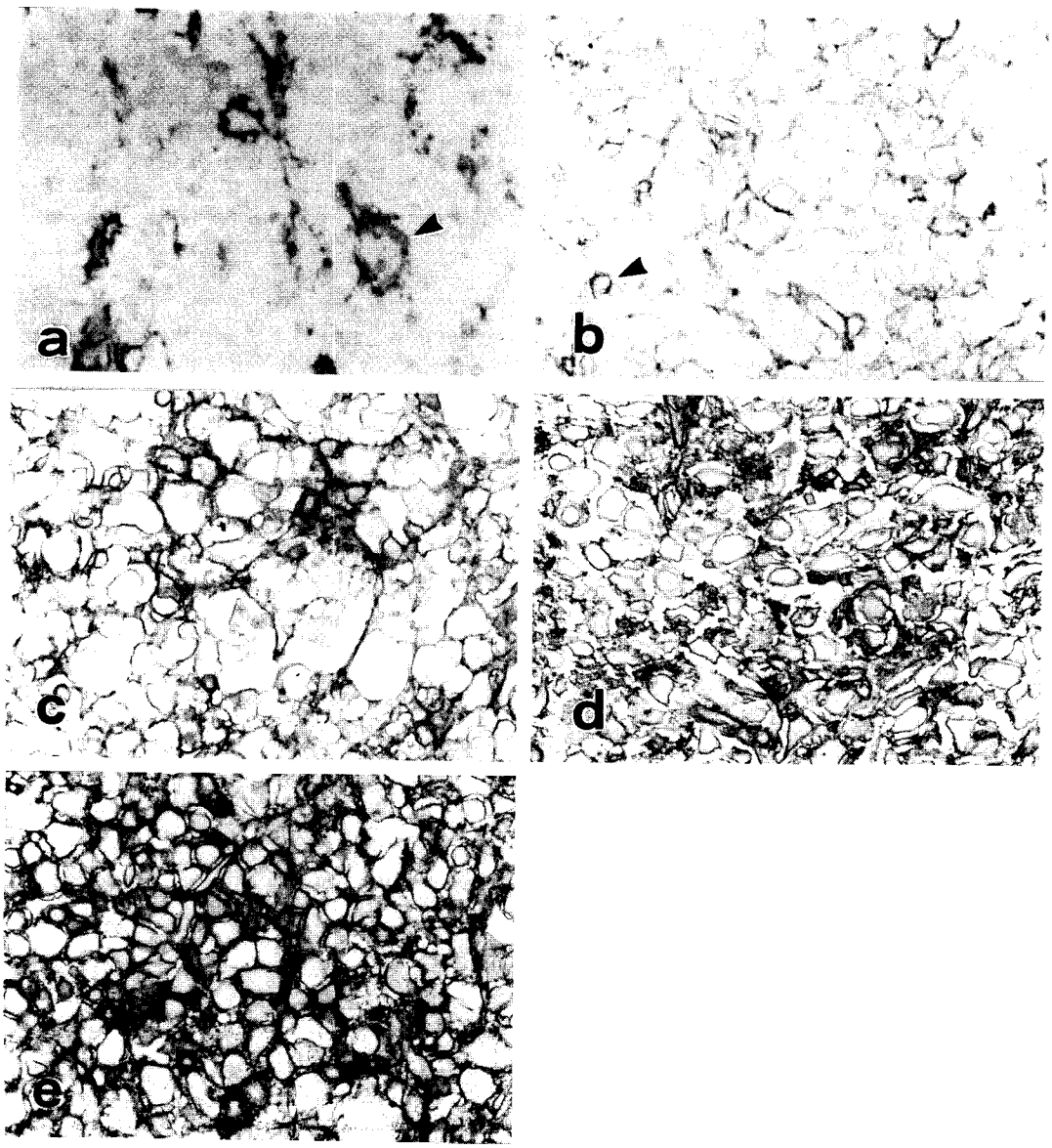

Fig. 1 Scoring of the immunostaining for CD44.
a: Nodal diffuse large B-cell lymphoma assessed as Grade 0. The lymphoma cells are completely negative. Only endothelial cells of with the surrounding conective tissues are positive (arrow). × 400
b: Nodal diffuse large B-cell lymphoma assessed as Grade 1. The lymphoma cells are faintly positive, but the stainability is equivocal. Intermingling lymphoid cells are positive (arrow). × 400
c: Nodal diffuse large B-cell lymphoma assessed as Grade 2. The lymphoma cells are weakly positive. Intermingling lymphoid cells and connective tissues are strongly positive. × 400
d: Nodal diffuse large B-cell lymphoma assessed as Grade 3. The lymphoma cells are modertely positive. × 400
e: Nodal diffuse large B-cell lymphoma assessed as Grade 4. The lymphoma cells are strongly positive. × 400

discriminate and had to be carefully evaluated immunohistochemically.

Two-colour flow cytometric analysis. Flow cytometric analysis was performed in 5 cases. Single cell suspensions were prepared from the lymphoma tissues and diluted with phosphate-buffered saline containing 2.5 % normal horse serum (Life Technologies, Inc., Tokyo, Japan), 20 mM Hepes buffer (Boehringer Mannheim Yamanouchi Co., Tokyo, Japan), and 0.1 % NaN_3 (PBS solution). A pellet of 0.5 to 1.0×10^6 cells was allowed to react with 5 μl of Hermes-3 for 30 min at 4 °C. A negative control was prepared by the addition of mouse IgG_2 (UPC10; Sigma Chemical Company, St. Louis, MO, USA). The cells were then washed once with cooled PBS solution. After centrifugation, cell pellets were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Cappel, Durham, NC, USA) for 20 min at 4 °C. After being washed and centrifuged again, the cells were incubated with 25 μl normal mouse serum for 10 min at 4 °C to prevent nonspecific binding of second antibodies. The second staining was performed by incubation with phycoerythrin (PE)-conjugated anti-CD3 (20 μl), anti-human immunoglobulin (50 μl, 1:25), anti-Ig κ (50 μl, 1:25) or anti-Ig λ (50 μl, 1:25) for 30 min at 4 °C. Flow cytometric analyses were performed using an EPICS 753 analyzer (Coulter Electronics, Hialeah, FL, USA).

Statistical analysis. Statistical comparison between the groups for the frequency of CD44 expression was carried out using the chi-square test or Fisher's exact test, the mean was calculated using t-test, and their confidence levels were determined using a chi-square and *t*-table, respectively.

Results

Tables 2 and 3 show the immunohistochemical reactivity of NHLs with Hermes-3. Of 153 cases, 117 (76 %) were positive for CD44; Grades 2 to 4. None of the 12 nodal follicular lymphomas (FLs) were positive for CD44 (Fig. 2a), whereas 28 of the 38 nodal diffuse B-cell lymphomas (74 %) were positive ($p < 0.0001$). This finding was also confirmed by flow cytometry (see below). Therefore, FLs were characteristically distinct from diffuse lymphomas (DLs) in CD44 expression in this study.

In nodal T-cell lymphomas, both low grade (angioimmunoblastic, T-zone) and high grade (pleomorphic) lymphomas expressed the CD44 antigen in a high frequency in over 90 % of cases (Fig. 2b). The mean CD44 Grade tended to be higher in high grade lymphoma (3.1) than in low grade lymphoma (2.6), but the difference was not statistically significant.

In nodal diffuse lymphomas, 93 % of the T-cell lymphomas were positive for CD44, significantly higher than the 74 % of diffuse B-cell lymphomas ($p < 0.04$), and the mean CD44 Grade of the former (2.9) was higher than that of the latter (2.2) ($p < 0.02$). There was no statistically significant difference in CD44 expression between T-cell and B-cell lymphomas for extranodal

Table 2 CD44 expression in B-cell lymphomas

Histologic subtype	Number of tested	Number of $CD44^+$	(%)	Mean grade
Nodal				
Follicular small cleaved	7	0	(0)	1.0
Follicular mixed	4	0	(0)	0.5
Follicular large	1	0	(0)	1.0
Diffuse medium	5	4	(80)	2.2
Diffuse mixed	6	6	(100)	2.8
Diffuse large	25	16	(64)	2.0
Immunoblastic	2	2	(100)	3.0
Subtotal	50	28	(56)	1.9
Extranodal				
Small lymphocytic	6	6	(100)	2.7
Follicular small cleaved	4	3	(75)	2.5
Diffuse medium	9	8	(89)	2.9
Diffuse mixed	11	10	(91)	2.6
Diffuse large	21	17	(81)	2.5
Immunoblastic	5	3	(60)	2.6
Burkitt	1	0	(0)	1.0
Subtotal	57	47	(82)	2.6
Total	107	75	(70)	2.3

Table 3 CD44 expression in T-cell lymphomas

Histologic subtype		Number of tested	Number of $CD44^+$	(%)	Mean grade
Nodal					
Angioimmunoblastic		7	7	(100)	2.6
T-zone		5	4	(80)	2.6
Pleomorphic	Medium	4	3	(75)	2.3
	Mixed	11	11	(100)	3.3
	Large	3	3	(100)	3.3
Subtotal		30	28	(93)	2.9
Extranodal					
Lymphoblastic		1	1	(100)	2.0
Pleomorphic	Medium	2	2	(100)	2.0
	Mixed	4	2	(50)	1.3
	Large	3	3	(100)	2.3
CTL[a]		6	6	(100)	3.0
Subtotal		16	14	(88)	2.3
Total		46	42	(91)	2.7

a: Cutaneous T-cell lymphoma.

Table 4 Clinical stage and CD44 expression in diffuse lymphomas

Clinical staging	Stage I			Stages II–IV		
	Number of tested	Number of CD44+	(%)	Number of tested	Number of CD44+	(%)
Nodal	5	3	(60)	63	53	(84)
Extranodal	23	20	(87)	46	38	(83)
Total	28	23	(82)	109	91	(83)

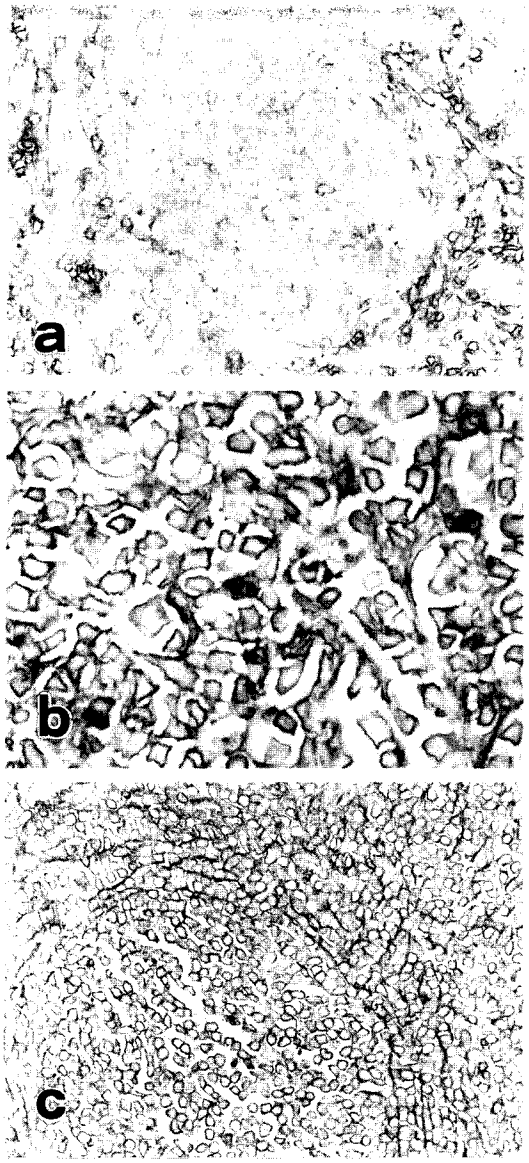

Table 5 CD44 expression in Hodgkin's disease

Histologic subtype	Number of tested	Number of CD44+	(%)	Mean grade
Mixed cellularity	9	7	(78)	2.3
Lymphocyte predominance	1	1	(100)	4.0
Nodular sclerosis	3	1	(33)	1.7
Lymphocyte depletion	1	0	(0)	0.0
Total	14	9	(64)	2.1

diffuse lymphomas.

In diffuse lymphomas, nodal and extranodal cases showed no statistically significant difference in the CD44 expression. However, interestingly, 3 out of 4 extranodal FLs were distinctly positive for CD44 in contrast to nodal FLs (Fig. 2c).

We compared the CD44 expression between diffuse lymphomas in stage I and those in stages II–IV. In nodal lymphomas, CD44 antigen was more frequently expressed in stages II–IV than in stage I, but the difference was not statistically significant. The extranodal cases in both stage I and stages II–IV showed similar frequency (Table 4).

Nine of 14 cases of Hodgkin's diseases belonged to Grades 2–4 (Table 5); *i.e.*, 50 % of stage I and 67 % of stage II–IV cases with no statistically significant difference.

The data of flow cytometric analysis were consistent with the results of immunohistochemical analysis. CD44-positive cells in nodal FLs were identified as reactive T cells (Fig. 3A). In the case of diffuse large B-cell lymphoma assessed as Grade 1 immunohistochemically,

Fig. 2 **a**: Nodal follicular lymphoma, large cell type. The lymphoma cells are negative for CD44, but the lymphocytes in the mantle zone and interfollicular area are reactive. × 50 **b**: Pleomorphic T-cell lymphoma, medium and large cell type, assessed as Grade 4. × 400 **c**: Follicular lymphoma of the duodenum, small cleaved cell, assessed as Grade 3. × 50

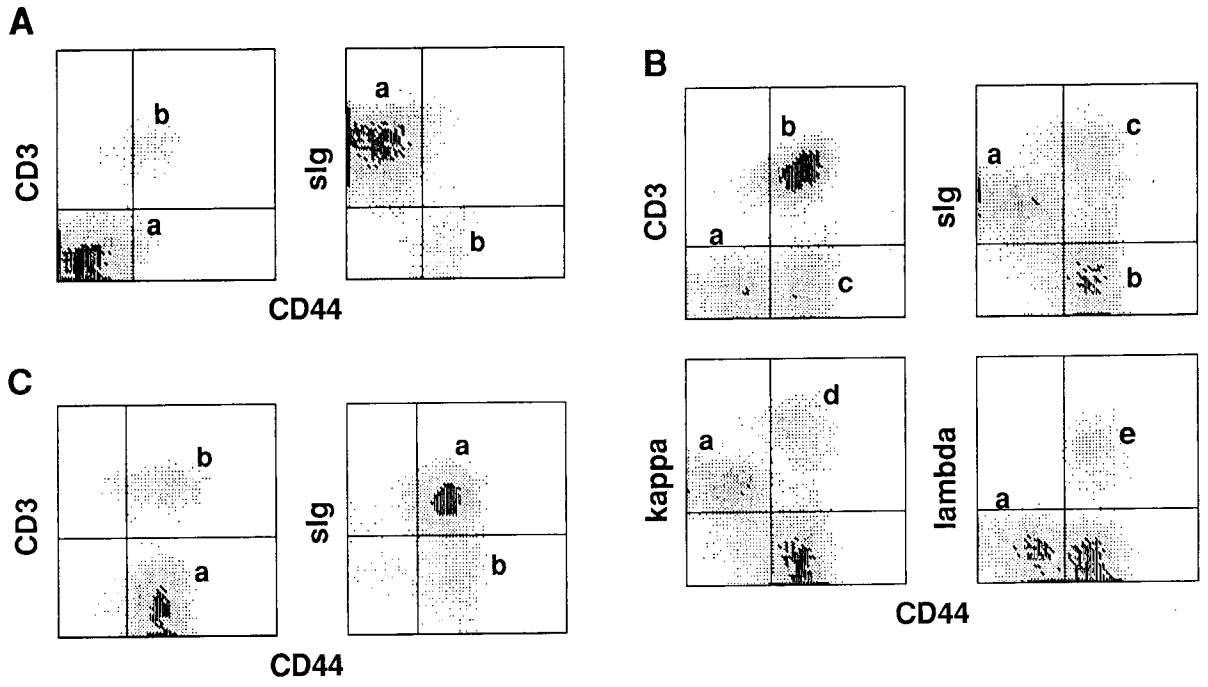

Fig. 3 Dual color flow cytometric analysis of the lymphoma cells. CD44 antigen (x-axis: green fluorescence) versus lymphocyte differentiation markers including CD3 and surface immunoglobulins (y-axis: red fluorescence) plotted in logarithmic units.

A. Follicular lymphoma, Grade 0.
  a: Lymphoma cells, which are sIg^+, $CD3^-$ and $CD44^-$.
  b: Intermingling reactive T cells, which are $CD44^+$.

B. Diffuse large B-cell lymphoma, Grade 1.
  a: Lymphoma cells, which are $sIg\kappa^+$, $sIg\lambda^-$, $CD3^-$, and $CD44^-$.
  b: Intermingling reactive Tcalls, which are $CD3^+$ and $CD44^+$.
  c, d, e: Intermingling small-sized B lymphocytes, which are $sIg\kappa^+$, $sIg\lambda^+$, $CD3^-$, and $CD44^+$.

C. Diffuse large B-cell lymphoma, Grade 3.
  a: Lymphoma cells, which are ,sIg^+ $CD3^-$, and $CD44^+$.
  b: Intermingling reactive T cell, which are $CD3^+$, sIg^-, and $CD44^+$.

CD44-positive cells were small B cells showing bitypic expression of Ig light chains and T cells. CD44-negative cells expressed surface Ig κ chain monotypically and were identified as neoplastic B cells (Fig. 3B). Surface immunoglobulin-positive neoplastic B cells as well as other nonneoplastic small B- and T-lymphocytes expressed the CD44 antigen in Grade 3 B-cell lymphomas (Fig. 3C).

Discussion

NHL is the neoplastic counterpart of lymphocytes and often manifests immunophenotypic or functional characteristics similar to those of normal lymphoid populations. This mimicry suggests that the same mechanism regulating normal lymphocyte 'homing' operates in NHLs and influences their spread.

The most interesting feature of the correlation of CD44 expression with histological subtypes of NHL was that none of the nodal FLs expressed CD44 antigen. This finding is compatible with the previous studies which demonstrated a much lower expression of CD44 in MLs with a morphologic or immunologic resemblance to germinal center cells (21, 22). These findings may be relevant to the fact that normal germinal center B cells were weak or negative for the CD44 antigen expression (21, 22). Our flow cytometric analysis revealed that CD44 positive cells in FLs were reactive T cells. In contrast, Picker *et al.* (21) reported that some FL cells in the mantle or

interfollicular zones were CD44 positive, suggesting that the follicular microenvironment influences the CD44 expression. One of our cases of diffuse B-cell lymphoma with partial follicularity expressed the CD44 antigen. Generally, FLs show low or no CD44 antigen expression. Interestingly, 33 % of FLs belonged to stage I, whereas only 7 % of DLs belonged to stage I. The lack of CD44 antigen in FLs may relate to the clinical stage. CD44 may be an accessory molecule to lymphocyte-endothelial cell recognition, and assist other homing receptors in the recognition. Other adhesion molecules or other mechanisms in the homing process are possibly present in the dissemination of FLs. Interestingly, we have recently found that FLs show a strikingly higher expression of α_4 -integrin compared with other MLs (data not shown).

Diffuse small lymphocytic B-cell lymphomas have been shown to be strongly positive for CD44 (21, 22), but such cases were not included in this study. Approximately two-thirds of the cases of nodal diffuse large B-cell MLs expressed CD44 as in previous studies (21, 22).

In nodal diffuse lymphomas, CD44 expression of T-cell MLs was higher than that of B-cell MLs. Thus, the dissemination of T-cell MLs may be more strongly influenced by the CD44 molecule, but the CD44 expression did not correlate with their histological subtypes.

Concerning the relation of CD44 expression to primary site, there was no notable difference between nodal and extranodal cases. Three of four FLs of the GI tract were clearly positive for CD44 in contrast to nodal FLs, MLs originating in the mucosal regions are thought to have distinct characteristics that differ from those of peripheral lymph nodes, and these MLs are defined as "MALToma" (31). Different adhesion molecules may operate in mucosal FLs compared within nodal ones, or the expression of CD44 antigen may be influenced by the microenvironment. Hermes-3 selectively blocks lymphocyte binding to mucosal HEVs, and has been thought to be involved in lymphocyte homing to the mucosal lymphoid region (11). In addition, low grade GI tract lymphomas have CD44 antigen. These things suggest that CD44 antigen is related to localized tumor involvement of such lymphomas.

In nodal lymphomas, both B- and T-cell lymphomas in stages II to IV tended to express a higher level of CD44 antigen than those in stage I. CD44 expression seems to be correlated to a high clinical stage and poor prognosis in the previous studies (21–25), but statistical significance was not established in our studies. In our series, only 5 out of 68 cases belonged to stage I. More cases are needed to clarify this point. However, nodal diffuse B-cell lymphomas in stage IV expressed the CD44 antigen less frequently than those in stages II and III (data not shown); nonetheless 2 of these 8 nodal lymphomas in stage IV involved bone marrow and possibly showed a leukemic change. Furthermore, one case of Burkitt's type, which shows an immature phenotype and is highly malignant, did not express CD44 antigen as shown by others (21, 22). Therefore, NHLs of an immature type or showing a leukemic changes might lose the CD44 antigen, and their dissemination might be regulated by other mechanisms. Similarly, LFA-1, one of the adhesion receptors belonging to an integrin family, tended to be negative in NHLs with bone marrow involvement (32). Most extranodal NHLs clearly expressed the CD44 antigen, and there was no correlation between the CD44 expression and clincal stage.

In Hodgkin's disease, 64 % of the cases expressed the CD44 antigen, but the CD44 expression was not correlated with clinical stage. As Hodgkin's disease is known to show contiguous spreading (33), some other mechanisms may regulate this characteristic expansion.

In conclusion, the CD44 expression in DLs is not as clearly to clinical stages and prognosis as indicated in previous studies. However, CD44 expression seemed to differ in some histological sugtypes and primary sites. CD44 antigen levels were absent or low in nodal FLs and immature MLs. On the other hand, the low grade GI tract lymphomas highly expressed the CD44 antigen. These findings may suggest that the relation between the CD44 antigen and the extension or dissemination of MLs is not a key-hole. It is necessary to investigate the participation of other adhesion molecules such as α_4-integrin or mechanisms other than adhesiveness in the extension or dissemination of MLs.

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