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Lymphatic Patterns of Colorectal Liver Metastases

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Background. **Hematogenous spread is considered the predominant pathway for development of colorectal liver metastases (CRLM) and subsequent further tumor dissemination portal nodal involvement is also frequently observed in such cases, suggesting that lymphatics may have a role in the spread of CRLM. The role of lymphatics in the development of liver metastases is, however, controversial. The lymphatic patterns of CRLM were determined using a well established murine model.**

Methods. **CRLM were induced using a well established murine intrasplenic colorectal cancer model. Tumors were assessed at varying stages of development, and lymphatic patterns were determined in tumors and liver by immunohistochemistry staining for podoplanin and LYVE-1. Blood vessels were characterized using the vascular marker CD-34. Assessment was undertaken using digital microscopy and image analysis.**

Results. **Peri- and intratumoral lymphatic vessels were identified by podoplanin staining in all metastases and significantly increased with tumor growth. LYVE-1 staining was also noted but was variable. There was a concurrent significant increase in portal lymphatic staining within the normal liver with increasing growth of CRLM.**

Conclusion. **There is increased expression of lymphatics within CRLM and normal liver with increasing tumor growth. Lymphatic development is likely to play a significant role in the intrahepatic and periportal dissemination of CRLM.**

Key Words: **lymphatics; colorectal cancer; liver metastases; LYVE-1; podoplanin; lymphatic immunohistochemistry.**

INTRODUCTION

Studies on the development and dissemination of liver metastases have focused predominantly on the hematogenous spread of tumor cells. There is little information regarding the role of the lymphatic system in this process. Recent evidence suggests that the spread of liver metastases may be enhanced by the formation of peritumoral lymphatics *via* the process of lymphangiogenesis [1–5]. In patients with colorectal isolated liver metastases (CRLM), the presence of macroscopic or microscopic periportal nodal involvement may be as high as 20% to 28% [6, 7].

The finding of periportal lymph node metastases in patients with CRLM, without the finding of lymph node metastases adjacent to the site of primary disease supports the spread of hepatic metastases to the periportal nodes through lymphatics [8, 9]. The lymphatic development of CRLM at different stages of tumor growth and concurrent changes within the liver have not been previously characterized as far as is known.

One of the limiting factors in previously characterizing lymphatic vessels had been the lack of specific lymphatic vascular markers. Antibodies to LYVE-1, a hyaluronan receptor analogue, and podoplanin, a glomerular podocyte transmembrane mucoprotein have been shown in several studies to have specific binding to lymphatic endothelium. The patterns of lymphatic development were defined at different stages of growth of CRLM in both tumor and normal liver tissue using a well characterized murine model of CRLM.

METHODS

Animals

Male inbred CBA strain mice, 6–8 wk of age, were used in these experiments (The University of Adelaide, SA, Australia). Animals were housed in standard cages with access to irradiated food and water

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ad libitum and exposed to a 12 h light/dark cycle. All procedures were performed according to the guidelines of the Austin Hospital Animal Ethics Committee. A total of 28 animals were used for this study.

Liver Metastases Model

Colorectal liver metastases were induced by intrasplenic injection of dimethyl hydrazine (DMH) derived primary murine colon cancer cells (MoCR), by methods previously described [10]. The growth, vascular patterns, and histology of tumors in this model have similar characteristics to human tumors. Briefly, a cell suspension (1×10^6 cells/mL) was prepared by standard techniques. Mice were anaesthetized *via* intraperitoneal injection of ketamine 100 mg/kg (Parke Davis, Sydney, Australia) and xylazine 10 mg/1 (Bayer, Perth, Australia). The spleen was exteriorized through a left subcostal incision and 0.05 mL of colon cancer cell suspension (25,000 cells) was slowly injected into the body of the spleen using a 25 gauge needle. A splenectomy was then performed and wounds closed in layers. Liver metastases become macroscopically visible at approximately 10 d following induction. This was followed by a rapid growth phase within the liver, reaching a plateau phase by 21 d following induction.

Experimental Design

Groups of seven animals with liver metastases were killed by anaesthetic overdose at the following time-points after tumor induction: 10, 16, 19, and 21 d. The livers of animals containing metastases were removed entirely, immediately fixed in 10% formalin (Sigma-Aldrich, Castle Hill, NSW, Australia) for 48 h and then preserved in 50% ethanol. Each liver was sliced into 1.5 mm slices to obtain representative cross sectional samples. Between 6 and 10 representative slices per liver were processed for paraffin sections. A minimum of 10 colorectal liver metastases were assessed per animal.

Assessment

Immunohistochemistry

Formalin fixed paraffin-embedded sections (4 μm) were cut and mounted on Superfrost slides (SuperFrost Plus, Freiburg, Germany) and used for immunohistochemical staining, using a standard indirect polymer linked secondary antibody method. The primary antibodies used were monoclonal rat anti-mouse CD-34, for detection of tumor blood vasculature (MEC14.7; DAKO Cytomation, Copenhagen, Denmark), Syrian hamster anti-mouse podoplanin (DM3501; Acris Antibodies, GmbH, Herford, Germany) and rabbit anti-mouse LYVE-1 (ab14917; Abcam Sapphire Bioscience, NSW, Australia) for detection of lymphatic vessels.

LYVE-1 Immunostaining Technique: Lymphatic Structures

Antigen retrieval was carried out for LYVE-1 using heated citrate buffer solution (pH 6), by a standard technique. Tris buffered saline (TBS) was used for all washes or diluents unless otherwise specified. Endogenous peroxidases were blocked using 3% H_2O in TBS for 30 min. Nonspecific binding was then blocked using normal goat serum (20% NGS/TBS; 30 min) prior to the addition of the primary antibody. Sections were incubated in rabbit anti-mouse LYVE-1 antibody (5 μg/mL) for 30 min at room temperature. Sections were then incubated with a linking antibody, rabbit anti rat IgG (1:500; Abcam Sapphire Bioscience, NSW, Australia) for 30 min at room temperature. This step was followed by the protocols set by DAKO Envision plus Kit (DAKO Cytomation) containing a polymer linked horseradish peroxidase (HRP) conjugated secondary antibody (30 min incubation), and followed by 3,3'-diaminobenzidine (DAB) as the chromogen (8–10 min exposure). Sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted in DePeX mounting medium (Chem Supply, Gillam, SA, Australia) using standard techniques. Negative controls were included with each staining run.

Podoplanin Immunostaining Technique: Lymphatic Structures

Enzymatic digestion was used to retrieve antigens for podoplanin immunostaining. Sections were incubated in Proteinase K (20 μg/mL in TBS, Roche Diagnostics, Victoria, Australia) for 30 min at 37°C. Nonspecific binding was blocked with fetal calf serum (20% FCS/TBS; 30 min). Sections were then incubated with the primary antibody, Syrian hamster anti-mouse podoplanin, 1 μg/mL) overnight at 4°C. After this, the sections were incubated for 30 min at room temperature with rabbit anti-Syrian hamster IgG secondary antibody in 5% murine serum.

CD-34 Immunostaining Technique: Blood Vessels

Antigen retrieval was carried out for CD-34 using heated citrate buffer solution (pH 6) using a standard technique as described for LYVE-1. Sections were incubated overnight with the primary antibody (2 μg/mL) at 4°C. Negative controls were included with each staining run.

Qualitative and Quantitative Staining Assessment

Immunostained sections for LYVE-1 and podoplanin were used to investigate the localisation and distribution of lymphatic structures in relation to tumor location. Positive lymphatic structures were identified as luminal structures containing one or more brown stained cell, and no red blood cells in the lumen. Sections stained for the vascular marker CD34 were used to investigate the presence and localisation of tumor vasculature. Each liver sample was studied using a stereologic sampling technique using a Nikon Coolscope digital microscope (Nikon, Tokyo, Japan) in conjunction with the Image Pro Plus Image analysis program (Media Cybernetics, Bethesda, MD). First, a descriptive analysis of the staining patterns for each antibody was performed. Second, a number of quantitative assessments for the presence and degree of staining for lymphatics and vascular structures were undertaken.

Lymphatic Staining Within Normal Murine Liver

The number of positively stained portal triads within the normal liver at each time-point in tumor progression was quantitated. A positive portal triad was recorded when the portal triad contained one or more structures distinctly stained. The percentage of positive portal triads was determined. A total of five livers per group were examined by investigating 10 sections per liver. Every portal triad present in each liver was assessed.

The cross-sectional luminal area of the lymphatic structures within normal liver was assessed using the podoplanin stained sections. This measure is associated with the extent of functional lymphatics. Two independent researchers performed the quantitation in a blinded manner. Five portal triads per liver slice were randomly selected using the Nikon Coolscope at a magnification of 200 μm (Nikon) and images captured. A total of five livers per group were examined by investigating 10 sections per liver. Every portal triad present in each liver was assessed. The images were then analysed using Image Pro Plus analysis software. The cross sectional area of each positive structure within each image was then measured.

Lymphatics in Colorectal Liver Metastases

The localization of lymphatic structures were analyzed in relation to the tumors present within the liver samples. Tissues stained with LYVE-1 and podoplanin were used. Their presence was assessed as being in either intratumoral: (throughout the central areas within the tumor), or peritumoral; encompassing the periphery of the tumor.