Tumorigenesis and Neoplastic Progression

Tumor Cell Transendothelial Passage in the Absorbing Lymphatic Vessel of Transgenic Adenocarcinoma Mouse Prostate

Giacomo Azzali

From the Lymphatology Laboratory, Section of Human Anatomy, Department of Human Anatomy, Pharmacology, and Forensic Medicine, School of Medicine, University of Parma, Parma, Italy

The distribution and fine structure of the tumor-associated absorbing lymphatic vessel in the tumor mass of prostate adenocarcinoma and of seminal vesicle metastasis in transgenic mice was studied for the purpose of understanding the modality of tumor cell transendothelial passage from the extravasal matrix into the lymphatic vessel. In the tumor mass, two main cell populations were identified: stromal tumor cells and the invasive phenotype tumor (IPT) cells, having characteristics such as a highly electron-dense matrix rich in small granules lacking a dense core and massed nuclear chromatin, which is positive to immunostaining with anti-SV40 large T antigen antibody. Based on the ultrastructural pictures of different moments of the IPT cell transendothelial passage by ultrathin serial sections of the tumor-associated absorbing lymphatic vessel, the manner of its transendothelial passage through the intraendothelial channel, without involving intercellular contacts, was demonstrated. The presence of IPT cells in the parenchyma of satellite lymph node highlights its significant role in metastatic diffusion. The intraendothelial channel is the reply to the lack of knowledge regarding the intravasation of the tumor cell into the lymphatic circulation. The lymphatic endothelium would organize this channel on the basis of tumor cell-endothelial cell-extravasal matrix molecular interactions, which are as yet unidentified. (*Am J Pathol* 2007, 170:334–346; DOI: 10.2353/ajpath.2007.060447)

It is widely believed that the lymphatic vascular system, interconnected to the blood vascular system, plays a fundamental role in the homeostasis of interstitial fluid, in returning proteins filtered from the capillaries to the blood, in immunological functions, and in the metastatic

dissemination of the tumor cell. In the second half of the 1900s, research on the lymphatic vascular system conducted using injection methods of coloring agents (Prussian blue and so forth), resins (Neoprene, metacrilate, Mercor, and so forth), and fluorescence micrography, the confocal microscopy, and lymphangioscintigraphy made significant contributions regarding the spatial distribution of lymphatic networks in different human organs and those of various animals.^{1–3} Interesting results were also obtained using cinematographic documentation *in vivo* on the kinetics of valves and lymph flow.⁴ In the last decade, the use of a series of molecules such as LYVE-1, Prox-1, podoplanin, D2-40, and vascular endothelial growth factor (VEGF) receptor-3 for identifying the lymphatic vessel^{5–8} has made interesting contributions on the nature and function of the lymphatic vessel and the microenvironment surrounding it. These results have increased our knowledge of the biology of the lymphatic vessel⁹ as well as of the structural and functional characteristics of the various components of the lymphatic vascular system.¹⁰ On the basis of morphological and experimental findings, two distinct sectors of lymphatic vascularization were identified: one of highly absorbing lymphatic vessels (essential for draining interstitial fluid, macromolecules, and cells) and the other formed instead of vessels whose main function is that of lymph conduction and flow.^{11,12} Furthermore, special attention has been focused on the molecular mechanisms involved in the regulation of lymphangiogenesis induced by the overexpression of VEGF-C, VEGF-D, VEGF-A, and by the platelet-derived growth factor (PDGF)-BB.^{13–16} The results obtained have clarified the role played by tumor lymphangiogenesis in the hyperplasia of intratumoral lymphatic vessels and in the metastatic dissemination of

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Address reprint requests to Prof. Giacomo Azzali, M.D., Ph.D., Emeritus Professor and Director of the Lymphatology Laboratory, Section of Human Anatomy, Department of Human Anatomy, Pharmacology, and Forensic Medicine, University of Parma, Via Gramsci, 14 (Ospedale Maggiore), 43100, Parma, Italy. E-mail: giacomo.azzali@unipr.it.

the tumor cell.^{17,18} There is little information instead on the mechanisms through which transendothelial migration takes place, and not much is known about how the tumor cell abandons the primary tumor and enters the tumor-associated absorbing lymphatic (TAAL) vessel. According to Streit and Detmar,¹⁹ an important role in favoring tumor cell access to the lymphatic system is played by tumor-associated lymphangiogenesis induced by the overexpression of VEGF-C and VEGF-D by means of hyperplasia of the peritumoral and marginal lymphatic vessels sufficient, in the view of Padera and colleagues²⁰ and Williams and colleagues,²¹ to permit lymph nodal metastatic diffusion, as well as by the involvement of endothelial cells in chemotactic recruitment and in intra-lymphatic transendothelial transport, as formulated for the melanoma cell. Pepper and Skobe,²² Cassella and Skobe,²³ and He and colleagues²⁴ maintain, however, that the mechanisms mediating the transmigration of the tumor cell through the lymphatic endothelium, essential for its metastatic dissemination, are still wholly unknown. According to Timar and colleagues,²⁵ at the basis of the tumor cell's migratory process and intravasation (entry to vessel) are the tumor cell-lymphatic endothelial cell interactions, or a passive transport of tumor cells, to (and perhaps into) lymphatic channels that constitutively express CCL21, resembling that of the dendritic cells.²⁶ On the contrary, according to Ji and Kato²⁷ and Ramjeesingh and colleagues,²⁸ the transendothelial passage of tumor cell would take place through the open interendothelial junctions after dissolution of the junctional adhesion complexes. For Carr,²⁹ the tumor cell's intravasation would take place after destruction of the endothelial wall's integrity, favored, in the opinion of Cao and colleagues,¹⁶ by the high interstitial pressure inside the tumor. In adenocarcinoma and melanoma xenograft in nude mice, Azzali³⁰ demonstrated the transendothelial migration of the tumor cell into the TAAL vessel through the intraendothelial channel, a morphological entity totally independent of the interendothelial contacts.

The purpose of this work is to study the distribution and fine structure of the TAAL vessel in the transgenic adenocarcinoma mouse prostate (TRAMP) and the process by which the tumor cell passes through the endothelial barrier and gains entrance into the lymphatic vessel. The TRAMP model was chosen because, unlike previously studied experimental models,³⁰ this one possesses characteristics of very high penetrability, rapid progression, and metastatic reproducibility that are comparable with spontaneous or induced models.³¹

Materials and Methods

Transgenic Animals

TRAMP mice, heterozygous for the PB-Tag transgene, were maintained in a C57BL/6 background. Transgenic males for the studies were obtained as [TRAMP × C57BL/6]F offspring. For the present research, 17 TRAMP mice and five C57BL/6 wild-type mice, 6 and 4 months old, respectively, were used. The animals had

access to laboratory chow *ad libitum*. The mice were sacrificed after ether anesthesia. Prostate and seminal vesicles from wild-type mice and from TRAMP mice with tumor and metastatic lesions, as well as para-aortic and parailiac lymph nodes, tributaries of the prelymph nodal collector lymphatic vessels of the above organs, were quickly removed, fixed, and embedded for light and transmission electron microscopy (TEM). Isolation of mouse tail DNA and polymerase chain reaction (PCR)-based screening assays to identify transgenic mice were performed as described previously.³²

Light and Transmission Electron Microscopy

The prostate and seminal vesicle specimens and para-aortic and parailiac lymph nodes were excised and fixed in 10% formalin for light microscopy and in 1% osmic acid-sodium veronal buffer, pH 7.4, for 1 hour for TEM. After the pieces (4 mm diameter) were dehydrated in alcohol or acetone, they were embedded in paraffin wax or Durcupan (Fluka Chemie, Buchs, Switzerland), respectively. Paraffin histological sections (5 μ m thick) were stained with Mayer's hematoxylin and with the main histochemical reactions of reduction of silver nitrate, of argentamine and argentomethenamine salts, and of azo-reaction and alkaline thioindoxyl method to investigate the composition of invasive phenotype tumor (IPT) cell dense granules.^{33–35} Semithin sections (1 μ m thick) were stained with toluidine blue 0.5%. Ultrathin sections were stained with uranyl acetate and lead citrate³⁶ and observed with a Philips 300 electron microscope (Eindhoven, The Netherlands).

Three-Dimensional Reconstruction

In addition to the absorbing lymphatic vessels of the prostate and seminal vesicles of wild-type mice, the study involved 109 TAAL vessels from TRAMP mice, 78 of which had IPT cells embedded in the lymphatic endothelial wall. Twelve TAAL vessels from the prostate and seminal vesicles of TRAMP mice showing significant aspects of IPT cell transendothelial passage were cut into ultrathin serial sections (from no less than 60 to 820), observed by TEM, photographed, and reconstructed in three-dimensional models using wax disks according to Born's technique,³⁷ modified by Werner's.³⁸ We further modified this method using different colors³ to improve the distinction between the different endothelial cells. We used a Faber-Castell 2B pencil (Faber-Castell, Stein, Germany) to transfer the profiles of each endothelial cell component from serial electron microscopy photographs onto a sheet of tracing paper (60 g), with the help of a small epidiascope with a potent light source. After the transfer, the cellular components were colored with crayons (Schwan-Stabilo 8740; Schwan-STABILO Schwanhuer GmbH & Co. KG, Weienburg, Germany). The sheet of paper was then treated with tetrahydronaphthalene, spread out on a lithographic slab, impregnated with liquid wax-paraffin, and gauged to a thickness of 1 mm with a steel cylinder at 100C. After the paper was

Figure 1. Morphological aspects useful to compare shape and sizes of prostate and seminal vesicles of wild-type and transgenic mice. **A:** Prostate (P) and seminal vesicles of 4-month-old wild-type mouse. **B:** Prostate (P) and hypertrophic seminal vesicles with metastatic area (frame) of 6-month-old TRAMP mouse.

cooled, the borders of the cells were excised with a lancet, and single serial wax disks were assembled and fixed with paraffin drops to stabilize the three-dimensional model. When properly performed, this wax-disk technique provides the most reliable three-dimensional reconstruction of lymphatic vessels and an accurate stereoscopic view of cellular elements.

Immunohistochemistry

Prostate and seminal vesicle tissues were removed from TRAMP mice, fixed in 10% formalin, and embedded in paraffin wax. Before staining with mouse anti-SV40 large T antigen monoclonal antibody (BD PharMingen), sections (4 μ m) were dewaxed. Antigen retrieval was performed by microwaving (power 9) for 37 minutes in 10 mmol/L citric acid, pH 6.0, and allowed to cool for 30 minutes. Endogenous peroxidases were quenched with 3% hydrogen peroxide in methanol for 10 minutes.

Sections were then blocked by incubation with blocking reagent from LSAB kit (LSAB kit; DAKO, Glostrup, Denmark) for 30 minutes at room temperature. Incubation was performed with mouse anti-SV40 large T antigen monoclonal antibody [1:400 in bovine serum albumin 0.2% in phosphate-buffered saline (PBS)] in a cold room overnight. After washing with PBS, slides were incubated with biotinylated secondary anti-rabbit and anti-mouse immunoglobulin from the LSAB kit for 60 minutes. After washing with PBS, the streptavidin detection system was used (LSAB kit; DAKO) with diaminobenzidine (DAKO) chromogen for 3 minutes before counterstaining with Mayer's hematoxylin. As negative controls, primary antibodies were omitted.

Results

The prostate and seminal vesicles of the 4-month-old wild-type mice have normal dimensions (Figure 1A), as well as a tubule-acinous-alveolar structure with cubic-cylindrical cells resting on a stroma of smooth muscle fibrocells, fibroblasts, and collagenous fibrils. The absorbing lymphatic vessels found in the intertubular stromal pontics reveal the same morphological and ultrastructural characteristics as those described in the

absorbing lymphatic vessels of normal organs in various micromammals.

In transgenic mice with transgenic adenocarcinoma mouse prostate (TRAMP), sacrificed when 6 months old, the prostate was voluminous and the seminal vesicles seemed to be highly hypertrophic, with brownish metastatic areas (Figure 1B). The tumor mass of the prostate and the metastatic mass of the seminal vesicles showed obvious subversion of the tubule-acinous-alveolar structure, where the epithelial base had completely disappeared. The cell population, distributed below the fibromuscular stroma, was mainly represented by voluminous cells having a central nucleus and abundant cytoplasm, with a strong dye affinity for toluidine blue, located for the most part close to the endothelial wall of the lymphatic vessel (Figure 2, A and B). With the TEM, two distinct types of cells were found: the stromal tumor cell (Figure 3A), which predominated numerically, and the tumor cell evolved into the invasive phenotype (IPT), numerically inferior (Figures 2B and 3A). The stromal tumor cell had a voluminous cytoplasmatic matrix with large vacuoles of the rough endoplasmic reticulum, small and medium electron-dense granules homogenous in content, free ribosomes uniformly distributed in the light matrix, and a central nucleus with irregularly dispersed lumps of heterochromatin (Figures 3A and 4A). The IPT cell (Figure 2, A, C, and D; and Figure 3A), negative to popular argentaffin reaction techniques (hexamin-silver method) and azo-reaction, devoid of ultrastructural aspects of enteroendocrine and neuroendocrine cells, was characterized instead by 1) a mostly fragmented nucleus with the heterochromatin massed along the peripheral margin of the karyoplasm and fine granules of euchromatin distributed in a moderately electron-dense central karyoplasm. This nucleus expressed immunohistochemically the oncoprotein SV40 large-T antigen (Figure 2E), contrary to the nuclei of the remaining epithelial cells and of the stromal tumor cells, colored only by Meyer's hematoxylin; 2) small round granules 220 to 240 nm in diameter with homogeneous content, devoid of dense core, were uniformly distributed in the rather electron-dense cytoplasmatic matrix; and 3) thin ectoplasmic filopod- and pseudopod-like protrusions were also observed. TAAL vessels were found only in the peripheral portion of the prostatic tumor mass and of the seminal vesicle metastatic mass (Figure 2, A and B), the same as in the peritumoral area tissue composed of smooth muscle fibrocells and fibroblasts. It should be noted that these lymphatic vessels were not present in the central part of the above-mentioned tumor masses. The TAAL vessel (Figure 3A) appeared to be formed of a single layer of endothelial cells lacking a continuous basement membrane, pores, and open junctions. The endothelial cells had a globe-like central body containing the nucleus and nonnuclear peripheral cytoplasmatic expansions joined to each other by overlapping-type contacts (Figure 3A); interdigitating and end-to-end-type contacts were rarely found. The lymphatic endothelial wall of the TAAL vessel, unlike that of the absorbing lymphatic vessel in normal tissues, was characterized by a regularly alternating distribution of endothelial cells with an electron-dense cyto-

Figure 2. Histological, histochemical, and ultrastructural aspects of prostate tumor mass of 6-month-old TRAMP mouse. **A** and **B**: Peripheral part of the tumor mass with peritumoral fibromuscular stroma (P) containing a tumor-associated lymphatic vessel TAAL (L) surrounded by tumor cells (**circles**), cytoplasm of which have a strong dye-affinity for toluidine blue. **C** and **D**: IPT cells (Th) characterized by filopods, undulopods, and by peculiar ultrastructural aspects of the cytoplasmic matrix that is rich in dense granules, nucleus of which (**E**) is immunohistochemically positive to SV40 large T-antigen protein (colored dark brown, **arrows**). Coloration is toluidine blue, original magnification: $\times 1920$ (**A**); $\times 3100$ (**B**); $\times 17,500$ (**C**); $\frac{1}{4}$ original magnification, $\times 26,500$; $\frac{1}{2}$ original magnification (**D**). Mayer's hematoxylin, original magnification, $\times 920$ (**E**).

plasmatic matrix and cells with a rather light matrix (Figure 3, B and C). The cytoplasmic matrix of the body of the lymphatic endothelial cells with an electron-dense matrix showed free ribosomes, Golgi complexes with dilated cisterns, and a fairly developed rough endoplasmic reticulum (Figure 3D). The electron-dense matrix of the nonnuclear cytoplasm expansions (Figure 3, B and C) likewise had free ribosomes, rough endoplasmic reticulum tubules, and medium-size vacuoles (300 to 570 nm in diameter), some of which contained small synaptic-type vesicles 35 to 50 nm in diameter (Figure 3, D and E). The immunocytochemical identification of the content of these microvesicles was hindered by technical problems, because preservation of the ultrastructural morphology by fixation with denaturing and crosslinking aldehydes strikingly reduces the antigenicity of these structures.

In contrast, the endothelial cells with a rather light matrix have rough endoplasmic reticulum and Golgi apparatus of modest entity, nonnuclear cytoplasmic expansions characterized by few mitochondria, polyribosomes, small rough endoplasmic reticulum tubules, noncoated vesicles, and sporadic vacuoles that appear empty (Figure 3C). Often, secondary extensions that originate in the expansions of the nonnuclear cytoplasm of an endothelial cell continue into the interstitial matrix and, after circumscribing a certain space of the interstice itself (Figure 3C), adhered to the abluminal wall of the adjacent endothelial cell, fixing themselves there by means of the tight and gap-type junctions. This behavior, as will be

demonstrated later with three-dimensional reconstructions, precludes the formation of an intraendothelial channel. There are few endo- and exocytotic invaginations in the abluminal and luminal plasmatic membrane of the lymphatic endothelial cells. A TAAL vessel under the oblique direction of the section plane, with alternate distribution of endothelial cells having an electron-dense matrix and cells with a light matrix fixed by tight and gap junctions, is shown in Figure 3A. Moreover, this ultrastructural picture shows, near the lymphatic vessel, stromal tumor cells and tumor cells evolved in the invasive phenotype (Figures 3A and 4A) easily identified by their particular ultrastructural characteristics. Although the stromal tumor cell is irregularly distributed in the extravascular matrix, the IPT cells could be distributed rather close to the abluminal wall of the TAAL vessel. The ultrastructural pictures also frequently point out the presence of an IPT cell in the lymphatic vessel lumen (Figure 4, A and B). The way in which an IPT cell is inserted into the endothelial wall can be perceived in Figure 4, A and C, which illustrates the behavior of the nonnuclear cytoplasm expansion extensions of adjacent endothelial cells without participation of the interendothelial contacts, which are fixed by tight and gap junctions (Figure 4C). A further example of the above morphological behavior chosen from ultrathin serial sections is given in Figure 5, A and B, where the nonnuclear secondary cytoplasmic extension of adjacent endothelial cells circumscribes at different levels of the endothelial wall of a TAAL vessel, a

Figure 3. Ultrastructural aspects of TAAL vessel and its relationship with stromal tumor cells and IPT cells. **A:** TAAL vessel (L) of the prostate tumor mass, under oblique direction of the section plane (small inset at bottom), formed of two endothelial cells whose nonnuclear cytoplasmic expansions are joined by overlapping contacts (**arrows**) fixed by tight and gap junctions. T, stromal tumor cell; Th, IPT cell close to the lymphatic endothelial wall. **B:** TAAL vessel (L) of the metastatic mass of the seminal vesicles, characterized by the peculiar alternating disposition of endothelial cells with an electron-dense matrix and cells with a light matrix, whose ultrastructural characteristics (**rectangle**) can be better seen in C. In the same figure, the behavior of the cytoplasmic expansions of adjacent cells in circumscribing an interstitial space (S) can be observed. **Arrows:** overlapping contacts between interendothelial cells fixed by tight and gap junctions; Lu, lymphatic vessel lumen. **D:** Lymphatic endothelial cell with electron-dense cytoplasmic matrix expansion having many vacuoles (**arrows**), some of which contain synaptic-type microvesicles (**E**). N, nucleus; Lu, lymphatic vessel lumen. Original magnifications: $\times 12,960$ (**A**); $\times 14,500$ (**B**); $\times 26,500$ (**C**); $\times 14,000$ (**D**); $\times 41,500$ (**E**).

space of the interstitial extravascular matrix. In fact, the cytoplasmic extension of the endothelial cell with an electron-dense matrix is indicated in Figure 5A by 1, and a dashed line in successive ultrathin sections appears lengthened in Figure 5B and then attaches itself to the secondary cytoplasmic extension of the adjacent endothelial cell. A three-dimensional reconstruction of the above morphological pictures enables us to understand the formative process of an intraendothelial channel, through which interstitial fluid and the particles suspended in it are drained. In the three-dimensional model, this intraendothelial channel looks something like a mountain tunnel, with entrance (abluminal) and exit (luminal) orifices. The transendothelial passage manner of an IPT cell into the TAAL vessel, a process often called intravasation, can be observed in the ultrastructural pictures of Figure 6, A to C. In addition to documenting the morphological characteristics of the stromal tumor cells and the IPT cells, these pictures illustrate the peculiar alternating distribution of endothelial cells with a light matrix and those with an electron-dense matrix. They also

show the location of an IPT cell between the nonnuclear cytoplasm expansions of adjacent endothelial cells in the moment of its transendothelial passage into the lymphatic vessel through an intraendothelial channel under the sagittal direction of the section plane (Figure 6C). It should be pointed out that the interendothelial junctions have never been involved in the formation process of the intraendothelial channel. The frequency of IPT cell transendothelial passage is significantly documented in the ultrastructural picture of Figure 7A, where different moments of the passage of two IPT cells have been fixed. The sequence of ultrastructural pictures in Figure 7, B to D, regarding transendothelial passage of the IPT cell in Figure 7A, was chosen among 480 ultrathin serial sections of the TAAL vessel in Figure 7A. These figures bear witness to the various morphological aspects of IPT cell insertion and of the behavior of the nonnuclear cytoplasmic expansions (colored yellow and light blue) of adjacent endothelial cells. Moreover, the tumor cell's adaptation to the diameter of the intraendothelial channel (~ 1.8 to $2.2 \mu\text{m}$) is evident, as cell body

Figure 4. Metastatic tumor mass of the seminal vesicle with TAAL vessel in almost sagittal section plane surrounded by stromal tumor cell (T) and with an IPT cell embedded in the endothelial wall. **A:** In **frame 1**, the IPT cell (Th) appears embedded in the endothelial wall, whereas in **frame 2** the IPT cell is inside the lumen of the vessel. **B** and **C:** The ultrastructural aspects can be observed. The IPT cell of frame 1 in **C** is inserted between the nonnuclear cytoplasmic expansions 1 and 2 of adjacent endothelial cells (colored yellow and light blue). **Arrow**, interendothelial contact fixed by tight and gap junctions. Original magnifications: $\times 3100$ (**A**); $\times 19,000$ (**B**); $\times 21,000$ (**C**).

restriction attests (Figure 7D). A further morphological picture of the transendothelial passage of an IPT cell (Figure 7A) is given in Figure 7, E and F, which documents, by three-dimensional reconstruction as well (Figure 7G), the initial stage of its transendothelial passage into the lymphatic vessel lumen. The behavior observed in the ultrastructural pictures of different moments of the transendothelial passage of the IPT cell through the intraendothelial channel is summarized in the schematic drawing in Figure 7H, which illustrates how the formation of the channel in the TAAL vessel endothelial wall occurs.

The para-aortic and parailiac lymph nodes (tributaries of prostate and seminal vesicle lymph) in 4-month-old wild-type mice appear tapered (1.4 mm in diameter and 3.2 to 3.3 mm in length) and show a normal structure of

the parenchyma without IPT cells by TEM. The lymph nodes of 6-month-old TRAMP mice are instead somewhat hypertrophic, 1.9 to 2.4 mm in diameter and 6 to 7 mm in length. The parenchyma, its structure overturned in the cortical, paracortical, and medullar zones by cords of voluminous lymphocytes with electron-dense liposome-type inclusions, is characterized by the frequent sighting of IPT cells distributed even in the marginal sinus (Figure 8, A–F).

Discussion

The distribution and fine structure of the absorbing lymphatic vessel in prostate adenocarcinoma and in the metastatic mass of seminal vesicles in TRAMP mice were

Figure 5. Ultrastructural pictures of a TAAL vessel (L) chosen from 96 ultrathin serial sections to demonstrate the manner of intraendothelial channel formation. **A** and **B**: The secondary extension (marked 1) of the nonnuclear cytoplasmic expansions of an endothelial cell and its elongation (**dashed line**) in the interstitial matrix circumscribing a space (S), ending in the secondary extension of the adjacent endothelial cell (marked 2). This behavior anticipates the formation of an intraendothelial channel. **Arrows**, overlapping interendothelial contacts fixed by tight and gap junctions. Original magnifications: $\times 26,500$ (**A**); $\times 12,500$ (**B**).

studied to determine how and to what degree the tumor cell relates to and crosses the endothelial wall for its passage into the lymphatic system. The TAAL vessel is present only in the peripheral part of the tumor and metastatic masses and in the peritumoral tissue, as found in murine T-241 fibrosarcoma and in B16-10 murine melanoma.²⁰ Similar findings were described in Kaposi sarcoma³⁹ as well, in human colorectal cancer,^{40,41} and in the experimental models of T84 colon adenocarcinoma and B16 melanoma overexpressing VEGF-C in nude mice.^{30,42} According to Helmlinger and colleagues,⁴³ the absence of TAAL vessels in the central portion of the tumor masses is attributable to the high interstitial pressure present inside the tumor, which would prevent penetration into the stroma. Intratumoral lymphatic vessels, which could contribute to lymph nodal metastasis, were found in head and neck tumors⁴⁴ and in breast and thyroid carcinomas^{21,45}; according to Leu and colleagues⁴² and Padera and colleagues,²⁰ however, these vessels would not be functional for transporting fluid because of the high interstitial pressure existing inside the tumor.⁴⁶

The TAAL vessel, unlike the blood vessel, has a single-layer endothelium lacking a continuous basement membrane, pores, pericytes, and open junctions, with endothelial cells joined to each other by overlapping intercellular junctions fixed by tight and gap junctions. These ultrastructural characteristics make a concrete contribution to the lack of information on the biology of the lymphatic vessel in the tumors noted by Jackson.⁹ Unlike the absorbing lymphatic vessel of wild-type mice, this is characterized by endothelial cells having a highly electron-dense matrix alternating with endothelial cells having a light matrix. This morphological aspect was not found in the TAAL vessels of the tumor masses of T84 colon adenocarcinoma and B16 melanoma xenografts expressing VEGF-C in nude mice.³⁰ The singular disposition mentioned above of the endothelial cells in the wall of

the TAAL vessel could, in my opinion, be the expression of an active involvement of the lymphatic endothelium,^{19,22} in freeing the interstitial matrix molecules that would guide the recognition, orientation, and chemoattraction of the IPT cell toward the lymphatic vessel. This would be supported by the wealth of ribosomes, of rough endoplasmic reticulum, and by the presence of vacuoles containing small, synaptic-type vesicles in the electron-dense matrix of the nonnuclear cytoplasmic expansions of the endothelial cells. In this connection, according to Entschladen and colleagues,⁴⁷ particularly important growth factors such as serpentine and the neurotransmitters also play a significant role in the active migration of the tumor cell. Gunn and colleagues²⁶ and He and colleagues²⁴ believe that the lymphatic endothelium would send signals by liberating chemokines such as secondary lymphoid chemokine (CCL21/SLC) expressed in the high endothelial venule of the lymph nodes, in the T-lymphocyte-rich paracortical areas of the spleen, and in the lymphatic vessel endothelium of many organs. Likewise, the chemokine CXCL4, together with its receptor, would guide the formation of pseudopod-type ectoplasmatic protrusions⁴⁸ and the phenomenon of the tumor cell's adhesion as found in the blood vessels by Orr and colleagues⁴⁹ as well. Moreover, if the CCL21/SLC has the role of mediating the attraction of the lymphocytes in the lymphatic vessels, as Pepper and Skobe maintain,²² it can be hypothesized that with the expression of CCR7 it could operate to recruit the tumor cell into the TAAL vessel.

Regarding the cell population of the TRAMP tumor mass, we share the hypothesis of Carr and Orr⁵⁰ that the tumor cell, only after being released from the primary mass, differentiates itself mainly into the stromal tumor cell (prevailing element) and the IPT cell. The latter is easily identified by the peculiar morphological aspects of its nucleus and its cytoplasm, of electron-dense granules, and by its numerous filopods and pseudopod-like

Figure 6. Metastatic tumor mass of the seminal vesicles with TAAL vessel (L) surrounded by stromal tumor cells (T) and by two IPT cells (Th), one of which is attached to the endothelial abluminal wall and the other shown in a moment of its transendothelial passage through an intraendothelial channel. **A**, **B**, and **C** demonstrate, in an ultrathin serial section at a different level, the same Th cell (frame) in its passage between the nonnuclear cytoplasmic expansions of adjacent endothelial cells (**arrow** and **arrowhead**); this morphological aspect results from the sagittal direction of the section plane of the intraendothelial channel (bottom right **inset**). Original magnifications: $\times 4700$ (**A**); $\times 11,200$ (**B**); $\times 26,500$ (**C**).

protrusions. These features are totally different from the ones found in enteroendocrine and neuroendocrine cells described in prostate and urethra epithelia, in both normal and carcinomatous tissues of humans and mammals.^{51,52} The pseudopod-like protrusions would justify the individual-type migration of the IPT cell from the interstitial matrix toward the lymphatic vessel. Concerning a hypothetical displacement in the extravascular matrix of the tumor cell, Friedl and Wolf⁵³ and Timar and colleagues²⁵ maintain that multiple environmental factors could play a role, as does a wide spectrum of mecha-

nisms, including tumor cell-lymphatic endothelial cell and tumor cell-extravascular matrix cell interactions.⁵⁴ Moreover, IPT cell motility would be favored by the loss of E-cadherin,⁵⁵ by the low peripheral pressure gradient with respect to that inside the tumor,⁵⁶ and by the degradation of the extravascular matrix, because the IPT cell is able to bind proteolytic enzymes such as eserin, protease, elastase, metalloproteinase, and so forth.²⁵ In this regard, Wang and colleagues⁵⁷ have identified certain genes that could contribute to the motility and the chemotaxis of cancer cells in tumors.

Figure 7. TAAL vessel (L) under sagittal direction of the section plane to show two IPT cells embedded in the endothelial wall. **A:** **Frames 1** and **2** show the passage of IPT cell through the endothelial wall at different moments. **B, C,** and **D** show ultrastructural pictures chosen from 220 ultrathin serial sections of the transendothelial passage of the IPT cell (Th) in **frame 1** of **A** into the lymphatic vessel lumen (Lu) through the intraendothelial channel (see Results for details). **Inset** in **D** denotes sagittal direction of the section plane. **Arrows** show the cell body restriction. **E** and **F** show serial sections of the IPT cell in **frame 2** of **A** in the initial phase of its transendothelial passage from the extravascular matrix into the lymphatic vessel lumen (Lu), as documented also by the three-dimensional reconstruction in **G, H:** Schematic drawing of a TAAL vessel to illustrate, through synthesis of the various ultrastructural pictures of cytoplasmic expansions of adjacent endothelial cells, how the intraendothelial channel is formed without the participation of intercellular junctions. The **dashed line** and the **arrow** underlie the abluminal-luminal direction of the cancer cell transendothelial passage. Original magnifications: $\times 3100$ (**A**); $\times 14,500$ (**B**); $\times 12,000$ (**C, D**); $\times 8100$ (**E**); $\times 9300$ (**F**).

Concerning transendothelial migration and the resulting metastatic dissemination of the IPT cell, the general opinion is based on the hyperplasia of the lymphatic

vessels induced by overexpression of the endothelial growth factors VEGF-C, VEGF-D, and VEGF-A, and of PDGF-BB.⁵⁸ Findings relating to the molecular mecha-

Figure 8. Ultrastructural pictures of parailiac lymph node parenchyma of 6-month-old TRAMP mouse demonstrating the presence of IPT cells. **A** and **B**: IPT cells (Th, small circles) distributed between the endothelium (en) that covers the cells of the cortical area, and the marginal sinus (S). L, small and medium lymphocytes; m, macrophage with dense bodies (phagosomes, lysosomes); r, reticular phagocytic cell; e, eosinophil leukocyte; **arrows**, connective capsule. **C–E**: IPT cells (Th) distributed between cells forming the cortical and medullar areas of the parailiac and para-aortic lymph nodes of 6-month-old TRAMP mice. L, lymphocytes; P, plasma cell; en, medullar sinus endothelium. Original magnifications: $\times 11,900$ (**A**); $\times 12,000$ (**B**); $\times 8100$ (**C**); $\times 12,000$ (**D–E**).

nisms through which the IPT cell interacts with the endothelium of the TAAL vessel are scarce, and nothing is known of how this cell gains entrance into the lymphatic circulation.⁵⁹ Mandriota and colleagues⁶⁰ hypothesize that the entry of tumor cell into the TAAL vessel is regulated by multiple factors produced by tumor cell itself. In my opinion, this dearth of information is attributable to the difficulty in sighting the IPT cell in the lymphatic endothelial wall using the optical microscope and immunofluorescence, as well as to the lack of studies performed using the TEM. I believe that only the TEM's high power of resolution and the observation of three-dimensional models based on serial ultrathin sections are able to provide a clear reading of the intravasation modality of the IPT cell into the lymphatic system. It should also be noted that with TEM it is impossible to detect in the same morphological picture the entire migratory transendothelial process of the IPT cell because it can be detected only by the observation of several ultrastructural pictures of TAAL vessels with IPT cells during various moments of their passage through the endothelial wall.

The sum of these ultrastructural pictures and their reconstruction in three-dimensional models demonstrated that the transendothelial passage of the IPT cell, in the TRAMP model as well, passes through an intraendothelial

channel in a way that is wholly independent of the inter-endothelial junctions. This is formed as a result of a secondary cytoplasmatic extension of the nonnuclear cytoplasm of an endothelial cell, which after circumscribing a space in the interstitial matrix (phenomenon that is manifested in an area 6.5 to 7.2 μm long of lymphatic endothelial wall) settles on and attaches itself to the abluminal wall of the adjacent endothelial cell. This is made possible, according to Ottaviani,⁶¹ by the extraordinary plasticity of the lymphatic endothelial cell and, in the view of Witte and colleagues,⁶² by the lymphatic vessel endothelial wall's predisposition to intravasation for both tumor and immune cells.

In the three-dimensional reconstruction from ultrathin serial sections (in a number varying from 60 to 820) of the TAAL vessel this intraendothelial channel has the aspect of a mountain tunnel 1.8 to 2.2 μm in diameter (detailed analysis of the intraendothelial channel in Azzali¹²). The constitution of this intraendothelial channel is on the whole exactly like that found in the TAAL vessel of the T84 colon adenocarcinoma and the B16 melanoma xenografts in nude mice.³⁰ This morphological behavior in common would bear out Bogenrieder and Herlyn's⁶³ affirmation that the molecular and cellular phases in cancerogenesis would be alike in all tumors. Moreover, the

large number of intraendothelial channels observed in the TRAMP model with respect to that found in the TAAL vessels of the adenocarcinoma and melanoma xenograft models³⁰ inclines us to consider the intraendothelial channel as a dynamic and nonstatic morphological entity. Through this intraendothelial channel, the passage of the IPT cell into the TAAL vessel takes place, probably aided by the various degrees of hydrostatic and oncotic pressure.^{64,65} The above transendothelial passage of the IPT cell does not uphold the hypotheses formulated regarding entry into the lymphatic circle as a result of breaking or the "disorganized and tortuous" structure of the TAAL vessel endothelial wall^{16,66} with unmodified interendothelial junctions,⁶⁷ or through open interendothelial junctions,⁵⁶ because the structure and the contacts between adjacent endothelial cells have always been intact. The possible sighting of interruptions of the endothelial wall in the TAAL vessel are, I believe, attributable to ultrastructural pictures of intraendothelial channels fallen by chance under the sagittal direction of the section plane (more detailed in Azzali^{12,30}) or to adulterations resulting from incorrect use of fixation and sample conservation methods of the tissue studied.

Is the TAAL a new vessel formed by the overexpression of growth factors VEGF-C and VEGF-D¹⁴ or the result of the activation of peritumoral lymphatic vessels suited to lymph nodal metastasis, as claimed also by Tille and colleagues⁶⁸ and Wong and colleagues?⁶⁹ The reply calls for more exhaustive studies. I am inclined, on the basis of a subjective evaluation of the numerical density of the lymphatic vessels in the TRAMP model compared with the significant density described for other tumors by Papoutsis and colleagues,⁷⁰ Mandriota and colleagues,⁶⁰ Oliver and Detmar,⁷¹ Stacker and colleagues,⁵⁸ and Hirakawa and colleagues,¹⁵ to find the hypothesis of the activation of the peritumoral lymphatic vessels more likely. The latter could be the consequence of the vessel englobement because of the rapid tumor growth. These vessels, in the opinion of Tille and colleagues⁶⁸ and Detmar and Hirakawa,⁷² are involved in the chemotaxis and the metastatic diffusion of the tumor cell, as noted by Zeng and colleagues⁷³ for human prostate cancer as well. In the TRAMP model, unlike the results obtained by Tsurusaki and colleagues⁷⁴ in human prostate carcinoma, the overexpression of VEGF-C did not result in an increase in diameter of the lymphatic vessel but showed a rise in their numerical density instead, as pointed out also by Jeltsch and colleagues⁷⁵ in transgenic mice.

Regarding the functional degree of the peritumoral TAAL vessel involved in metastatic diffusion, the metastases found in the seminal vesicles of TRAMP mice testify to its active participation, thus dispelling the doubts expressed in this regard by Padera and colleagues.²⁰ Frequent sightings inside the lymphatic vessel lumen of IPT cells wholly lacking in cytological aspects of metabolic sufferance also point to the existence, in the TAAL vessel lymph, of conditions suitable for their survival and after metastatic dissemination. The latter is supported in the TRAMP model by the discovery of many IPT cells present in the sinuses and the parenchyma of the para-aortic and parailiac lymph nodes. According to Wiley and col-

leagues,⁷⁶ the CCR7 receptor may help to keep tumor cells (malign) in the lymph nodes because the T-cell paracortical areas are rich in CCL21 and CCL19 ligands for CCR7. This confirms a significant correlation between the expression of VEGF-C and lymph node metastatic dissemination, as noted in human prostate carcinoma by Tsurusaki and colleagues⁷⁴ and Trojan and colleagues.⁷⁷ In spontaneous prostate adenocarcinoma of transgenic mice, Wong and colleagues⁶⁹ suggest that the tumor-secreted VEGF-C and, to a lesser degree, VEGF-A, are needed for inducing an intratumoral lymphangiogenesis, which is "unnecessary for lymph nodes metastasis." According to Skobe and colleagues,⁷⁸ the considerable diffusion of the IPT cell in the lymph nodes is an indication of tumor aggressiveness. Could the high number of IPT cells in the act of entry into the TAAL vessel be related to adhesive and malignant tumor power? On this subject, Bevacqua and colleagues⁷⁹ point out a correlation between malignancy and adhesive power in human melanoma and breast cancer cell cultures. In the TRAMP model, IPT cells have never been found, either singly or in groups, attached to the luminal wall of the TAAL vessel; Balch and Lange⁸⁰ consider this weak proof of their survival.

Conclusion

The morphological findings obtained by the study of the TRAMP model tumor mass represent an accurate demonstration of the transendothelial migratory modality of IPT cell from extravasal matrix toward the TAAL vessel and of its presence inside the satellite lymph node. This passage occurs by a formation called the intraendothelial channel, a transient dynamic entity originating on the basis of the peculiar plasticity of TAAL vessel endothelium with high absorption capacity, and probably on the basis of tumor cell-lymphatic endothelial cell molecular interactions, which unfortunately are still unknown. The lack of knowledge of molecular bases regulating the intraendothelial channel organization represents a critical point as well as a signal to pay more attention to the structure-function relationships in the still little-known TAAL vessel. Furthermore, to show the active communication between tumor cell and lymphatic endothelium,⁵⁹ the intraendothelial channel represents a different route in comparison with that of lymphatic expansion that promotes metastasis, referred also in experimental and clinical-pathological studies.^{81,82} Once achieved the knowledge of the above-mentioned molecular bases, new strategies could be formulated to enhance or inhibit the formation of the intraendothelial channel, aiming at increasing lymphocyte homing and tissue homeostasis in lymphedema, or at blocking metastatic dissemination of tumor cell to regional lymph nodes via lymphatics, respectively.

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