A Transformative Approach to Cancer Metastasis: Primo Vascular System as a Novel Microenvironment for Cancer Stem Cells

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Metastasis is the principal cause of death among cancer patients. Despite intensive research with the application of genetic and molecular biology, its prevention and management remain far from satisfactory. More innovative approaches are needed. The major routes for tumor dissemination are the two circulatory systems, lymphatic vessels and blood vessels, so the metastases occurring through them are referred to as lymphatic and hematogenous metastases, respectively. Surprisingly, a new circulatory system, named the primo vascular system (PVS), which is composed of very thin primo vessels (PVs) and primo nodes (PNs), has recently emerged as a third circulatory system. Thus, a natural conjecture would be that the PVS might be associated with carcinogenesis and metastasis, and, indeed, that conjecture has been supported by strong evidence of metastasis in rodents with xenograft tumors. In fact, a study showed that migration of tumor cells to secondary sites was more efficient in the PVS than in the lymphatic system. In addition, the PVS is also a conduit for a fluid, which, according to proteomic analyses contains remarkably high levels of carbohydrate metabolic derivatives that are usually associated with stem cells, cancer cells and differentiated myeloid cells. Thus, the PVS has the potential to transport growth and communication factors between primary and secondary tumor sites, thereby enhancing the oncogenicity of tumor cells at secondary sites. Furthermore, the study of a tumor-derived PVS in murine xenografts of human histiocytic lymphoma has suggested that the PVS may provide a safe haven for a select population of cancer stem cells. These pieces of evidences for the involvement of the PVS in cancer metastasis is call for more intensive investigations of its possible role as an incubating microenvironment for tumor formation, propagation, sustenance, and relapse.

Keywords: Cancer Metastasis; Cancer Stem Cell; Primo Vascular System; Microenvironment


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Introduction

Cancer cells move long distances by floating in the fluid that flows through the vascular systems; thus, the blood and lymph systems are major routes of cancer metastasis. Recently, a new fluid conduit called the primo vascular system (PVS), which is comprised of primo nodes (PNs)
and primo vessels (PVs), emerged as a hitherto unnoticed anatomical structure. Because it is a fluid-conducting system, it might be another metastasis route of cancer, and, indeed, strong evidence supports such a hypothesis [1].

The PVS was first discovered in the early 1960’s [2], but was ignored until it was reinvestigated and confirmed by the Seoul National University group in 2002 [3]. The presence of the PVS was observed on the surfaces of internal organs like the liver, stomach, large intestine and bladder [4]. This subset of the PVS is called the organ-surface PVS (OS-PVS). Another major subset, which has been observed, is the PVS floating in the flow of blood [5] and lymph [6] or in the cerebrospinal fluid of the brain and the spinal cord [7].

The flow of fluid in the PVS was directly demonstrated by injecting Alcian blue into a primo node in the dorsal skin of a rat and observing the flow of the dye in the OS-PVS on the surface of the stomach [8]. The flow speed of the
Alcian blue solution injected in to the OS-PVSs of rabbits was measured to be $0.3 \pm 0.1 \text{ mm/s}$ [9]. The ultra-structure of the OS-PVS, as revealed by using cryo-scanning electron microscopy and high-voltage electron microscopy, showed (1) globular clumps of matter inside the sinus of the channel with thin strands of segregated zones, which is microscopic evidence of primo-fluid flow, and (2) sinuses with wall structures surrounded by extracellular matrices of collagen-like fibers. The flow in the PVS might be due to peristaltic motion, which can be verified with bioelectric measurement. The resting potential of the OS-PVS was measured, and the electrical signals resembled those found in smooth muscles. The effects of acetylcholine and pilocarpine, neurotransmitters of the autonomic nervous system, and nifedipine, an antagonist of Ca$^{2+}$ channels, on the membrane potential of cells in the OS-PVS were investigated. The results indicated that PVS cells have voltage-dependent Ca$^{2+}$ channels and can be relaxed by cholinergic activation of muscarinic receptors. This result supports the contractility of the PVS and its circulatory function being subject to cholinergic regulation [10, 11].

The PVS floating in the lymph ducts has been extensively studied in rabbits [12], rats [13], and mice [14]. The lymph PVS is visible \textit{in vivo} and \textit{in situ} with suitable staining dyes like Alcian blue [15] and fluorescent nanoparticles [13]. Detailed protocols for the observation of the lymph PVS in the abdomen or on the skin were provided for those who need to reproduce the experiments [16]. The lymph PVS is particularly interesting because lymph ducts are the major routes of cancer metastasis. Thus, an immediate question is, how do moving cancer cells interact with the PVS that is floating in the lymph flow?

The physiological functions of the PVS have hardly been studied yet because the anatomical and the structural information on the PVS is not sufficient to enable such studies. However, some medically-significant points have already been suggested. Abundant immune cells exist in the OS-PVS and the lymph PVS, which indicates that the PVS is one of the important parts in the immune system [17]. The presence of small embryonic-like stem cells in the PVS [18, 19] and hematopoietic stem cells [20] seem to support the original claim by BH Kim about the hematopoietic function of the PVS [21]. So far, the most important connections with diseases have been the findings that the PVS might be deeply related to cancer biology, in general, and to the metastasis of cancer, in particular.

**Cancer-associated primo vascular system**

Even early pioneers of the PVS conjectured about its possible connection with cancer [22, 23], but the first observation of a cancer-associated PVS (C-PVS) was made with nude mice into which human lung cancer cells had been in occluded [24]. Two to eight weeks after tumor cell inoculation, the skin on the xenografted tumor was removed to expose the tumor with an intact membrane. Trypan blue solution was poured on the membrane of the exposed tumor drop by drop and washed off with warm saline, and the C-PVS appeared.

The tumor tissue grown under the skin of a nude mouse is shown in Fig. 1A, where the PVS can be seen on the surface of the tumor after skin resection. After the Trypan blue staining had been applied, the PV and the PN emerged as blue threadlike structures. Blood vessels or fascia were not stained, but the PV and the PN were prominently stained as shown in Figs. 1B and 1C. PVs sometimes entered nearby fat tissues, as indicated by the double arrow in Fig. 1C.

The PVS was not only densely populated in proximity of xenografted tumors, but also more OS-PVSs were found in the abdominal cavities of tumor-bearing mice than in the abdominal cavities of normal mice [25]. Akers \textit{et al.} found PVs extending to and from the abdominal viscera, often
disappearing in adipose tissues before resurfacing in a more distal region. In addition, some PVs of the tumor were loosely attached to the surface of the tumor, and some segments appeared to be within the serosal tissue or invading into the tumor [26].

Heo et al. developed a melanoma model with a green fluorescent protein (GFP) expressing mouse by inoculating melanoma cell lines into the abdominal region. The C-PVS of the tumor evolved endogenously from the host animal, not the exogenous tumor tissue [27]. The inner part of the tumor was investigated by examining sections of the tumor mass. The PVSs were observed as green structures. The PVs were very thin, threadlike formations with branches. A PV sprouting from a blood vessel was observed; a PN was also seen. These PVs were difficult to observe with optical microscopy. In contrast, fluorescence microscope images showed an abundance of PVs inside the tumor.

Other works on the C-PVS [28-33] have followed sporadically. However, the most interesting and immediate concern remains the potential role of the PVS as an extra path for cancer metastasis.

Cancer metastasis

Metastasis is the most ominous feature of cancer, accounting for greater than 90% of human cancer deaths. The process of metastasis consists of a stepwise sequence of events that give rise to an outgrowth of secondary lesions. These steps include the shedding of tumor cells from a primary tumor into the circulation, survival of those cells in the circulation, their arrest in distant organs, their extravasation on into the parenchyma, their adaptation to the new environment, initiation and maintenance of their growth, and vascularization of the metastatic tumor. Tumor metastases can occur via lymphatic vessels or blood vessels, referred to as lymphatic and hematogenous routes, respectively. Epithelial malignancies, or carcinomas, typically begin their dissemination via the lymphatic route whereas bone and soft tissue tumors, or sarcomas, metastasize via the hematogenous route.

Signaling pathways in hematogenous metastases have been studied at the level of individual proteins by using molecular biology techniques since Judah Folkman’s revolutionary article proposing that all tumors were angiogenesis dependent [34]. Targeted therapies against mediators of tumor angiogenesis were designed to achieve clinical applications for the control of metastases. Although anti-vascular endothelial growth factor (VEGF) therapy was approved to combat metastasis, recent studies in mice paradoxically revealed an increased risk of metastasis associated with this therapy [35]. These preclinical findings corroborate results from recent clinical trials [36] showing no overall survival benefit for the VEGF inhibitor in the treatment of various cancers.

The tumor’s microenvironment has emerged as a crucial player for tumor progression and metastasis [37]: Tumor stromal cells, tumor-associated macrophages [38], mesenchymal cells [39], and cancer-associated fibroblasts [40] have been shown to affect the metastatic behavior of cancer cells. Beyond the contributions of specific cell types to metastasis, the extracellular matrix (ECM) also has a capacity to control cancer initiation and progression. Other microenvironment properties, such as vessel integrity and vascular anatomy, have an ability to influence metastatic tumor-cell dissemination to and growth at secondary sites.

Despite the tremendous accumulation of knowledge on metastasis acquired through the application of genetic and molecular biology, we still have little understanding of how to control and manage metastasis. Studies on the microenvironments of cancer cells need some paradigm-shifting visions. Recent advances in molecular imaging and in vivo microscopy have opened new avenues of investigation to address the question as to how tumor cells actually migrate via specific routes and how metastatic cells interact with stromal cells and other anatomical environments. One notable finding with these state-of-the-art technologies is the presence of a C-PVS around a tumor, which is a novel microenvironment for cancer cells that may provide an innovative approach to cancer metastasis.

Transport of cancer cells through the PVS

As soon as the C-PVS was discovered, an immediate conjecture was that it might be an extra path for cancer metastasis because the PVS is a system through which a liquid flows [8, 9]. This conjecture was supported by the observation of the transport of cancer cells from a primary tumor to secondary tumors at a distance through a PV [1]. Frontier technologies were employed to detect the PVS-mediated metastatic process: near-infrared quantum-dot electroperoration and multispectral fluorescence imaging. The in vivo imaging of quantum-dot-labeled lung cancer cells inside the PVS around a superficially grown tumor and abdominal organs provided evidence for the PV as a metastatic route. These in vivo observations were later confirmed by histological analyses [1].

Figure 2 shows an exemplary case of cancer-cell migration through both a PV and a lymph vessel from a primary tumor to a secondary tumor. In this particular case, more cancer cells moved through the PV than through the lymph vessel.

Human lung cancer cells labeled with the fluorescent nanoparticles or GFP gene transfection were injected into testicular parenchyma in rats in order to check whether cancer cells could move a long distance via the PVS from one organ to another, [41]. Twenty-four hours after injection, the OS-PVS was observed with multi-photon microscopy. Some of the injected cancer cells were detected inside the
OS-PVS and in the intestinal parenchyma into which the OS-PVs had entered. The result showed that the PVS might be a transportation path for cancer cells to other organs from the test is; thus, it could be a metastasis route of testicular cancer, which is the most common solid tumor in young adults and whose incidence is rising in both the United States and Europe [42].

Cancer stem cells

Cancer stem cells (CSCs) have been the subject of intensive investigations for both oncology and stem cell biology over the past 10 years [43]. CSCs refer to a subpopulation of tumor cells that has the ability to self-renew and generate the diverse cells that comprise the tumor. These cells have been termed cancer stem cells to reflect their ‘stem-like’ properties and ability to continually sustain tumor growth. Only this minority population of tumorigenic cells can drive tumorigenesis and disease progression, through therapy resistance and metastasis, while most other cancer cells have little or no capacity to contribute to tumor growth.

The first evidence for the existence of CSCs came from acute myeloid leukemia, in which a rare subset comprising 0.01-1% of the total population could induce leukemia when transplanted into immunodeficient mice [44]. There is now increasing evidence for the presence of CSCs in a variety of solid tumors: acute and chronic myeloid leukemia, breast cancer, glioblastoma, colorectal cancer, pancreatic cancer and ovarian cancer [45]. The observation that only rare, phenotypically-distinct tumorigenic cells are capable of generating a malignant population when transplanted into immune deficient mice is the basis of inferring the existence of a CSC population. The other crucial point to satisfy the CSC model is that tumorigenic cells give rise to non-tumorigenic progeny. CSCs from the above-mentioned cancers have been found to form more tumorigenic cells, as well as non-tumorigenic cells, demonstrating their capability to form heterogeneous tumors by undergoing epigenetic changes, akin to the differentiation of normal stem cells [45]. Thus, CSCs share important properties with normal tissue stem cells, including self-renewal (by symmetric and asymmetric division) and differentiation capacity.

The CSC concept has a significant clinical implication that future cures might require strategies that will eliminate CSC populations. CSCs are more resistant to chemotherapy and radiotherapy in various cancers [46]. An understanding of the complex and patient-specific evolutionary process by which human malignancies develop their genetic and epigenetic diversity will most likely be crucial. Accordingly, the CSC concept is of key importance in achieving a sustained disease-free outcome or even a definitive cure.

PVS hypothesis: novel micro environmental niche for cancer stem cells

Normal stem cell behavior is modulated by the external signals that the cells encounter, and this is generally orchestrated in vivo within protectivive niches that are composed of micro environmental cells. For example, neural stem cells lie within a perivascular niche in which endothelial cells regulate stem cell self-renewal. Evidence that CSC behavior may be similarly modulated in vivo by tissue-specific, ‘niche’ microenvironments is accumulating with advanced in vivo imaging technology. Therefore, a functional and contextual analysis of intact CSC niches is required for a proper understanding of the role of CSCs in tumor formation and metastasis.

Several lines of evidence have been reported to highlight the PVS as specific micro environmental niche for normal stem cells, as well as CSCs. Central to the PVS is the existence of primo microcells that are thought to function like very small embryonic-like adult stem cells. Studies to observe the growth and the budding of primo microcells have been tried using primary culture techniques and cellular imaging [47, 48]. More comprehensive data using morphological and immunochemical analyses have strengthened the hypothesis that the PVS is a favorable niche for primo microcells, rare populations of stem-like cells with self-renewal and broad differentiation potential [49]. Primo microcells have very small, approximately 3-4 µm in diameter, extremely dense, eccentrically located, round nuclei surrounded by basophilic cytoplasm. Several embryonic stem cell markers, including CD133, Oct-4 and Nanog, have shown high expressions by immune staining analysis.

In addition to the evidence obtained from normal animal studies to show the presence of stem-like cells within the PVS, attempts using xenograft models to identify and characterize the cells in the PVS showed a significant link between the PVS and CSCs. Islam and colleagues [28] reported the presence of a tumor-derived PVS in murine xenografts of human histiocytic lymphoma (U937) in the vicinity of the primary tumor. The qRT-PCR analysis of mRNA isolated from the cells in the PVS showed high levels for the expressions of human-stem-cell-specific transcription factors, including KLF4 and NANOG, which are characteristics of a pluripotent and undifferentiated state of stem cells. Such evidence for the existence of “stem-like” cells within the PVS supports the hypothesis that the PVS provides a unique “niche” environment for CSCs and may play a critical role in tumor growth and metastasis.

Interaction of CSCs with their microenvironment (CSC niches) is likely to have growing importance to elucidate treatment-resistance mechanisms and to refine therapeutic strategies [50]. Tumor formation and metastasis involve the
co-evolution of CSCs together with complex microenvironments including the extracellular matrix, the tumor vasculature, and immune cells. The PVS could be a specific anatomical structure that nurtures CSCs and enables them to contribute to tumorigenesis. If CSCs depend upon aberrant PVS microenvironments, then the PVS might represent a target for the treatment of cancer. However, the PVS is also a unique pool of immune cells, including macrophages, which have the functions of recognizing infections and damage, of protecting tissues from those infections and damage, and preventing tumor growth. Accordingly, a more sophisticated strategy for the targeting PVS, a hidden haven for CSCs, will be required considering the diverse capacities of the PVS to induce both beneficial and adverse consequences for tumorigenesis. Re-education of the cells within the PVS, rather than targeted disruption, may be a surrogate approach to induce anti-tumorigenic effects.

**Other prospects**

The hypothesis that the PVS is a novel microenvironment for stem cells and cancer stem cells was first suggested by a proteomic analysis of the PVS. A proteomic analysis of the tissues and liquid from the OS-PVS of rabbits [51] showed the presence of biochemicals that are usually associated with stem cells [52, 53], cancer cells [54], and differentiated myeloid cells [55]. These cells with vigorous proliferation show a similar abundance of carbohydrate- or energy-related processes. In addition, the primo fluid flowing in the PVS may be important in tumor growth as it contains hyaluronic acid, adrenalin and noradrenalin hormone, albumin, and primo-microcells (or Sanals). Thus, investigating whether tumor tissues use the chemicals provided by the PVS would be desirable.

Interestingly, the C-PVS often enters the adipose tissues around the tumor [25-27]. In fact, the OS-PVS of normal animals is frequently hidden in intestinal fat tissues so that tracing or imaging it is usually prevented by the opaqueness of fat cells [56]. Obesity with its derivative disease like diabetes due to adipose tissues is one of the most threatening health problems only second to cancer. Now both cancer and obesity might be closely connected through the PVS. The PVS on the tumor coexisted with blood vessels, and the PVSs were strongly coupled with the white adipose tissues around the tumor tissue.

A hypothetical use of the PVS connected to tumor tissues is its possible use as a drug delivery path for anti-cancer medicine. Cancer treatment with acupuncture or other traditional Chinese medicine is also plausible because the PVS is an anatomical structure of acupuncture collaterals [21]. A host of items, such as migration, invasion, angiogenesis, proliferation, and growth of cancer tissue, may be newly illuminated by the light of traditional Chinese medicine in conjunction with the PVS.

**Conflicting interests**

The authors have declared that no competing interests exist.

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