A Pilot In Vivo Model of Human Microcystic Lymphatic Malformations

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Objective: To develop an in vivo mouse model of human microcystic lymphatic malformations (LMs) and provide a tool for investigating the biological mechanisms and treatment of microcystic disease.

Design: Animal model and histologic analysis.

Setting: Tertiary referral center.

Subjects: Fresh microcystic LM from human subjects were harvested and xenografted in the immunologically naive nude mice (Athymic Nude-Foxn1

Main Outcome Measures: Specimens were divided (5 x 5 x 5 mm) and secured in 4 quadrants subcutaneously along the dorsum of 4 nude mice. Weekly observations for volume, color, and texture of the grafts were performed with sequential harvesting from each quadrant at 30-day intervals. All grafts (n=16) were sectioned and stained with hematoxylin-eosin. Comparative pathologic evaluation of the grafts and native LM was performed by 2 blinded pathologists. Immunohistochemical analysis for D2-40 (a known lymphatic endothelial cell marker), Ki-67, and human-specific nuclear antigen was performed.

Results: Near complete microcystic LM xenograft survival (n=13 [81%]) was achieved in the mouse irrespective of the period of implantation. Xenografts underwent a brief growth phase to day 20 to 30 and were quiescent until approximately day 65 but ultimately had a gradual loss of volume following transplant. Histologic analysis revealed structural characteristics matching the native LM tissue. Immunohistochemical analysis found that 10 (77%) of the surviving xenografts (77%) were positive for D2-40, 9 (69%) were positive for human-specific nuclear antigen, and 8 (62%) were positive for Ki-67.

Conclusions: This preliminary in vivo model suggests that microcystic LM can survive in the athymic nude mouse. The presence of markers for human antibodies, lymphatic endothelium, and cellular proliferation demonstrates the stability of native tissue qualities within the xenografts.


LYMPHATIC MALFORMATIONS

( LM s) are one of the most common childhood head and neck masses. They arise from anomalous lymphatic vascular development and represent one of several types of congenitally derived vascular malformations (VMs). Consisting of ectatic lymphatic networks, LM may occur anywhere in the body. Present at birth, LMs are known to grow commensurate with their host with periodic expansion secondary to trauma, local infection, or dependency. Nearly 75% of LMs occur in the head and neck, with 30% of patients demonstrating obvious disease at birth. The incidence of LM has been approximated between 1 in 2000 and 1 in 4000 newborns with no obvious sexual or racial predilection.

Although LMs are regarded as benign vascular anomalies, they rarely resolve spontaneously and become problematic with age because of growth and mass effect on surrounding tissues. Lymphatic malformation is classified into microcystic or macrocystic disease based on the size of the cystic lymphatic spaces occupying the lesion. The etiology and pathogenesis, however, are not clear and current therapeutic modalities are limited. In particular, microcystic LMs have a high rate of persistence and recurrence despite available medical and surgical interventions. These entities are often very difficult to manage and can involve notable soft-tissue and boney distortion of the head and neck. Regions commonly affected include the mandible, floor of mouth, cervical soft tissue, and upper aerodigestive tract.

Questions surrounding LM in humans have remained elusive partially owing to the paucity of experimental models available for investigating their pathogenesis and treatment. On the basis
of previous in vivo investigations of malignant and vascular tumors, we aimed to develop a similar approach to examining LM in an animal model. The long-term goal of this pilot project is to achieve a greater understanding of LM development and provide a tool for exploring novel therapies. We present herein the preliminary results of an animal model of microcystic human LM.

METHODS

SPECIMENS

This study was approved by the institutional review board of University of Arkansas for Medical Sciences and Institutional Animal Care and Use Committee. After obtaining informed consent, fresh surgical specimens of microcystic LM were obtained from 3 patients. Representative samples of microcystic disease from two 12-year-old boys and one 30-year-old man were obtained for the purpose of this study. Clinical and radiographic confirmation of microcystic LM was established prior to harvest. Histologic examination by a pathologist experienced with vascular anomalies confirmed the diagnosis for each patient at the time of resection. Immediately at the time of harvest, surgical specimens were transported to the vascular anomalies research center. Specimens were then divided for xenograft implantation and formalin fixation (10%) with paraffin embedding.

THE MODEL

Four female 6- to 8-week-old nude mice (Athymic Nude-Foxn1tm; Harlan) were anesthetized with intraperitoneal injection of 2.22 mL/Kg of ketamine hydrochloride (100 mg/mL), xylazine hydrochloride (40 mg/mL), and phosphate-buffered saline solution (PBS) at premixed 1:1:2.3 volume ratios. Fresh human LM specimens were sectioned into approximately 5 × 5 × 5-mm pieces and inserted subcutaneously into 4 quadrants along the dorsum of the nude mice. A single section was placed at the upper-left, upper-right, lower-left, and lower-right quadrants. A total of 4 mice were used to place 16 xenografts in this experiment.

OBSERVATION, MEASUREMENT, AND HARVESTING

Xenograft appearance was observed and recorded at 7-day intervals for volume, color, and texture. Simultaneously, the size of each graft was recorded using vernier calipers and using the average of the 2 largest diameters (a and b). The volume \( V \) of grafts was estimated as \( V = \pi/6(a \times b)^{3/2} \). Each graft was harvested at essentially serial 30-day intervals, removing 1 graft at each of 4 time points (30, 60, 90, and 120 days). This was performed with the mice under anesthesia, again with intraperitoneal injection of 2.22 mL/Kg of ketamine (100 mg/mL), xylazine (40 mg/mL), and PBS at premixed 1:1:2.3 volume ratios. In 1 mouse, 2 grafts were harvested on the 45th day. All nude mice survived the entire experimental protocol and were retained throughout the experiment until the last graft was harvested.

HISTOLOGIC ANALYSIS

Harvested grafts from the nude mice were formalin fixed and paraffin embedded. For both the fresh human samples and posttransplanted mice specimens, 4-μm sections were deparaffinized and stained with hematoxylin-eosin (HE). Representative slides were examined by 2 blinded pathologists (C.-Y.F. and A.G.S.) who had experience with vascular anomalies.

IMMUNOHISTOCHEMICAL ANALYSIS

After deparaffinization and rehydration, sections were heated to 97°C for 20 minutes in a water bath in the presence of antigen retrieval solution (CITRA, pH 6.0; Invitrogen) and cooled for 30 minutes. The endogenous peroxidases in all sections were blocked with hydrogen peroxide for 10 minutes and washed with PBS (pH 7.4; SIGMA). Sections were preincubated with 2% nonfat milk for 30 minutes at room temperature. Then the sections were incubated in primary D2-40, human-specific nuclear antigen, and Ki-67 antibodies for 20 hours at 4°C. After washing with PBS, the sections were incubated in primary antibody enhancer (Thermo Fisher Scientific) for 10 minutes and HRP Polymer (Thermo Fisher Scientific) for 15 minutes at room temperature. After washing the sections in PBS, they were incubated with diaminobenzidine (DAB; Thermo Fisher Scientific) for 5 minutes at room temperature. The sections were counterstained with hematoxylin for 20 seconds. Sections were dehydrated through graded alcohol solutions and cleaned by xylene substitute. Then they were mounted with Permount medium (Thermo Fisher Scientific) and coverslips.

ANTIBODIES

D2-40 (mouse antihuman monoclonal antibody; Abcam Plc) was diluted at 1:400. Human-specific nuclear antigen (mouse anti-human monoclonal antibody; Millipore) was diluted at 1:400. Ki-67 (rabbit antihuman monoclonal antibody; Abcam Plc) was diluted at 1:100. Strong staining in greater than 10% of the cells indicated a positive receptor value. Xenografts, the native human specimens, and mouse skin with subcutaneous tissue, acted as experimental specimens, positive controls, and negative controls, respectively, for the stained antibodies.

RESULTS

XENOGRRAFT QUALITY

All nude mice survived the entire experimental protocol until the last graft was harvested. In mouse 3, 1 graft disappeared on the seventh day after transplant. Two grafts in mouse 4 became infected and were determined unsuccessful. The remaining 13 grafts (81%) survived the protocol and were retained throughout the experiment until the final harvest on day 120.

On average, the xenografts had a small but brief growth phase until day 20 to 30. Following this transition the xenografts underwent a gradual decrease in size. Graft size appeared quiescent from the 35th to 65th day after transplant (Figure 1A). Before transplant, the fresh human LM specimens were white and consistently soft in texture. Similarly, the graft appearance under the nude mouse skin remained white, flat, and soft until day 20 of transplant. At this point the grafts changed to a harder texture with blue discoloration. At 2 months, the grafts gradually turned purple. At the date of harvest, all the harvested grafts were round and vascular (Figure 1C).

HISTOPATHOLOGIC ANALYSIS OF GRAFTED LM

Pathologic investigation of the harvested xenografts revealed the presence of a vascular malformation but of unclear origin. The identification of lymphatic channels was noted in all grafts despite day of harvest. All 13 surviv-
ing grafts were deemed viable and showed no evidence of tissue necrosis.

The harvested grafts (n=13) were composed of capillaries and lymphatic channels. In the peripheral area of these grafts, the LM channels were most abundant. Tissue quality was better in younger grafts, which also had less capillary infiltration. Lymphatic channels in the grafts were lined by lymphatic endothelial cells and filled with acellular, pink, and homogeneous lymph, rather than by red blood cells that were seen in the veins and capillaries of the harvested specimens (Figure 2). Thirty days after transplant, in the center of the tissue, the cellular density was low and hyaline degeneration was found. At 60 days after transplant, fibrous and fat tissue was identified in the harvested specimens, which was retained until day 120.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Of the surviving xenografts (n=13), 77% (n=10) were positive for D2-40, as shown in Figure 2B and Figure 3. The number of lymphatic channels in LM grafts was highest at 30 days after transplant (Figure 3A). The grafts decreased in volume with time, but the number of lymphatic channels were maintained and appeared more densely packed by 120 days (Figure 3B). The preimplanted human specimens of LM were used as a positive control for D2-40 immunostaining. Unaffected mouse skin and subcutaneous tissue was also stained and confirmed negative for D2-40.

To confirm that the harvested xenografts were retained human specimens, immunohistochemical analysis for human-specific nuclear antigen was performed. Nine grafts (69%) were positive for human-specific nuclear antigen; 8 grafts (62%) were positive for Ki-67, a marker of cellular proliferation. Results from these stains can be observed in Figure 4A and B. Mouse myocardium was also used as negative control for human-specific nuclear antigen immunostaining. The preimplanted human LM tissue was negative for Ki-67 by immunostaining (Figure 4C).

**COMMENT**

Lymphatic malformations are the most common form of congenital vascular malformation and classified into macrocystic, microcystic, and mixed lesions.3,4 Previously labeled as “lymphangioma” or “cystic hygroma,” macrocystic LM are composed of single or multiple cysts 2 cm3 or larger. Microcystic LM are composed of single or multiple cysts smaller than 2 cm3. Mixed LMs include both macrocystic and microcystic components. Generally, microcystic LM are regarded as benign anomalies of the lymphatic system that can suddenly expand in size, infiltrate vital structures, impair function, or cause significant long-term sequelae.5 Local infection, trauma, and hormonal fluctuations have been attributed to disease expansion. Despite recent reports that macrocystic diseases can spontaneously resolve in a small percentage of patients, this has not been found in mixed or microcystic LMs.

Various therapeutic options have been explored for LM. They range from surgical excision to intralesional injection of sclerosing agents such as bleomycin, doxycycline, ethanol, and OK-432.4,6-13 Successful resolution of macrocystic LM by both surgical and intralesional treatment approaches has been found.11-14 Unfortunately, current therapeutic modalities have inconsistent success in microcystic and mixed lesions.3 At this point, there is limited evidence to suggest a role of intralesional therapy for microcystic disease.13 As a result, surgical excision is required to control microcystic LM. Carbon dioxide laser ablation can augment treatment of involved mucosa.11 Successful therapy of microcystic disease often requires multiple procedures that involved removal of infiltrated normal tissue. As a result of persistent growth...
or therapy, microcystic LM often leads to significant functional and aesthetic impairment of patients affected. More importantly, despite resection, microcystic lesions have a high rate of recurrence and retained ability to expand. The poorly understood etiology and pathogenesis of microcystic LM has curtailed new therapeutic approaches to this recalcitrant disease. At present, research dedicated to LM depends on tissue extraction and

![Figure 2](image_url) **Figure 2.** Histopathologic analysis of xenografts microcystic lymphatic malformation (LM). A, Ninety days after transplant, tissue at the periphery of grafts maintained features of LM, which is characterized by thin-walled spaces, lined by endothelial cells and filled with acellular, pink, and homogeneous lymph (hematoxylin-eosin, original magnification ×200). B, The endothelial lining of lymphatic spaces seen in surviving LM grafts stained strongly positive (black) for lymphatic endothelial marker D2-40 (immunohistochemistry, original magnification ×200).

![Figure 3](image_url) **Figure 3.** D2-40 immunostaining (black) for harvested lymphatic malformation (LM) xenografts is consistent despite period of implantation. A, Thirty days after transplant, the number of lymphatic channels in LM grafts is highest (immunohistochemistry, original magnification ×200). B, At 120 days after transplant, the LM grafts were composed of more densely packed lymphatic channels that remained positive for D2-40 (immunohistochemistry, original magnification ×100).

![Figure 4](image_url) **Figure 4.** Lymphatic malformation (LM) graft immunostaining for antihuman nuclear antigen and Ki-67 (immunohistochemistry, original magnification ×200). Positive marker of antihuman nuclear antigen at 30 days after transplant (A); Ki-67 was positive in transplanted graft (B) but negative in preimplanted native human LM tissue (C).
molecular analysis. To understand the development of LM and identify alternative treatment options, translational science for LM is necessary. In particular, animal models reflecting the pattern of LM development can improve our knowledge and approach to these lesions. In 1999, an experimental mouse “lymphangioma” model was developed by Mancardi et al. These authors reported that an LM could be developed in the mouse by intra-peritoneal injections of Freund’s incomplete adjuvant. This method has also been used to induce “lymphangioma” in rats. However, these models were later found to be better representations of oil-granulomas and plasmacytomas that contain lymphatics and blood vessels rather than true LM. More importantly, these models were not developed from native human LM, the ideal tissue sample for investigation of this disease. On the basis of a standard experimental approach to developing in vivo models, we suspected that microcystic LM could be xenografted. We aimed to achieve this goal by using the nude mouse as our vehicle for observing native human LM.

Nude mice are athymic and T-cell deficient. They have the ability to tolerate xenografts and have been widely used for pathologic and normal human tissue xenografting. More importantly, 2 independent researchers demonstrated the survival of human hemangiomata, a benign tumor of vascular origin, in the nude mouse. Because LMs are another type of vascular anomaly, we hypothesized that they may also survive in the nude mouse and likely to retain their principal morphologic and biological properties when grafted.

In the present study we demonstrate the survival of human LM in 13 of 16 xenografts (81%) harvested from 3 patients with microcystic disease. A brief period of growth occurred early but was not maintained as the samples became quiescent and subsequently diminished in size over a period of 120 days. Structural characteristics of human LM were retained and confirmed by pathologic examination, with the greatest number of lymphatic channels identified at 30 days after implantation. The lymphatic channels were found abundantly packed at the periphery of the specimens with focal hyaline degeneration and fibrosis at later intervals of harvesting (60 days). However, pathologic examination revealed that the number of lymphatic channels did not decrease until 120 days after implantation. Necrosis or tissue loss was not identified in any of the harvested grafts. These results suggest that xenografted human LM can survive but do not grow in the athymic nude mouse. Xenografted tissue appears ideally reflective of native LM at 20 to 30 days after transplant. However, how long these LM grafts can survive, and if they can be stimulated to grow, needs further research.

Lymphatic malformation is characterized by lymphatic vessels as thin-walled channels lined with endothelial cells, irregularly shaped, and heterogeneous in size. However, lymphatic channels lack clear light microscopic definition in current pathologic practice and require confirmation by specific immunostaining the lymphatic endothelial cell markers. D2-40 antibody is a specific LM marker that labels lymphatic endothelium of thin-walled vascular channels and does not react with blood vessel endothelium. In this experiment, the LM endothelial cells of the grafts revealed strong positive staining of D2-40 and maintained the same structural features of human LM. The D2-40 antibody was specific to human antigens. However, to confirm the presence of human we performed staining specific to human antigens. The strong positive staining for human-specific nuclear antibody for the majority of ours samples (69%) indicates that the lymphatic tissue in the mice xenografts originated from human tissue and was not induced by the nude mouse itself.

The specificity of D2-40 antibody for lymphatic endothelial cells is virtually 100%; whereas sensitivity is estimated at 60% to 65%. This suggests that the number of lymphatic channels that actually exist in the grafts likely includes more than just D2-40–positive channels. This observation regarding D2-40 immunostaining also explains why 3 of the 13 grafts in our sample were D2-40 negative. The specificity and sensitivity of other lymphatic endothelium markers for this animal model needs further investigation. Lymphatic endothelium markers such as Prox-1 and Lyve-1 are available but are not as sensitive, nor are they as specific, as D2-40 to small lymphatic channels. Therefore, we used and considered D2-40 as the best marker in this microcystic disease model.

We observed another interesting phenomenon in our research. To identify the potential presence of proliferating cells within the xenografts, we immunostained for Ki-67, a marker commonly used to indicate cellular turnover in in vivo models of carcinoma. Although LM is a slow-growing entity in humans, we suspected that a transplant may stimulate cellular division. In this study, 8 grafts stained positive for Ki-67. The original human specimens in this study, however, were negative for Ki-67, consistent with a report by Chen and colleagues that there is no expression of Ki-67 in either human microcystic or macrocystic LM. The expression of the human Ki-67 protein is strictly associated with cell proliferation. The antibody in our study, however, was not specific to human tissue, and potential antigen crossover may explain the presence of proliferating cells, potentially mouse, within our harvested specimens. Further investigation of cellular turnover in the grafted human LM tissue is necessary.

However, this result from Ki-67 immunostaining may also indicate that cellular proliferation is occurring in the tissue sample following transplant and explains the recurring mechanism of microcystic LM after surgical excision. Interestingly, Ki-67 has been used as a marker to demonstrate increased plasmacytoid dendritic cells in human LMs. Previous work suggests that immunomodulation associated with plasmacytoid dendritic cells may play a role in LM pathogenesis and recurrence.

In conclusion, in this pilot study we examined the potential for human microcystic LM, a recalcitrant vascular anomaly, to grow in vivo. To our knowledge this is the first reported attempt to observe native human LM in the nude mouse model. These preliminary results suggest that human microcystic LM can survive in nude mouse with structural and molecular characteristics similar to the original human specimens. This animal model may provide a new and promising research tool for human LM.
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Author Contributions: Dr Hou had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Hou, Dai, Shafirstein, Suen, and Richter. Acquisition of data: Hou, Buckmiller, and Richter. Analysis and interpretation of data: Hou, Dai, Shafirstein, Fan, Saad, Suen, and Richter. Drafting of the manuscript: Hou and Richter. Critical revision of the manuscript for important intellectual content: Dai, Buckmiller, Shafirstein, Fan, Saad, and Suen. Statistical analysis: Hou and Richter. Administrative, technical, and material support: Dai, Shafirstein, Suen, and Richter. Study supervision: Dai, Shafirstein, Fan, and Saad.

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REFERENCES