

LACK OF FUNCTIONING LYMPHATICS AND ACCUMULATION OF TISSUE FLUID/LYMPH IN INTERSTITIAL “LAKES” IN COLON CANCER TISSUE

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ABSTRACT

There is controversy as to whether intratumoral or peritumoral lymphatics play a dominant role in the metastatic process. The knowledge of how and where exactly tumor cells enter lymphatics is important for therapeutic targeting either the tumor core or peritumoral tissue with drugs or radiation. The basic questions remain: what is the morphological structure of intra- and peritumoral interstitium and lymphatics; what is their hydraulic conductivity?; and do these local physical conditions allow detached tumor cells to migrate to lymphatics? Identification of lymphatics has been based on immunohistochemical staining of lymphatic endothelial cells. This method does not, however, show the tissue fluid filled interstitial space and the shape of minute lymphatic vessels in tumors. We visualized the interstitial space and lymphatics in the central and peripheral regions of tumors using our original method of color stereoscopic lymphography in translucent tissue fragments and simultaneously with immunohistochemical staining of lymphatic and blood endothelial cells. The density of open and compressed lymphatic and blood vessels was measured in the intratumoral

“hot spots” and at tumor edge. Moreover, the intratumoral tissue hydraulic conductivity was measured to define force necessary for propelling tissue fluid to peritumoral lymphatics. We found very few rudimentary minor blind lymphatics in the tumor core and numerous minor fluid “lakes” in the interstitium with no visible connection to the peritumoral lymphatics. Lining of “lakes” did not express molecular markers specific for lymphatic endothelial cells. Ninety-five percent of structures of what looked like lymphatics had compressed lumen and the hydraulic conductivity was 3 powers of magnitude lower than in the adjacent non-tumoral tissue. It can be concluded that lack of functioning lymphatics in tumor foci manifested by accumulation of tissue fluid in “lakes,” low fluid conductivity and compression of lymphatics by tumor cells, and proliferating connective tissue may hamper escape of tumor cells. The most favorable site of entry of tumor cells to lymphatics seems to be the interface of the tumor and surrounding tissue with open lymphatics.

Keywords: tumor, lymphatics, hydraulic conductivity, metastasis, color stereoscopic immunohistochemistry, lymphography

Metastatic cancer cells can escape from their site of origin and spread to distant organs through invasion of the blood vascular and/or the lymphatic system, and this metastasis is the leading cause of cancer mortality. Tumor vascularization is widely accepted as a bona fide indicator of tumor growth, metastases, and patient survival. In 1996, Vermeulen (1) published a first international consensus on the methodology and criteria of the evaluation of angiogenesis quantification in solid tumors and 5 years later, a second consensus report appeared, in which new concepts and mechanisms of tumor vascularization were integrated (2). The third consensus was published in 2006 (3). Contrary to angiogenesis, the *de novo* formation of lymphatic vessels or lymphangiogenesis and its role in promoting the metastatic spread of tumor cells has only recently become a focal point of cancer research showing a relationship between patient survival and lymphatic density in different tumor types.

Although there is a large body of evidence that newly visualized lymphatics facilitate formation of metastases, it remains unclear whether the intra- or peritumoral lymphatics mainly participate in transport of detached tumor cells to the lymph nodes. Some studies prove peritumoral neolymphangiogenesis contribution to tumor metastases, however, opposite views exist as to whether intratumoral lymphatics have any role in tumor metastasis (4,5). The knowledge of where exactly tumor cells enter lymphatics would be important for therapeutic targeting either the tumor core or peritumoral tissue with drugs or radiation.

The basic questions remain on the morphological structure of intra- and peritumoral interstitium and lymphatics, their hydraulic conductivity, and consequently the local physical conditions allowing tumor cells to migrate to the lymphatics. If there are open initial lymphatics in the tumor core, tumor cells could easily enter them and flow with the lymph stream. However, there is a

large body of evidence that tumor tissue fluid pressure is high, and tumor cell mass and desmoplastic connective tissue compress lymph and blood vessels (6-8). Identification of lymphatics has been based on immunohistochemical staining of lymphatic endothelial cells. This method does not, however, show the tissue fluid filled interstitial space and the shape of minute lymphatic vessels.

To visualize the interstitial space and lymphatics in the central and peripheral regions of tumor, we used our own original color stereoscopic lymphography in translucent tissue (9,10) with simultaneous immunohistochemical stainings specific for lymphatic and blood endothelial cells. The density of open and compressed lymphatic and blood vessels was measured in the intratumoral "hot spots" and at tumor edge. Moreover, the intratumoral tissue hydraulic conductivity was measured to define the force necessary for propelling tissue flow to peritumoral lymphatics.

MATERIAL AND METHODS

Patients

Samples of human colon cancer and normal colon tissue were obtained from 50 patients and were classified according to the TNM staging (*Table 1*). Harvesting of tissues and their morphological evaluation were approved by the Warsaw Medical University ethics committee.

Processing of Specimens

Tumor containing colon tissue (3x5cm) was divided into two fragments for color lymphography and immunohistochemistry. One fragment was immediately injected with Patent Blue in chloroform suspension to visualize the interstitial space and lymphatic capillaries. Another fragment was snap-frozen at 70°C, sectioned, and stained with monoclonal antibodies. Fluid hydraulic conductivity was measured in 12 fragments.

TABLE 1
TNM Staging of Colon Cancer Patients

Stage	Number of patients
T1	2
T2	8
T3	38
T4	2
N0	26
N1	14
N2	10
M	0
Liver	10
Lung	0
Peritoneal dissemination	1

Color Lymphangiography

Briefly, a 2x3cm fragment of tissue was injected with Paris blue (artist paint) suspension (9,10). One gram of paint was suspended in 20ml of chloroform (Sigma, St. Louis, MO, USA), mixed for 1h, centrifuged for 20 min at 2,000 rpm, and filtered at 0.22 micron. A drop of suspension was checked under light microscopy for presence of clumps, and the solution should be clear blue. A glass insulin syringe with 23 gauge needle was used for injection because plastic syringes dissolve with chloroform. Injection of 1ml lasted for 3 min under low pressure. Subsequently, the injected tissue was fixed in 6% formalin for 1 day and dehydrated daily for 6 days in 60% ethanol (first day), 70% (second day), 80% (third day), 96% (fourth and fifth days) and 100% (sixth day). Then, tissue was placed in methyl salicylate (Sigma, St. Louis, MO, USA) for 24h and cut into 150-500 micron thick slices. Preservation in methyl salicylate makes the specimen transparent. Evaluation was done using stereomicroscope and magnification of 100x was routine. For higher magnification, specimens were cut thinner (50-100 microns).

Paris Blue only slightly stains the inner wall of vessels so that the anterior and posterior aspects and lumen can be observed at their entire length. To discriminate between lymphatics, veins and arteries of the same size, these two last vessels were injected through the stumps protruding from the specimens.

In order to further prove that the stained structures were not blood vessels, five-by-five mm thick Paris Blue injected fragments of tissue were snap frozen at -70°C and sectioned for immunohistochemical staining with monoclonal antibodies to LYVE-1 lymphatic endothelial cell hyaluronan receptor and FVIII- related antigen and CD31 to identify blood endothelial cells.

Immunohistochemistry of Tumor Specimens

The tumor fragments were fixed in 10% formalin for 24h at room temperature and embedded in paraffin. Five-micron -thick sections were cut and incubated with 10% normal rabbit serum in phosphate-buffered saline (PBS) solution, followed by treatment separately with biotinylated mouse anti-human FVIII-related antigen or CD31 (both Dako, Glostrup, Denmark), LYVE-1, podoplanin, and prox-1 (RELIATech, Wolfenbüttel, Germany) monoclonal antibodies followed by streptoavidin-alkaline phosphatase complex (LSAB 2 Kit AP, DAKO, Glostrup, Denmark). Counterstaining was performed with Mayer's hematoxylin.

Quantification of Blood and Lymphatic Vessels

Lymphatic vessel density (LVD) and micro-blood vessel density (MVD) were assessed on histological sections of the colon cancer as well as in normal colon tissue distant from the cancer (>5cm) (11). Presence of vessels in tissue sections was evaluated following staining with monoclonal antibodies as described above. Briefly, 6 area "hot spots" of maximal LVD and MVD were identified on the cancer sections by screening

(magnification x 40 and x100). Three areas in the vicinity of normal tissue (tumor periphery) and 3 towards the tumor center (inner tumor), and additionally, MVD and LVD were counted on 3 areas between normal colon tissue and adjacent tumor margin and 3 areas distant from the tumor (normal tissue). The number of vessels was counted within a counting grid at 200x magnification in 3 to 5 consecutive fields in each area. As defined by N. Weidner for the blood and lymphatic vessel count, "any stained endothelial cell or cell cluster separated from another microvessel structure was considered a countable microvessel" (11). The slides were reviewed independently by three observers (WLO, MS, MG) blinded to clinical details. MVD and LVD are expressed as the mean value of counted microvessels in 3 to 5 evaluated grids in areas of maximum vessel density.

Cross Section Area of Lymphatic and Blood Vessels

The cross section area ratio – the ratio of lymph and blood vessel area within the tumor and adjacent colon tissue to lymphatic and blood vessels area in the normal colon tissue – was counted using a computerized image analysis system (Olympus, Microimage, Japan).

Open vs Closed Lymph and Blood Vessel Ratio

The ratio of open vs closed lymph and blood vessels within the tumor and adjacent colon tissue to lymphatic and blood vessels in the normal colon tissue was counted using computerized image analysis system (Olympus, Microimage, Japan). Closed vessels had a lumen of less than 1 μ m in diameter.

Compression of Lymphatics in Tumor Tissue

The level of compression of lymphatic vessels was assessed by quantification of the aspect ratio – the ratio of maximum to minimum axes of perfused vessels. An aspect ratio of 1 represented a perfect circle. The

larger was the aspect ratio, the greater the amount of vessel compression. Aspect ratio data were calculated as mean \pm SD.

Histochemical Staining for Confocal Microscopy

Specimens were stained with monoclonal antibodies against LYVE-1, podoplanin, and prox-1. Secondary fluorochrome-bound goat-anti-mouse Alexa Fluor 633, and Alexa fluor 488 (Invitrogen, Oregon, USA) were applied. Cell nuclei were stained with Hoechst Bisbenzimidazole H 33258 (Sigma-Aldrich, Germany). Confocal microscope (Zeiss LSM 510) with helium-neon 633nm, and argon 488nm and 514nm lasers was used for visualization.

Tumor Hydraulic Conductivity

A routine method for measuring tissue hydraulic conductivity was applied (6,7). Hydraulic conductivity is a sum of conductivity of extravascular tissue and lymphatic and blood vessels. Fast increase in tumor cell mass and formation of dense connective tissue around tumor foci may cause deformation and obturation of lymphatics and make them nonconductive. Also blood vessels may undergo partial or total compression. Accumulation of tissue fluid increases its pressure. We recorded tissue fluid pressure using the wick-in-needle technique. An 8 gauge injection needle with a polyethylene tubing (OD 1.34mm) containing glass-wool wick protruding from the tubing tip at 5mm was introduced into the mid-portion of the specimen. The outer part of tubing was connected to the pressure transducer and recording was done using a 3 channel device and LabView software (National Instruments, Austin, TX, USA). Using 1 ml calibrated syringe, serum was injected under the pressure of 30, 50, and 70 mmHg, and volume/sec flow was measured. The injected serum filled the specimen, and there was no leakage from the wall surface

for at least 1 min. This time of flow recording was sufficient for obtaining data for calculation. Apparent hydraulic conductivity coefficient was calculated according to Darcy's law: $K_{app} = Q/(4\pi a_0 p_0)$, where Q is flow rate, p is perfusion pressure, a is initial radius of fluid cavity (6). Data were expressed in $\text{cm}^2 \text{min}^{-1} \text{mmHg}^{-1}$. The reference values of hydraulic conductivity were obtained from adjacent non-infiltrated colon wall.

Statistical Analysis

Statistical analysis was performed by using the student t-test and Mann-Whitney U test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Color Lymphography of Tumor and Adjacent Tissues

Color lymphangiographic pictures of the normal colon wall and of tumor center, edge and adjacent tissue were evaluated. The lymphatic vessels in a normal colon mucosa and submucosa were characterized by typical irregular architecture network, changing diameter of lumen in the course of vessel, and lack of di- or trifurcations (*Fig. 1A*). In contrast, normal arteries showed regular bifurcations and veins a branch merging pattern (*Fig. 1B,C*). In the tumor center, no ordinary shaped lymphatic vessels were seen. Sporadically, small blind fragments of lymphatics of a length of 50-100 μm could be detected (*Fig. 1D*). Under stereomicroscopy, 2-4 cells lining their lumen could be seen. Generally, the picture of tumor center was dominated by multiple oval or round, sometimes irregular shaped 10-20 micron diameter spaces with density of 100-200 in the "hot places" (*Fig. 1E*). No cells lining these spaces were seen. They retained their open shape presumably due to accumulated tissue fluid that could not be drained by

fragmented lymphatics. Some tumor fragments showed only blue dye between collagen fibers (*Fig. 1F*). At the tumor edge and in adjacent tissues, lymphatics of a normal shape were seen (*Fig. 1G,H*), some of them containing cells of an apparently macrophage or tumor cell phenotype.

In specimens stained with Paris Blue and subsequently hematoxylin-eosin, lymphatics were identified only in the peritumoral areas (*Fig. 1I*).

Immunohistochemistry of Tumor Lymphatics and Blood Vessels

Histological evaluation was carried out in N-normal colon wall, TE- tumor edge, TS- tumor superficial, and TC- tumor center regions. In 20 specimens, lymphatics were found in N in 100%, in TE in 70%, in TS in 50%, and in TC in 40%. Vessels in the TC regions were tiny, fragmented, and had their lumen of irregular outline in most cases closed. Similar pictures were seen on confocal microscopy (*Fig. 1J,K,L*). Some few vessel-like structures were LYVE1- and prox1-positive, whereas the multiple lake-shape structures were negative.

Density Ratio of Lymphatics to Blood Vessels

The mean values of density ratio of lymphatics (LVD) to blood vessels (MVD) are shown in *Table 2*. There were few lymphatics in hotspots compared with TE and N regions. There were also fewer blood vessels in hot spots compared with N region, however, a high density in the tumor edge.

Cross Section Area of Lymphatic and Blood Vessels

The cross section area of lymphatics in hot spots was 8-times lower than at tumor edge, whereas, at the edge was higher than in a normal wall. There was a high density of blood vessels at the tumor edge (*Table 3*).

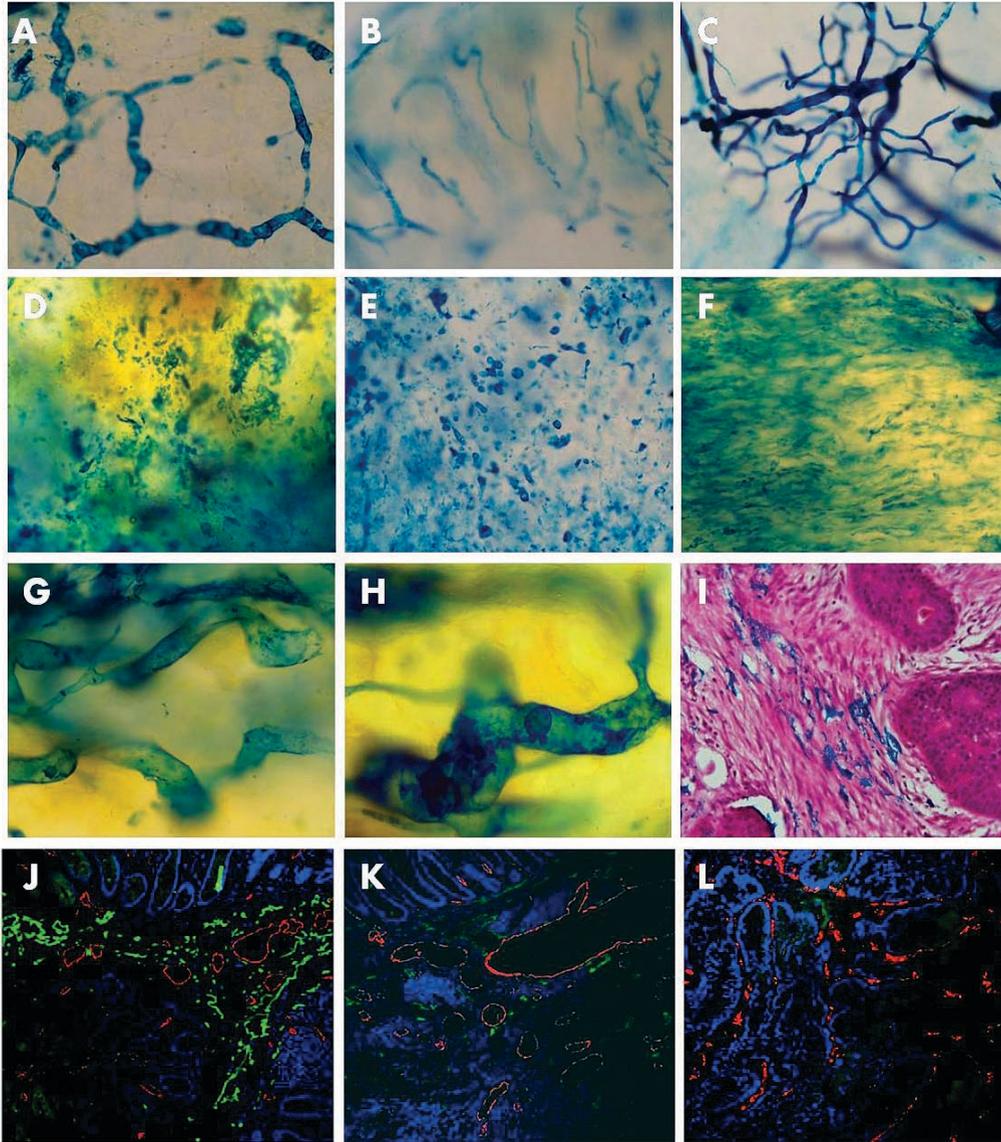


Fig. 1. Normal colon, peritumoral and tumoral lymphatics and blood minivessels. A to H stained by intra-tissue injection of Paris Blue. I stained both with Paris Blue and hematoxylin-eosin. J to L confocal pictures of lymphatics and blood vessels. A. Normal submucosal lymphatics. Irregular network, partly dilated, partly constricted vessels with unidirectional valves, x100. B. Normal mucosal arteries forming loops not observed in lymphatic network, x100. C. Normal submucosal veins merging in the centripetal direction with larger veins, a pattern different from that of lymphatics, x100. D and E. Intratumoral accumulation of Paris Blue in minute irregular, oval and round “lakes” most likely containing tissue fluid. Lack of lymphatic structures, x100. F. Fibrous tissues around a single tumor focus. Paris Blue fills microspaces between collagen bundles, x100. G. Peritumoral lymphatics forming a normal network, x200. H. A peritumoral lymphatic with large presumably tumor cell, x200. I. Paris Blue stained lymphatics in a fragment of fibrous tissue around a tumor focus. No lymphatics within tumor. J. Confocal picture of a normal mucosal and submucosal area. Red – blood vessels, green – lymphatics, x100. K. Confocal picture of the tumor edge. Red – blood vessels (stained for factor VIII related antigen), green – lymphatics (stained for LYVE1), x100. Note fewer lymphatics than in normal colon submucosa. L. Confocal picture of the tumor center. Red – blood vessels, green – lymphatics, x100. Note lack of lymphatics.

TABLE 2
LVD and MVD in Normal and Malignant Tissues of Colon

	N-normal	TE-tumor edge	T-tumor hot spots
LVD	3.74 ± 1.80	2.92 ± 2.37	0.94 ± 1.91*
MVD	8.40 ± 5.08	11.27 ± 3.08	4.49 ± 2.95

Data are the mean values ±SD of counted microvessels in 3 to 5 evaluated grids in areas of maximum vessel density, n=20. *hot spots vs TE and N p<0.05

TABLE 3
Cross Section Ratio of Open Lymphatic and Blood Vessels in Colon Cancer

	N-normal	TE-tumor edge	T-tumor hot spots
LV	4119.60 ± 2606.13	6848.01 ± 11568.92	870.37 ± 382.76*
MV	3708.62 ± 3673.31	18563.79 ± 34804.71	3666.81 ± 4721.57

Data are mean number of pixels covering vessel area ±SD, n=20, magnification x200.* hot spots vs TE and N p<0.05

Open vs Closed Lymphatic and Blood Vessel Ratio

Evaluation of the number of open lymphatics revealed that only 40% remained open at TE and 5% at T hot spots. Also a small percentage of blood vessels were found open in these areas (Table 4).

Compression of Lymphatic and Blood Vessels

The level of compression of lymphatic and blood vessels was assessed by quantification of the aspect ratio – the ratio of maximum to minimum axes of perfused vessels. An aspect ratio of 1 represents a perfect circle. The larger was the aspect ratio, the greater the amount of vessel compression. The ratio for lymphatics and blood vessels was between 4 and 5 in T hot spots (Table 5). This indicated that almost all vessels were compressed.

Tumor Tissue Hydraulic Conductivity

Hydraulic conductivity was measured in the central part of the tumor specimen with fat appendices as controls. The injection pressure initiating test fluid flow in the tumor was 35±10 mmHg and in controls, it was 14±6 mmHg (p<0.05). Hydraulic conductivity coefficient K_{app} increased from 1.2 to 7.0x 10⁻⁷ cm² min⁻¹ mmHg⁻¹ and 2.5 x 10⁻⁴ to 2.0 x 10⁻³ cm² min⁻¹ mmHg⁻¹ in controls compared to tumor tissue (p<0.05).

DISCUSSION

Lymphatic vessels are one of the pathways of spread of cancer. There are controversies whether the peritumoral or intratumoral lymphatics play a basic role in transport of detached tumor cells. The purpose of this study was to visualize peri- and intratumoral lymphatics in colon cancer, their density, anatomical shape, and hydraulic conductivity depending on lymphatic patency and deformation of tissue by accumulating tissue fluid. These factors create conditions

TABLE 4
Open Lymphatics and Blood Vessels in Colon Cancer Tissue

	N-normal	TE-tumor edge	T-tumor hot spots
LV	95%	40%**	5%*
MV	95%	70-80%	25%

Data are mean percentage of vessels with lumen to all vessels per microscopic field, n=20.*hot spots vs TE and N p<0.05, **TE vs N p<0.05

TABLE 5
Compression of Lymphatics and Blood Vessels

	N-normal	TE-tumor edge	T-tumor hot spots
LV	2.47 ± 0.67	2.66 ± 1.53	4.42 ± 3.0*
MV	1.89 ± 0.98	3.1 ± 1.81	5.44 ± 3.36

Data are mean±SD, n=20, *hot spots vs TE and N p<0.05

for tumor cell penetration into lymphatics and their flow to lymph nodes. For visualization, we applied an original method based on intra-tissue injection of Paris Blue dye specifically staining the inner surface of the lymphatic wall and making tissue translucent by immersion in methyl salicylate. In addition, tumor tissue was stained for lymphatic endothelial cell antigens. Also, hydraulic conductivity for tissue fluid/lymph dragging metastatic cells was measured.

The study provided the following information: a) the hot spots located in tumor center contained only a few lymphatics with compressed lumen, but multiple minute irregular, oval or round blind spaces (lakes), presumably containing tissue fluid; b) these spaces were not lined by endothelial cells; c) most lymphatics and blood vessels in tumor hot spots had compressed lumen; d) at tumor edge, lymphatics were visualized, however, their density was lower than in a normal colon submucosa, and some of these vessels

contained large tumor-like cells; e) the frequency of blood vessels at tumor edge was highest, and this was higher than in the hot spots and normal colon submucosa; and f) tumor tissue hydraulic conductivity was extremely low and may be accounted for by mechanical rigidity of tumor tissue resulting in compression of initial lymphatics and blood vessels.

There were fragments of blind lymphatics in the tumor center, however, the histological picture was dominated by multiple minute spaces or “lakes” in the interstitium. These spaces could only be detected by intra-tissue Paris Blue dye injection and could not to be identified on routine immunohistological pictures. They were presumably formed by capillary filtrate not drained away because of lack of patent lymphatics (12). The intra-tissue injected Paris Blue in chloroform suspension enters interstitial spaces and lymphatics but not blood vessels due to the large size of the dye particles, and sticks to

the internal surface of these structures. In translucent specimens, the dilated interstitial spaces and lymphatics can be easily seen including cells in their lumen. Moreover, chloroform does not destroy the molecular structure of the tissue, and staining with monoclonal antibodies is feasible. This is an excellent method for spatial visualization of the interstitial space and lymphatics in skin, subcutaneous tissue, lymph nodes, and all organs containing lymph vessels (9,10,12).

There is still a considerable debate about the role of intratumoral versus peritumoral lymphatic vessels in the pathology of primary human tumors (13,14). Several studies have shown that the density of lymphatic vessels located immediately adjacent to the tumor is associated with the presence of lymph node metastases (5). Moreover, in a retrospective prognostic study, it was found that the size of peritumoral lymphatic vessels was the most significant independent factor that correlates with LN metastasis in human malignant melanomas (15). However, other studies show that intratumoral and not peritumoral lymphatic vessels are vital for lymphatic metastasis (4,16).

Tumor-associated LVD (lymphatic vessel density) is most frequently assessed by counting the number of immunostained vessels in tumor sections, as defined by Weidner et al (11). Vascular "hot spots" containing lymphatic and blood microvessels are thought to represent localized areas of biological importance since they originate from tumor cell clones with the highest angiogenic potential which will predominantly enter the circulation and give rise to vascularized metastases. The problem is the lack of highly specific markers for lymphatic endothelial cells. At present, the most reliable ones are LYVE-1 and podoplanin (3,16). The major drawbacks of the visual MVD counting method are its inherent subjectivity and the difficulty of standardization among laboratories. Our specimens were evaluated by 3 independent observers.

To avoid subjectivity, the image

cytometry seems to be more objective and reproducible, and moreover, it provides additional information on vessel luminal area and vessel luminal perimeter. This is why we measured area and number of compressed lymphatic and blood vessels using the image cytometry. Ninety five percent of lymphatics in hot spots and 60% at tumor edge were obstructed. The level of compression of lymphatic vessels assessed by quantification of the aspect ratio – the ratio of maximum to minimum axes of perfused vessels was in hot spots around 5 in both lymphatics and blood vessels. The situation was different at tumor edge. Here, the lymphatic and blood vessel area was found to be higher than in normal colon submucosa.

High intratumoral interstitial pressure in hot spots and formation of dense connective tissue around tumor foci may cause deformation and obturation of lymphatics and make them nonconductive (6-8). This is due to deposition of large amounts of the so-called hydrophilic extracellular matrix (ECM), predominantly glycosaminoglycans (GAG). Tumor tissue may possess unique characteristics, attributable in part to an embryonic-like stage of development with extensive synthesis of ECM, which leads to substantial differences in composition and assembly compared with the host tissue. This may be the reason for low tumor hydraulic conductivity also observed in our study. Interestingly, hydraulic conductivity did not change much with time and pressure of intra-tumor serum injection as it did in the control tissue. This further strengthens our supposition that tissue fluid/lymph flow in tumor center cannot move detached tumor cells (13,14). However, penetration could take place at tumor edge where vessels were not compressed by tumor cell mass and fibrous tissue.

Taken together, our method for stereovisualization of the interstitial space and minor tissue lymphatics revealed very few fragmented initial lymphatics in the tumor tissue but numerous minor fluid lakes with no evident connections with the peritumoral

lymphatics. Lining of “lakes” did not express molecular markers specific for lymphatic endothelial cells. The hydraulic conductivity of tumor was much lower than reference tissue. Most open lymphatics were located adjacent to the tumor. Some of them contained in the transparent lumen single cells of a size larger than lymphocytes. It can be concluded that lack of effective lymphatic drainage in the center of tumor foci manifested by accumulation of tissue fluid in “lakes,” low fluid conductivity, and compression of lymphatics and blood vessels by tumor tissue and proliferating connective tissue may hamper escape of colon tumor cells. The most favorable site of entry of tumor cells to lymphatics seems to be the interface of the tumor and surrounding tissue with open lymphatics, and this peritumoral region should be the target for future therapeutic modalities.

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