LYVE-1 Is Not Restricted to the Lymph Vessels: Expression in Normal Liver Blood Sinusoids and Down-Regulation in Human Liver Cancer and Cirrhosis

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Abstract

Lymphatic vessel endothelial hyaluronan receptor (LYVE-1) is thought to be restricted to lymph vessels and has been used as such to show that tumor lymphangiogenesis occurs on overexpression of lymphangiogenic factors in mouse tumor models. However, these studies have not yet been corroborated in human tumors. Here we show, first, that LYVE-1 is not exclusive to the lymph vessels. Indeed, LYVE-1 is also present in normal hepatic blood sinusoidal endothelial cells in mice and humans. Surprisingly, LYVE-1 is absent from the angiogenic blood vessels of human liver tumors and only weakly present in the microcirculation of regenerative hepatic nodules in cirrhosis, though both vessels are largely derived from the liver sinusoids. Second, we propose a novel approach to identify lymphatics in human and murine liver. By combining LYVE-1 and Prox 1 (a transcription factor) immunohistochemistry, we demonstrate that lymphatics are abundant in cirrhosis. In contrast, in human hepatocellular carcinoma and liver metastases, they are restricted to the tumor margin and surrounding liver. The absence of intratumor lymphatics in hepatocellular carcinomas and liver metastases may impair molecular and cellular transport in these tumors. Finally, the presence of LYVE-1 in liver sinusoidal endothelia suggests that LYVE-1 has functions beyond the lymph vascular system.

Introduction

Molecular markers that unequivocally distinguish lymphatics from blood vessels are critical to further understand lymph vessel formation and function (1–3). The vascular endothelial growth factor receptor-3/FLT4 for vascular endothelial growth factor-C and -D was described originally as restricted to the adult lymphatic endothelia but later found in angiogenic blood vessels of retinas, wounds, and tumors and in fenestrated capillaries of normal tissues (4). Recently, LYVE-1, a CD44 homologue, was identified as the first HA receptor present on lymph vessels but completely absent from blood vessels (reviewed in Ref. 5). Subsequently, LYVE-1 was used as a marker to characterize lymphatics and lymphangiogenesis in mouse tumor models (reviewed in Ref. 3). Still unknown is whether lymphangiogenesis occurs in human cancer.

The only known ligand for LYVE-1 is HA, a ubiquitous extracellular matrix molecule with proinflammatory, angiogenic, and cellular migratory functions (6). The continuous turnover of HA involves clearance by tissue lymphatics, followed by degradation in the lymph nodes and LSECs. LYVE-1 functions in lymphatics, whereas the HARE functions in LSECs (7). But, in fact, the biochemical mechanisms of HA degradation remain largely unknown, e.g. it is still not known how cirrhosis and HCC lead to impaired HA degradation by LSECs, with concomitant increases in serum HA levels. A related question is which HA receptor is impaired in cirrhosis and HCC (8). HCC usually evolves from cirrhosis and is one of the most common malignancies worldwide, with a growing incidence of ≈1,000,000 new cases/year (9). A better understanding of the HA receptors within the liver microcirculation may provide critical information into the treatment of these diseases. On the basis of these observations, we hypothesize that LYVE-1 is also present in normal liver blood sinusoids. We studied LYVE-1 expression in the lymphatic and the blood microcirculation of both normal and diseased livers and provide the first supporting evidence for regulation of LYVE-1 in human liver cancer and cirrhosis. In addition, we present the first study on the distribution of lymphatics in HCC and liver metastases.

Materials and Methods

Human Tumor Specimens. Paraffin-embedded formalin fixed tissues obtained by surgical resection of 25 cases of HCCs and 17 cases of liver metastases (comprising 12 gastrointestinal, 3 pancreatic, and 1 ovarian carcinoma) were retrieved from the 1999–2001 surgical pathology files of the Massachusetts General Hospital. Sections were 4–6 μm thick. This study was performed under the guidelines of Massachusetts General Hospital.

Animals. Athymic NC/N Cr/Sed Nude, RAG2, CB-17/ICR/SCID/Sed, C3H/Sed, and FVB/NJ female mice, 8–9 weeks of age, were bred and maintained in our defined flora- and specific pathogen-free animal colony. C57BL/6J were obtained from The Jackson Laboratory (Bar Harbor, ME). All procedures were carried out following Institutional Animal Care and Use Committee approval.

IHC Analysis. IHC for LYVE-1 was done according to published methods (5) using the antimonous and anti-human LYVE-1 antisera, kindly provided by Dr. David Jackson (Oxford University, Oxford, United Kingdom). Mouse anti-human endothelial cell CD31 monoclonal antibody (clone JCT70A) was from Dako Corp. (Carpinteria, CA). IHC for Prox 1 was adapted to paraffin sections, from previous studies with frozen tissues (10, 11), as follows. Briefly, mouse tissues were fixed by vascular perfusion of 4% paraformaldehyde in PBS as published (12), dehydrated, and embedded in paraffin. Sections (5 μm thick) were dried for 4–10 h at 37 °C, dewaxed in xylene, and rehydrated in graded ethanol. After comparing six antigen retrieval methods, which produced equivalent results, we chose the following conditions: microwave retrieval [4 min on full power (950 W), followed by 6 min on 10% power] in low pH target retrieval solution (Dako Corp.), followed by 20 min at room temperature. Sections were rinsed in PBS [0.01 M phosphate buffer, 0.138 M NaCl, and 0.0027 M KCl (pH 7.4)] and soaked for 30 min at room temperature in methanol containing 3% hydrogen peroxide (Sigma Chemical Co., St. Louis, MO). Non-specific binding of antibodies to sections was blocked for 30 min at room temperature in PBS containing 10% goat serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), 5% BSA (Jackson Immunoresearch laboratories, Inc.), and 0.3% Triton X-100. We used three different Prox 1

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4 The abbreviations used are: LYVE, lymphatic vessel endothelial hyaluronan receptor; HA, hyaluronic acid; HARE, hyaluronan receptor for endocytosis; HCC, hepatocellular carcinoma; IHC, immunochemistry; LSECs, liver sinusoidal endothelial cells.

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antibodies, which were characterized previously (10, 11): (a) rabbit antiserum (1:1,000–1:10,000 dilution) obtained against a glutathione S-transferase-fusion protein containing homeo- and prospero domains of human Prox 1; (b) purified IgG fraction of this antiserum (1:500–1:6,000 dilution); and (c) purified IgG fraction (1:500–1:4,000) of antiserum raised only against the prospero domain of human Prox 1. The best signal-to-noise ratio was typically seen with the third one. Sections were incubated for 30 min to 1 h with primary antibodies diluted in blocking buffer. Negative controls were done by substituting the immune sera with nonimmune rabbit serum or negative control rabbit IgG (Dako Corp.) or by omitting the primary antibodies. No positive staining was obtained in these cases. Liver tissue-bound primary antibodies were detected using the Envision Peroxidase/Diaminobenzidine Systems (Dako Corp.). The Avidin Biotin Complex Vectastain Elite Peroxidase-based systems (Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine as the substrate (Sigma Chemical Co.) was used for all other tissues. Positive stainings were brown nuclei (Prox 1) and brown cell membranes (for LYVE-1 and CD31). Sections were counterstained with hematoxylin and mounted in Permount (Sigma Chemical Co.).

Lectin Perfusion. Biotinylated Lycopersicon esculentum lectin (Vector Laboratories) was injected systemically at 10 μg/gram of body weight 5 min before perfusion fixation. The livers were then processed as above. Vessel wall-bound lectin was detected with the Avidin Biotin Complex Vectastain Elite Peroxidase-based system.

Results

LYVE-1 Is Not Exclusive to the Lymphatic Endothelium but Also Present in Liver Blood Sinusoids. Because the liver sinusoidal endothelium plays a major role in HA catabolism (7), we hypothesized that LYVE-1 is present in the hepatic blood vasculature and not only in the lymphatics as is thought currently (3). To test this hypothesis, we used LYVE-1 IHC to stain livers from immune-deficient (RAG2, CB-17/ICR-SCID/Sed, and NCr/Sed-Nude) and immune-competent (C57BL/6J, C3H/Sed, and FVB/NJ) mice. Liver lymphatics stain for LYVE-1 (Fig. 1a). Our results show that, in all six strains, normal liver blood sinusoids also express LYVE-1 (Fig. 1, a and b); in addition, they stain for L. esculentum agglutinin, confirming blood perfusion of these LYVE-1-expressing vessels (Fig. 1c). The most intense sinusoidal LYVE-1 signal is seen in the metabolic Zone 2 of the murine liver acini (Fig. 1b).

In contrast, LYVE-1 expression is absent from the arterial and venular endothelium (Fig. 1, a and b). The same pattern is seen in normal human liver (Fig. 1d). Finally, we found that the LYVE-1 antigen is present in both the LSECs and Kupffer cells. Kupffer cells, which are resident liver macrophages located within the liver sinusoidal lumen, were identified as LYVE-1- and CD68-double-positive cells in adjacent liver sections (data not shown).
Importantly, in both mouse and human, blood sinusoids of the spleen and bone marrow do not express LYVE-1 (Ref. 5 and data not shown).

The Homeobox Protein Family Member Prox 1 Can Be Combined with LYVE-1 to Identify Lymphatics in Mouse and Human Adult Liver. We then examined LYVE-1 expression in diseased livers, both in the sinusoids and the lymphatics. Because we had found that LYVE-1 marks both blood and lymph vessels in the liver, we used Prox 1 to help distinguish between the two. Prox 1 is a transcription factor necessary for embryonic lymphangiogenesis (13) and restricted to the lymphatic endothelium of human melanomas grown ectopically in chick chorioallantoic membranes (14). However, its expression pattern in the mouse and human adult vasculature is not known. Thus, we proceeded to immunohistochemically stain a panel of adult tissues (liver, colon, lung, skin, pancreas, kidney, lymph nodes, and eyes) for LYVE-1 and Prox 1. Indeed, we confirmed both the presence of nuclear Prox 1 in the lymphatic endothelium and its absence from blood vessels (see Fig. 1e for data in the mouse colon). More pertinent to this study is the evidence that, in the liver, Prox 1-LYVE-1-double-positive vessels are only identifiable in the portal tracts and capsule (Fig. 1f and data not shown) and not in the parenchyma. This is the expected distribution for normal liver lymphatics (15). Thus, Prox 1 staining (but not LYVE-1) is a means to identify a specific vessel as being a lymphatic in the liver.

Angiogenic and Remodeling Blood Vessels of HCCs and Cirrhotic Nodules Show Lower LYVE-1 Expression than the Normal Sinusoidal Counterparts. Repeated liver injury by chronic hepatitis or drug or alcohol abuse can cause cirrhosis, the fibrous scarring of the liver (15). Elevated serum HA associated with cirrhosis is either caused by impaired degradation by the liver endothelium, increased output by activated Ito cells, or both (8). Thus, we hypothesized that decreased levels of the scavenging HA receptor LYVE-1 are associated with cirrhosis. Immunoperoxidase staining of adjacent sections for the endothelial marker CD31 (16) and for LYVE-1 revealed that, contrary to normal human hepatic sinusoids (Fig. 1d), LYVE-1 is only sparsely expressed in the parenchymal sinusoid-like vessels of cirrhotic livers (Fig. 2a). The LYVE-1-positive vessels within the fibrous tissue also express Prox 1 and, thus, are more likely to be lymphatics. Moreover, LYVE-1 is also typically absent from human HCC regardless of whether there is underlying cirrhosis in the surrounding liver (Fig. 2c; compare top and bottom panels) or not.
In liver metastases, the tumor vasculature is CD31 positive (Fig. 2d, top panel); however, LYVE-1 staining is not associated with the blood vessels either (Fig. 2d, bottom panel). Thus, CD31 is elevated and LYVE-1 expression in LSECs is decreased in both liver dysplasias, when compared with the normal liver.

Lymphatics Are Restricted to the Fibrous Connective Tissue Regions at the Margins of HCC and Liver Metastases. Next, we asked whether there are lymph vessels in HCC and in liver metastases (with and without underlying cirrhotic disease). We considered the Prox 1-LYVE-1-double-positive vessels as the only lymphatics identifiable in this study. Subsequently, we studied the distribution of such vessels in human liver tumors. Serial sections were stained for LYVE-1 (two sections per tumor), Prox 1 (two sections per tumor), and CD31 (one section per tumor). Fig. 3 shows a representative case of the results we obtained. Both in HCC (16 of 16) and in liver metastases (9 of 9), we found that lymphatics were not present within the tumor parenchyma or in the intratumor septa of connective tissue. However, they were visible in the fibrous areas that separate the tumors from the remaining liver (tumor capsule) and more abundant in the regions further away from the tumor nodules. In the cases where there was no clearly defined capsule, lymphatics were also restricted to the outer tumor margins. In other words, lymphatics were not seen within the core of liver tumors but were abundant in their immediate vicinity. These results were corroborated additionally by the absence of LYVE-1-positive vessels from a total of 25 HCCs and 17 metastatic adenocarcinomas (data not shown). We cannot exclude the possibility that there are lymphatics that do not stain for either Prox 1 or LYVE-1. Finally, because Prox 1-stained vessels typically stained with LYVE-1, these results also suggest that LYVE-1 is not downregulated in the lymphatics in cirrhosis and HCC.

Discussion

Role of LYVE-1 beyond the Lymphatic System. The findings we present here challenge the prevailing view that vascular expression of LYVE-1 is restricted to the lymphatics (3, 5) and suggest a role for LYVE-1 in hepatic blood vessels. The liver is the largest visceral organ in mammals (15), has the most richly perfused parenchyma, and is a major site for metastatic disease (17), the largest cause of mortality and morbidity in cancer patients (18). In addition, the liver microcirculation itself is unique in that its discontinuous, fenestrated (15, 19) endothelia also participate in extrathymic T-cell maturation and HA catabolism, the latter currently thought to be mediated by HARE (7). Interestingly, LYVE-1 staining of the hepatic sinusoids was most intense in metabolic Zone 2, the midzone of the liver acini, whereas the largest number and size of the characteristic LSEC fenestrae is seen in Zones 1 and 3. On the other hand, midzonal parenchymal cells are most susceptible to oxidative stress in ischemia reperfusion (20) and to nitric oxide synthase inhibitors in endotoxin-induced RBC arrest (21). Because both Kupffer cells and LSECs respond to HA by regulating nitric oxide synthase (22), it will be relevant to determine whether they do so via LYVE-1 signaling or through other cell-surface HA receptors, such as CD44 (23).
As an HA scavenging receptor, LYVE-1 could conceivably have a role in liver-specific functions. Considerations of kinetics and ligand-specificity make it unlikely that LYVE-1 is the main receptor for uptake of HA by LSECs (5) and more likely to function either in concert with HARE or in another HA-related process. Importantly, the HARE complex has not been sequenced or fully characterized yet, but HARE and LYVE-1 show very different tissue distribution patterns and kinetic properties, and antibodies to either molecule do not cross-react.5 LYVE-1 homology to CD44 is also a suggestive link to the immunoregulatory functions of LSECs (24).

Implications of the Differential LYVE-1 Expression in Cirrhosis and HCC. Whereas the reduction in LYVE-1 expression was evident in the blood vessels of HCC and cirrhosis, we did not observe a reduction in LYVE-1 in the lymphatics, suggesting cell-specific responses to the HCC and cirrhotic liver microenvironment. Interestingly, the most prominent difference in the liver vasculature in HCC is the occurrence of fenestrae, the formation of basement membranes underlying the LSECs (19), and the associated increase in the levels of serum HA. Transforming growth factor α has a major role in cirrhosis, inducing a decrease in the surface density of LSEC fenestrae and an increase in the synthesis of HA by Ito cells (reviewed in Ref. 25). Our results do not allow us to determine which aspect of sinusoidal “capillarization” leads to, or is influenced by, the altered LYVE-1 expression. Nevertheless, they do provide the first association between cirrhosis, HCC, and reduced levels of a cell-surface receptor for HA metabolism in vivo. In contrast to LYVE-1, CD44 is not normally expressed in LSECs but is up-regulated in rat cirrhotic liver (23) and in patients prone to cirrhosis because of alcoholic liver disease (26). Future studies are needed to evaluate how LYVE-1 expression correlates with the stage of liver disease in patients. Cirrhosis and tumor-induced alterations in LYVE-1 expression in the liver microcirculation could have important implications for the diagnosis and treatment of liver disease. Finally, it should be studied whether reagents that target LYVE-1 are a means for radioimmunodetection of cirrhosis and HCC.

Differential Lymphangiogenesis between Cirrhosis and Human Liver Tumors. Our present report is also the first study using two recently identified lymphatic markers, LYVE-1 and Prox 1, to characterize the distribution of lymph vessels in human liver tumors. Interestingly, because lymphatics are restricted to the portal tracts and the Glisson’s capsule of normal livers, their presence within the parenchymal fibrous areas that develop de novo in cirrhosis suggests that cirrhosis is accompanied by lymphangiogenesis. This is consistent with previous suggestions that increased lymph production in viral liver disease leads to lymphatic proliferation (27). On the other hand, we found only peritumoral lymphatics in liver cancer; lymphatics were not observed in the tumor parenchyma or between tumor nodules. These results are consistent with our previous lymphangiographic study in murine tumors (2). Intratumoral lymphatics have been identified in certain animal models (reviewed in Refs. 1 and 3), but, to date, no similar study has been done for liver tumors.

There is a remarkable contrast between the conspicuous presence of blood vessels throughout the liver (normal, cirrhotic, and cancer) and the concentration of the lymphatics in connective tissue. Lymphangiogenesis and lymph vessel survival may respond to mechanical and biochemical cues abundant within connective tissue; these cues may be absent from the intratumor microenvironment or overridden by lymphangiostatic factors (none of which has been identified to date), including the mechanical stress generated by cancer cells (28). These open questions, because the physiological and pathological stimuli driving lymph vessel proliferation are not known. We do know that angiogenesis is partly driven by oxygen and nutrient diffusion limits that establish a survival-based need for blood vessels at specific distances from normal cells. But is there any similar need for lymphatics? Is there any counterpart to hypoxia-driven angiogenesis in lymphangiogenesis? In other words, what are the sensors that trigger lymphangiogenic responses: pressure, pH, redox, or other possible indicators of a need for a drainage system? Future studies should address these questions, as well as determine whether the absence of lymphatics within HCCs and liver metastases is an impediment for molecular and cellular transport in human liver tumors.

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