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COMPARATIVE ANALYSIS OF D2-40 AND LYVE-1 IMMUNOSTAINING IN LYMPHATIC MALFORMATIONS

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ABSTRACT

Identification of lymphatic vessels in normal tissue and vascular malformations has been considerably enhanced by the recently discovered lymphatic endothelial markers D2-40 and LYVE-1. However, comparative analysis of these two antibodies in the evaluation of lymphatic malformations has not been widely reported. We evaluated twenty lymphatic malformations of skin/subcutis/soft tissue with immunostaining for D2-40 and LYVE-1. Ten high-power fields from each section were scored for total number of immunopositive vessels using identical fields with both markers. Vessels were grouped by diameter (<225 µm and >225 µm), with each vessel categorized according to the percentage of its lumen showing immunopositivity (<25, 26-75, or >75). Endothelial staining intensity was graded low or high in each case. We found no significant difference between total number of vessels stained with D2-40 or LYVE-1 or between the 2 markers in terms of the percentage of luminal circumference stained or intensity in vessels smaller than 225 µm. LYVE-1 stained a higher percentage of luminal circumference of channels greater than 225 µm at both low and high intensities. Large channels stained much less and sometimes not at all with either antibody. D2-40 and LYVE-1 are both effective for highlighting endothelium of lymphatic malformations, staining similar percentages of channels. LYVE-1 provides more luminal staining in channels larger than 225 µm but is less specific also staining macrophages and adipocytes. Both markers are expressed less strongly or sometimes not at all in large channels.

Keywords: D2-40, LYVE-1, lymphangioma, lymphatic malformation

The hyaluron receptor LYVE-1, podoplanin, the homeobox gene product Prox-1, and vascular endothelial growth factor receptor 3 are molecules that have been reported to play a role in lymphangiogenesis and are selectively expressed by lymphatic endothelium (1-3). D2-40 (which recognizes podoplanin) and LYVE-1 are commercially available antibodies used as lymphatic endothelial markers in histologic sections of paraffin-embedded tissue to visualize lymphatic channels (4-8). Histopathologic differentiation of lymphatic malformations from primarily venous malformations on conventional hematoxylin and eosin stained sections can at times be difficult or impossible and is of paramount importance because of differences in treatment. Both D2-40 and LYVE-1 have been reported to stain lymphatic malformations (4-6,8) but only the efficacy of D2-40 has been assessed in this regard (6). It would therefore be desirable to optimize evaluation with the best available antibody. We hereby immunostained lymphatic

malformations with D2-40 and LYVE-1 antibodies and conducted a comparative analysis with attention to pattern, intensity and reliability of staining.

MATERIALS AND METHODS

Case Selection

We studied 20 lymphatic malformations of the skin/subcutis/soft tissue retrieved from the pathology files of Children's Hospital Boston between 2001 and 2004. The 20 cases were selected based on the presence of channels smaller and greater than 225 microns within the same lesion. The study was conducted in accordance with the Children's Hospital Boston Institutional Review Board policy.

Samples were from 9 boys and 11 girls ranging from 3 months to 20 years in age with a mean of 8.7 years. Eleven lesions were from skin and subcutis (some with subjacent soft tissue) and 7 from subcutis/soft tissue. Fourteen lesion were proximally located (head, neck, trunk or genitalia) and 6 distally (extremities).

Immunostaining

The antibody to D2-40 was purchased from Signet Laboratories (Dedham, MA) and used for immunochemistry as previously described (9). The LYVE-1 antibody was purchased from Reliatech (Braunschweig, Germany) and used at a dilution of 1:300 with the immunostaining procedure similar to that of D2-40 with the exception of epitope retrieval. In detail, formalin-fixed paraffin-embedded sections were cut, dried, deparaffinized, and rehydrated. Epitope retrieval was performed with Tris buffer (at pH 9.5 for D2-40 and at pH 6 for LYVE-1). Slides were rinsed with phosphate-buffered saline (PBS), hydrogen peroxide blocked (10 minutes) and rinsed in PBS. Antibody was applied and incubated for 1 hour at room temperature. Slides were again rinsed in PBS and secondary antibody was applied and incubated for 10 minutes at room temperature. Following a rinse, labeling reagent was applied and incubated for 10 minutes at room temperature, slides were rinsed, and chromogen was applied and incubated (20 minutes for D2-40, 15 minutes for LYVE-1). Finally, slides were then rinsed in water and counterstained with hematoxylin.

We grouped vessels into either smaller or larger than 225 microns. This size was chosen because it was the diameter of the high power field in the microscope utilized (vide infra). The percentage of the circumference immunostained in each lymphatic vessel was determined and they were divided then into three groups; 0%-25%, 26%-75% and 76%-100%. In addition, intensity of staining in each vessel was visually assessed as low or high. Determinations were performed in 10 high power fields (x400) with a light microscope (NIKON Y-200) using an identical light intensity and attempting to score identical fields for each marker. Determinations were done by one observer (AF-G).

Statistical Analysis

The percentage of the luminal circumference of each vessel occupied by immunopositive cells was categorized into three levels: 0-25%, 26-75%, and 76-100%. A Poisson log-linear model was used to compare total number of lymphatic vessels stained with D2-40 and LYVE-1. The distribution of immunopositivity across the three levels was compared between D2-40 and LYVE-1 using the Pearson chi-square test separately for small and large vessels at each of the two intensities. Therefore, an observed chi-square value exceeding 5.99 with 2 degrees of freedom was required to conclude that the markers differed significantly in the amount of lymphatic endothelial staining (10). Statistical analysis was performed using the SPSS statistical package (version 14.0, SPSS Inc., Chicago, IL). Two-tailed values of $p < 0.05$ were considered statistically significant.

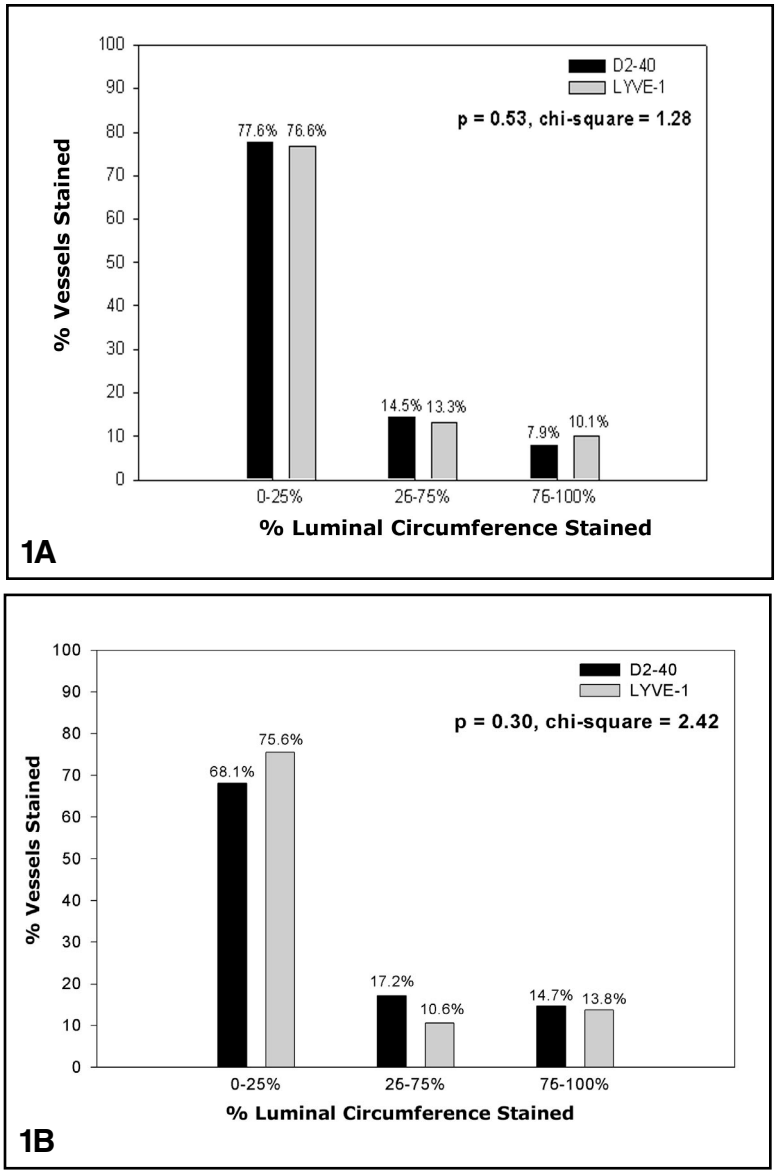

Fig. 1. Small vessels. There were no significant differences in the percentage of lymphatic endothelial staining between D2-40 and LYVE-1 for small vessels at low (A) or high intensity (B).

RESULTS

Histopathologic Features

All lesions exhibited features typical of lymphatic malformation, with channels of varying size lined by flat endothelial cells. The smallest channels had only endothelium whereas the larger channels had a variable and irregular smooth muscle coat. Occasionally, the largest channels had a smooth muscle/fibrous coat. The lumina were either empty or contained a lacy network of protein, clusters of lymphocytes, and/or occasional

hemosiderophages. Involvement of the papillary dermis was usually accompanied by epidermal elevation and hyperplasia and sometimes ulceration and inflammation. The stroma often contained clusters of lymphocytes, lymphoid follicles and plasma cells, and fibrosis of the dermis and subcutaneous fibrous septa was usually apparent.

Immunohistochemical Findings

The total number of immunopositive vessels was 1164 for D2-40 and 1208 for LYVE-1 in the ten fields (x400) from the 20 lymphatic malformations. A Poisson log-linear model was used to compare total number of lymphatic vessels stained with D2-40 and LYVE-1, and no significant differences were observed ($p = 0.98$). Thus, we conclude that overall the two markers stained a comparable number of vessels. Neither arterial nor obvious venous endothelium stained with D2-40 or LYVE-1. With LYVE-1, there was staining of macrophages and faint staining of adipocytes that was not observed with D2-40.

There were no significant differences in percentage of lymphatic endothelial staining between D2-40 and LYVE-1 for small vessels at low intensity ($p = 0.53$, chi-square test = 1.28) (*Fig. 1A*) or high intensity ($p = 0.30$, chi-square test = 2.42) (*Fig. 1B*).

The pattern of staining for D2-40 and LYVE-1 markers of large vessels at low and high intensities indicated some differences. At both intensities, the LYVE-1 marker stained a higher percentage of the luminal circumference compared to D2-40. At low intensity (*Fig. 2A*) the difference was borderline significant ($p=0.06$) and clearly significant at high intensity ($p<0.001$) (*Fig. 2B*).

These immunopositivity results for each marker according to vessel size and intensity are presented in *Table 1*. Comparing proximally versus distally located lesions, there were no significant differences in the percentage of cells stained with D2-40 or LYVE-1 (D2-40: 956/2979 = 32.1% vs. 212/642 = 33.0%, $p = 0.64$; LYVE-1: 979/3009 = 32.5% vs. 245/709 = 34.6%, $p = 0.31$)

Figure 3A and *3C* show a lymphatic malformation immunostained with D2-40. The lymphatic endothelium is stained in primarily small channels. *Figure 3A* and *3D* show a section (contiguous to the one depicted in *Fig. 3A* and *3B*) immunostained with LYVE-1 depicting staining of lymphatic endothelium in primarily small channels as with D2-40. The intensity is slightly greater with LYVE-1.

DISCUSSION

Lymphatic development is initiated during the sixth and seventh weeks of embryonic life with lymphatic endothelial markers such as VEGF-C, VEGF-A, VEGF-D, Prox-1, and LYVE-1 being expressed at different embryological ages (11).

D2-40 is an antibody that was raised against dysgerminoma and recognizes a 40,000 kDa O-linked membrane sialoglycoprotein M2A (12,13). It is also expressed in a variety of normal tissues (6,14), certain soft tissue tumors (15), mesothelioma (16) and a variety of brain tumors (17). It has been recently shown that this antibody recognizes podoplanin (7). D2-40 reacts with normal lymphatic endothelium but not hemovascular endothelium (15) and shows immunopositivity in lymphatic malformations, Kaposi sarcoma, kaposiform hemangioendothelioma, and some angiosarcomas (5,9,15). D2-40 has been reported to stain lymphatic endothelium more effectively in smaller vessels in lymphatic malformations (6).

LYVE-1 is a homologue of CD44, a member of the LINK superfamily, having a great affinity for hyaluronan and reported to stain lymphatic endothelium but not hemovascular endothelium (18,19). Lack of lymphatic endothelial specificity is reflected by immunopositivity in hepatic sinusoidal cells, splenic endothelium, high endothelial cells in lymph nodes, and activated tissue macrophages (20). Immunopositivity has

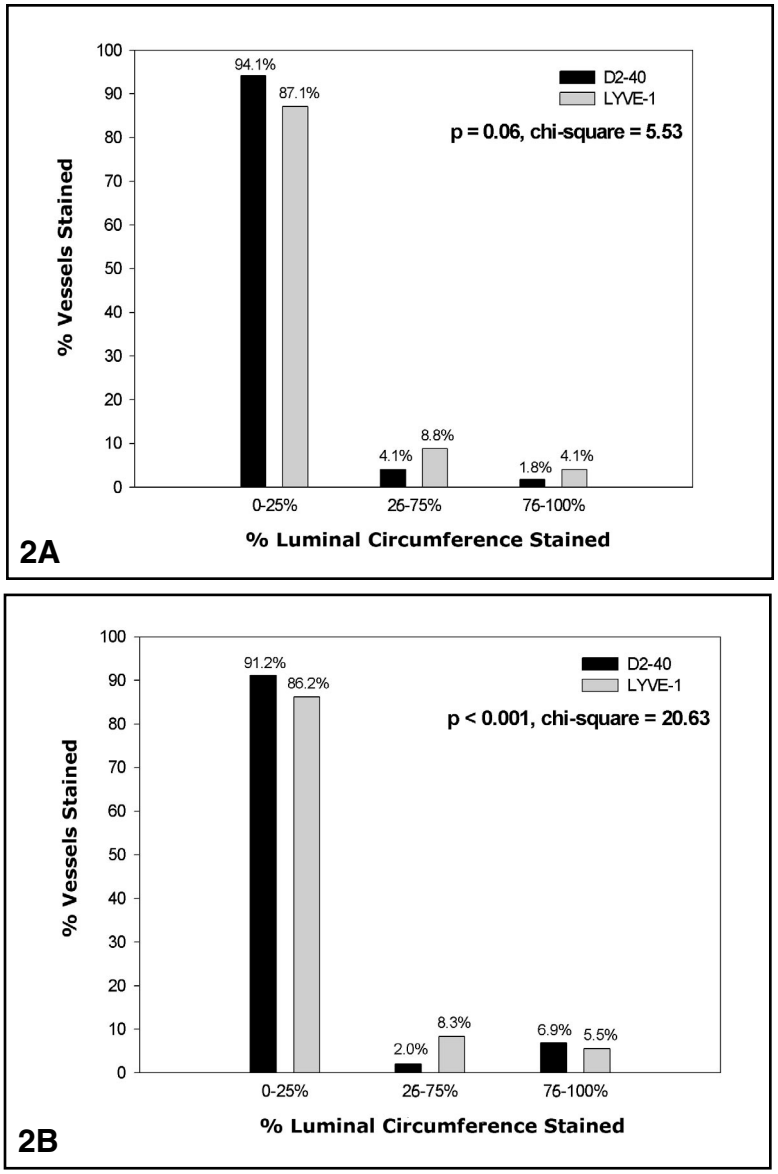

Fig. 2. Large vessel. At both intensities, the LYVE-1 marker stained a higer percentage of the luminal circumference compared to D2-40. The difference was borderline significant at low intensity (A) and clearly significant at high intensity (B).

been described in lymphatic malformations, Kaposi sarcoma, angiosarcoma (8), and infantile hemangioma (21,22).

Distinguishing lymphatic vessels, particularly of the small variety, from venous channels in normal tissues and in vascular malformations can be difficult (6). In particular, distinguishing lymphatic from venous malformations by conventional histopathology can be a vexing problem since wall structure and luminal contents may be indeterminate or misleading, respectively. The recent identification of immunohistochemical markers that reliably stain normal lymphatic

TABLE 1
Lymphatic Endothelial Immunopositivity for D2-40 and LYVE-1 According to Vessel Size and Staining Intensity

Vessel Size, Intensity	Lymphatic endothelial staining (% of vascular circumference)	D2-40	LYVE-1	p value
Small, Low	0-25	78% (246)	77% (386)	0.53
	26-75	15% (46)	13% (67)	
	76-100	8% (25)	10% (51)	
Small, High	0-25	68% (79)	75% (93)	0.30
	26-75	17% (20)	11% (13)	
	76-100	15% (17)	14% (17)	
Large, Low	0-25	94% (208)	87% (128)	0.06
	26-75	4% (9)	9% (13)	
	76-100	2% (4)	4% (6)	
Large, High	0-25	91% (465)	86% (374)	<0.001*
	26-75	2% (10)	8% (36)	
	76-100	7% (35)	6% (24)	

Small vessels were defined as <225 microns and large vessels as >225 microns. Numbers of vessels are shown in parentheses.
*Statistically significant based on Pearson chi-square analysis.

endothelium but not hemovascular endothelium in paraffin-embedded sections has considerably helped in the evaluation of certain vascular malformations. Correct identification of lymphatic malformations and their distinction from other vascular lesions is important not only for the proper classification of these various entities but also for their appropriate treatment (23).

A recent study has shown that D2-40 was a useful marker in identifying lymphatic vessels in vascular malformations, and that in the majority of cases more than half the lymphatic channels displayed staining. The authors also stated that smaller channels tended to stain more consistently than larger ones (6). Although our morphometric method differed somewhat, our results closely parallel theirs. The weak or even absent staining in the large channels is possibly related to other factors such as altered gene expression secondary to rheologic differences (24-26).

Our study shows that D2-40 and LYVE-1 are both effective in staining endothelial cells in lymphatic malformations and stain a similar percentage of channels. LYVE-1 stains a greater percentage of the luminal circumference in larger channels but has higher background staining because of immunoreactivity of adipocytes and macrophages. The reason why both markers are suboptimal in staining large channels is not fully understood.

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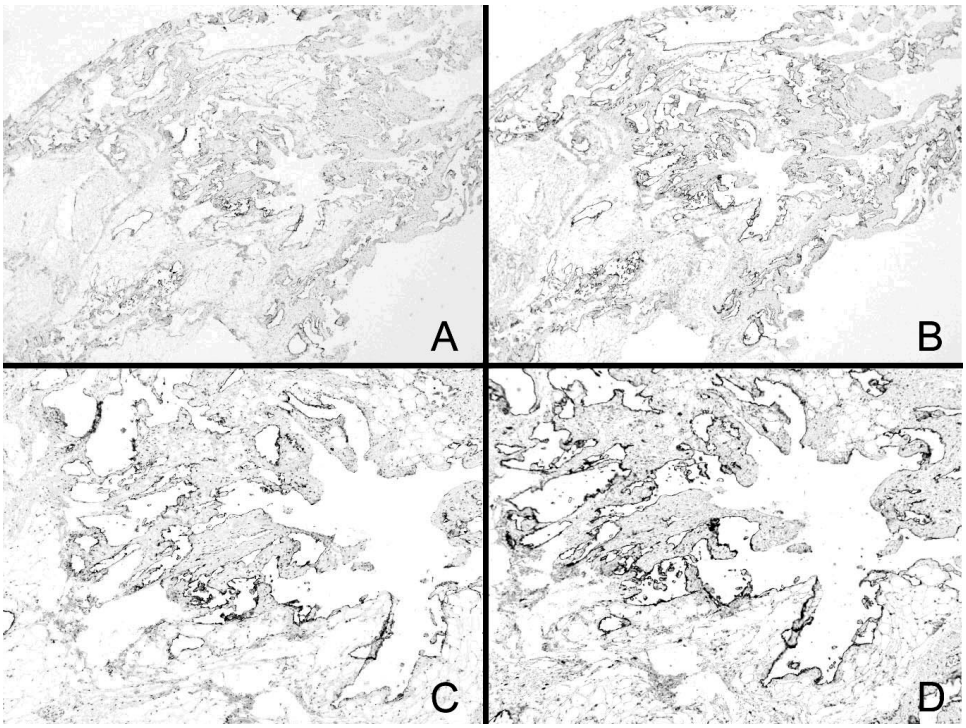

Fig. 3. Representative lymphatic malformation. A) D2-40 lymphatic endothelial immunopositivity is present in mainly small vessels (x4). B) LYVE-1 lymphatic endothelial immunopositivity is seen with a distribution essentially similar to that obtained with D2-40 is seen in a section contiguous to that in a (x4). C, D) D2-40 and LYVE-1 immunostains respectively at greater magnification show satisfactory lymphatic endothelial immunopositivity of small lymphatic channels but diminished or absent staining in large channels (x10). The intensity of lymphatic endothelial immunopositivity is minimally greater with LYVE-1.

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