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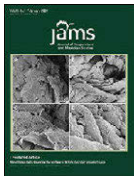

Research Article

Mesothelial Cells Covering the Surface of Primo Vascular System Tissue

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Abstract
The primo vascular system (PVS) is reported to have a periductium composed of cells with spherical or spindle-shaped nuclei and abundant cytoplasm. However, little is known about these periductium cells. In this study, we examined the morphological features of cells covering the PVS tissue isolated from the surface of abdominal organs of rats. By hematoxylin and eosin (H&E) staining, we observed a layer of dark nuclei on the basement membrane at the borders of the sections of primo node (PN), primo vessel (PV), and their subunits. The nuclei appeared thin and linear (10-14 μm), elliptical (8-10 × 3-4 μm), and round (5-7 μm). The borders of the PVS tissue sections were immunostained with a selective antibody for mesothelial cells (MCs). Areas of immunoreactivity overlapped with the flattened cells are shown by hematoxylin and eosin staining. By scanning electron microscopy, we further identified elliptical (11 × 21 μm) and rectangular squamous MCs (length, 10 μm). There were numerous stomata (~200 nm) and microparticles (20-200 nm) on the surface of the PVS MCs. In conclusion, this study presents the novel finding that the PVS periductium is composed of squamous MCs. These cells tightly line the luminal surface of the PVS tissue, including PNs, PVs, and small branches of the PVs in the abdominal cavity. These results will help us to understand the physiological roles such as hyaluronan secretion and the fine structure of PVS tissue.

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1. Introduction

The primo vascular system (PVS), a novel circulatory system, was first introduced by Bong-Han Kim in 1963 as the anatomical structure of acupuncture meridians [1]. The PVS is composed of primo vessels (PVs) that connect primo nodes (PNs). PVS tissue has been identified on the serosal surface of internal organs (i.e., the large intestine, small intestine, and liver) and inside the blood and lymph vessels in several animal species [2].

It is known that PVS tissue on the surface of abdominal organs is packed with heterogeneous immune cells, such as mast cells, eosinophils, neutrophils, and lymphocytes [3-5]. Recent studies have revealed that there are also erythrocytes and reticulocytes in the PVS on organ surfaces [6,7]. Numerous small round cells ($<$10 μm) and fibrous structures have also been observed on the surface of PVS tissue [8].

In 1965, Kim described the periductium of the PV, which contains cells with spherical or spindle-shaped nuclei (6∼12 μm) and abundant cytoplasm [9,10]. Since then, cells with such large nuclei have not yet been identified on the surface of PVS tissue.

In 2013, Lim *et al*. [6] reported that the outer layer of PN slices (200 μm in thickness) from abdominal organ surfaces was densely stained with hemacolor, a rapid staining dye for cells in the blood smear. This observation implies that cellular density was higher in the outer layer of the PVS section. Currently, it is unknown whether the dense outer layer is due to the nuclei of small round cells [3-8] or due to cells with large nuclei, as proposed by Kim [9,10].

In this study, we examined cells on the surface of the PVS by hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), and scanning electron microscopy (SEM). We identified mesothelial cells (MCs) on the PVS that is known to line the serosal cavity [11,12].

2. Materials and Methods

Male Sprague-Dawley rats weighing 100-180 g (Orient Bio, Gyeonggi-do, Korea) were used for this study. The rats were kept in an isolator maintained at a constant temperature-controlled environment (22°C-26°C) with atmosphere humidity (40%-60%) under a 12-h light:12-h dark cycle (light on at 9:00 AM) and allowed free access to water and standard rodent chow until sacrifice. All the experiments were performed in accordance with the Guide for the Laboratory Animal Care Advisory Committee of Seoul National University and approved by the Institute of Laboratory Animal Resources of Seoul National University (SNU-160622-5).

To obtain samples of the PVS tissue on the surface of abdominal organs, the rats were anesthetized with an anesthetic cocktail (alfaxalone, 30 mg/kg; xylazine, 10 mg/kg) administered by intramuscular injection. The abdomen of each rat was incised along the linea alba, and milk-colored and semitransparent PVS tissue samples composed of PNs and PVs were collected under a stereomicroscope with × 10-20 magnification [6].

The PVS tissue samples were stored in saline and then in 4% paraformaldehyde. For H&E staining to characterize the cellular morphology, the PVS tissue samples were initially fixed overnight in a tissue processer, embedded into paraffin, and sectioned to 4-μm-thick slices using a microtome. The resulting PVS tissue slices were stained with H&E (Harris hematoxylin solution, Eosin Y solution; Sigma-Aldrich, St. Louis, MO, USA) as a part of the routine processing. For IHC, the tissue specimens were cut into 30-μm sections on a cryostat. The primary antibody, hector battifora mesothelial-1 (HBME-1) (M3505, CiteAb Ltd., Bath, UK), which is known to interact with an unknown target in the microvilli of MCs [13,14], was diluted (1:1000) in phosphate-buffered saline with Triton-X (5%) and further labeled with Alexa 488 for 1 hour. All the other procedures were performed in accordance with the procedures followed by Kim *et al*. [15]. For SEM, samples were fixed, dehydrated, dried with hexamethyldisilazane, and observed in a scanning electron microscope (Sigma, Carl Zeiss, Jena, Germany), as reported previously [8].

3. Results

PVS tissue samples containing PNs and PVs were obtained from the surface of abdominal organs, as illustrated in Fig. 1-A1-A3. The isolated tissue samples were usually bent or coiled within the saline (Fig. 1-A3). In H&E staining, we observed dark nuclei aligned with the border of the cross section of the PNs (Fig. 1-B1) and the longitudinal section of the PVs (Fig. 1-C1). Such dark nuclei were also identified at the border of the subunits of the PNs and the PVs (marked by gray arrows). It is also noteworthy that both the PNs and PVs were composed of multiple subunits. At higher magnification, a single layer of flattened cells was located along the boundary of the PNs (Fig. 1-B2 and 1-C2, arrows). The nuclei at the border of the PNs and PVs were thin and linear (10-14 μm), elliptical (8-10 × 3-4 μm), and round (5-7 μm; Fig. 1-B3 and 1-C3, arrowheads). A thin structure resembling the basement membrane was located directly under the cells with flattened nuclei (Fig. 1-B3 and 1-C3, arrowheads). The estimated thickness of these cells appeared about 2-4 μm. The flattened nuclei and basement membranes appeared to demarcate the subunits of the PNs and the PVs. The observations made based on the images shown in Fig. 1 suggest that the cells on the edges of the PN and PV sections were MCs, which cover the surface of abdominal organs and the wall of the abdominal cavity [12].

To confirm this possibility, we immunostained the PVS tissue using HBME-1 antibody, which is a selective biomarker for MCs [13,14]. HBME-1 immunoreactivity densely appeared on the borderline of the PNs and PVs, as shown in Fig. 2. Fig. 2A illustrates the fluorescence image visualizing the interaction of HBME-1 antibody and target antigen in a PN section. We observed bright fluorescence at the boundaries of two parts of PN section (indicated by arrows). The upper part of Fig. 2A is likely to be a part of the same PVS tissue irregularly aligned during cryo-cutting. If the tissue was aligned straight, the shape of the PN section would be round as reported previously [6]. When the square areas in Fig. 2A are examined at higher magnification, we further confirmed the strong green immunofluorescence at the boundaries (Fig. 2B-2D). Similarly, we also observed the immunofluorescence in the PV section as shown in Fig. 2E and identified dense fluorescence at the

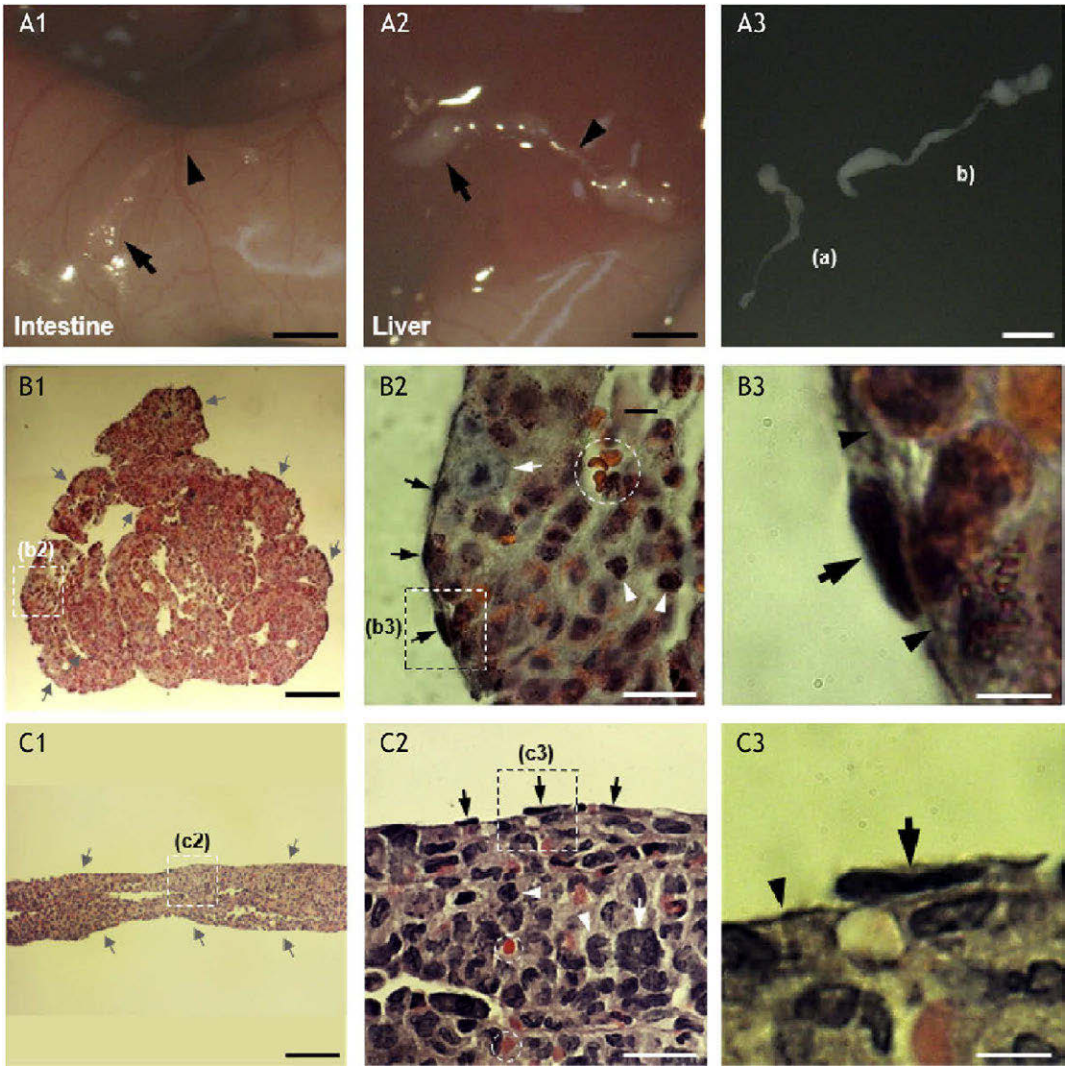

Figure 1 Hematoxylin and eosin staining of sectional (4 μm in thickness) micrographs of the organ surface primo vascular system (PVS) tissue. (**A1 and A2**) PVS tissues composed of primo nodes (PNs, arrows) and primo vessels (PVs, arrowheads) on the surface of the small intestine (A1) and liver (A2). (**A3**) The same PVS tissue samples in saline. [PN: (A1), 0.60 × 0.44 mm; (A2), 1.24 × 0.45 mm]. Scale bars: 1 mm. (**B1 and C1**) Cross section of a PN (B1) and longitudinal section of a PV (C1). Note the dark nuclei covering the border (gray arrows). Scale bars: 100 μm. (**B2 and C2**) Magnified view [squares in (B1) and (C1)] showing flattened nuclei of the cells (arrows) located along the boundary of a PN (B2) and PV (C2). There are mast cells (open arrows), white blood cells (open arrowheads), red blood cells (circles) inside the PN and PV. Scale bars: 20 μm. (**B3 and C3**) Magnified view [squares in (B2) and (C2)] showing cucumber-like nuclei (arrows) on the thin basement membranes (arrowheads) in the PN (B3) and PV (C3). Scale bars: 5 μm.

boundary of the PV section (Fig. 2F). The results of the IHC experiment indicate that the PVS tissue is covered by MCs. To confirm this observation, we investigated the surface of the PVS tissue by SEM.

Fig. 3 shows representative SEM images of the squamous MCs located on the surface of the PVS tissues. As shown in Fig. 3A, the squamous MCs that covered an area broader than the cell size were thin and elliptical or halibut-like in shape. The longer and shorter axes of the MCs were 20.7 ± 4.96 μm and 11.1 ± 3.75 μm, respectively (n = 7). It seems that some cells were about to be peeled off and there was another cell layer below those cells, as shown in Fig. 3A. In the other area, including the surface of small processes, the squamous MCs were aligned side by side or wrapped the entire surface of the processes, as shown in Fig. 3B. Those MCs appeared rectangular, with a length of 10.0 ± 3.00 μm (range, 5.17-13.79 μm, n = 15). The nucleus of the cell in Fig. 3C measured 4.5 μm, which is comparable with the size of the round nuclei measured in the H&E-stained section (Fig. 1). We observed microvilli on the surface and the intercellular borders (Fig. 3C and 3D). The width of the microvilli on the cell surface was ~100 nm. In addition, there were many cells with a thick extracellular matrix, but without prominent microvilli (Fig. 3D). On the extracellular matrix, we also observed numerous stomata (~200 nm; marked by arrowheads), and

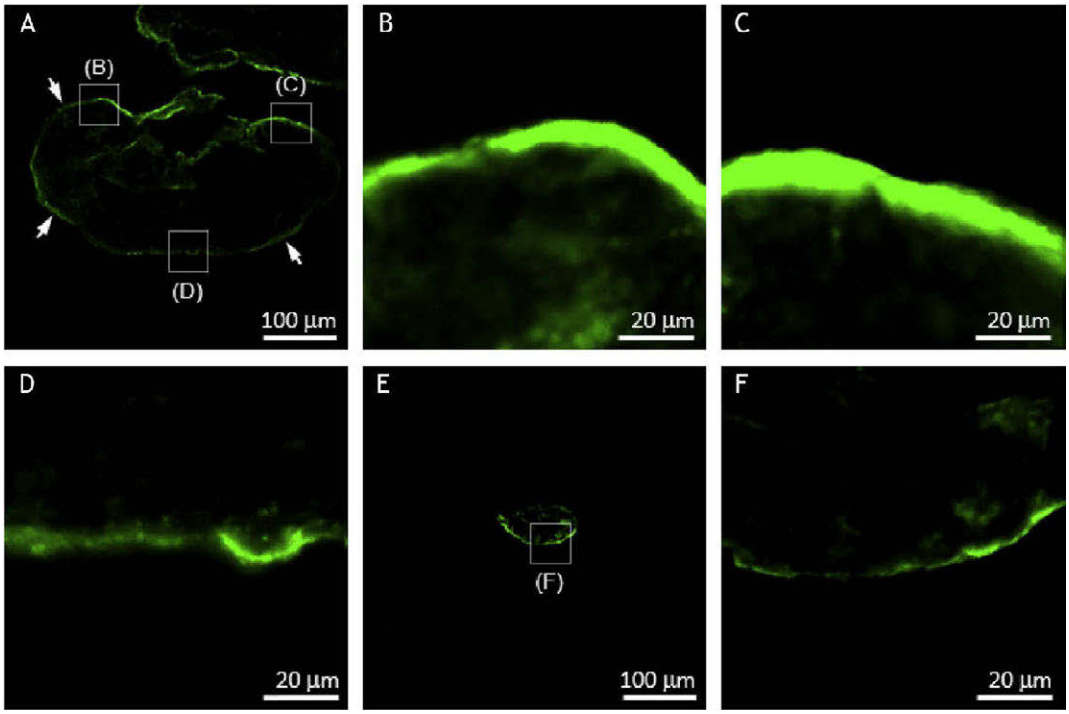

Figure 2 Immunoreactivity against the HBME-1 antibody (a marker of mesothelial cells) in a cross section of primo vascular system tissue (30 μm in thickness). (**A and E**) HBME-1 immunoreactivity (arrows) located on the border of the PN (A) and PV (E). (**B-D and F**) Magnified view of the squares in (A) and (E) showing immunostaining by the mesothelial cell marker from PN (B-D) and PV (F) sections.

in some cases, nanoparticles (20-100 nm) were present on or around the stomata (marked by arrows).

4. Discussion

The results of this study indicated that the surface of PVS tissue, including small-diameter branches of PVs, was tightly covered by squamous MCs. This finding is supported by (1) the morphology of MCs obtained from H&E staining, (2) the immunoreactivity of the surface layer to an MC-selective antibody (HBME-1) [13,14], and (3) the typical morphology of squamous MCs shown at high magnification by SEM. To our knowledge, this study is the first to report MCs in PVS tissue.

In this study, the immunofluorescence with HBME-1 appeared as a thin band at the boundary of the PVS tissue, and individual cells were not readily recognized by the immunofluorescence (Fig. 2). This is likely due to the nature of the antibody that bind to the unknown targets in the microvilli [13] and glycoconjugates [14] together with an overlapping fluorescence from multiple layers of thin MCs in 30-μm sections of the PVS tissue. In addition, SEM revealed that the MCs on the PVS were rather large and elliptical or halibut-like in shape. The MCs had microvilli on the surface and stomata on the extracellular matrix and the intercellular borders (Fig. 3). These cytological features are similar to those of flat or squamous MCs on the omentum of rats [11,16], human peritoneal MCs isolated from the omentum [17], and the proepicardium cells that develop from the mesothelium in quails [18]. The oval shape of the nuclei of the MCs shown in Fig. 3C is similar to that of the nuclei in the periductium of PVs, as reported by Kim [9,10]. Collectively, these observations indicate that the sheet-like cells covering the surface of the PVS tissue were MCs [12,19].

PVS tissue has been studied using electron microscopy. For example, Lee *et al.* [3] showed that PVS tissue had a bundle structure and that PNs had a coarse and fenestrated surface. Lim *et al.* [8] showed extensive fibers and nanoparticles on the surface of PVS tissue. The presence of MCs was not stated in previous studies, including those 2 studies using SEM. In the present study, we were not able to observe MCs in some specimens. In other specimens, we observed only parts of MCs, and so we could not obtain a complete picture of the mesothelial covering, as shown in Fig. 3. This lack of consistency may have been due to disruption of the MCs during various procedures of SEM experiments, including isolation from the rat body, incubation, and fixation for electron microscopic visualization. It is likely that the SEM images in previous studies [3,8] show the submesothelial connective tissue layer [11,20]. Alternatively, there may be different forms of the PVS tissue in the abdominal cavity. Further study is needed to resolve this discrepancy.

Interestingly, the PVS MCs were found to be tightly wrapped around the small-diameter processes of the PVS tissue (Fig. 3A and 3B). The MCs seemed to exist in more than one layer (Fig. 3A). We were not able to find any description of such a distinctive alignment of MCs in previous studies on other tissues. Further study is also needed to confirm whether such property is specific to PVS tissue in vivo.

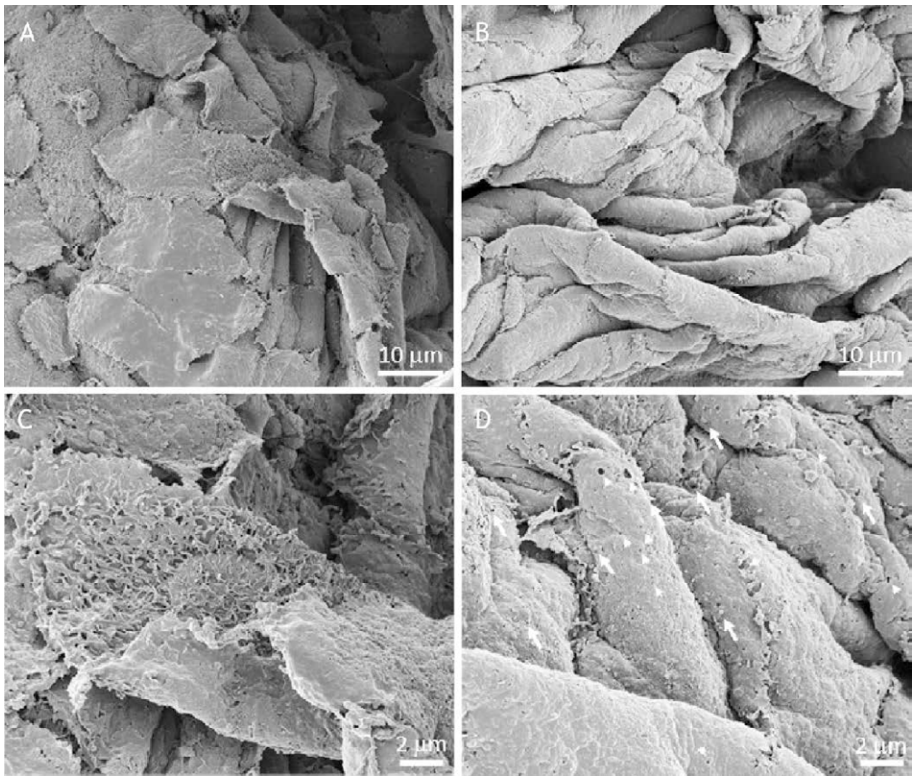

Figure 3 Scanning electron microscopy of mesothelial cells on the surface of primo vascular system (PVS) tissue. (**A and B**) Squamous mesothelial cells covering the body (A) and wrapping the small branches of the PVS tissue (B). (**C**) Magnified view of the cells in (A) showing the microvilli and round nuclei (3.3 × 4.7 μm). (**D**) Mesothelial cells wrapping the PVS branches at a higher magnification. Note the protrusions along the cell borders, numerous stomata (arrow heads) through the extracellular matrix, and the particles near or over the stomata (arrows).

Recent studies have indicated that PNs and PVs are composed of multiple subunits [3,6,21]. In this study, we observed that the subunits of the PVS tissue, including the small branches of the PVs, were covered by MCs. This finding indicates that MCs could be used as a marker for the identification of individual subunits in a PN and/or PV.

The functions of MCs are well known and have drawn particular attention in the field of tissue regeneration. MCs are known to synthesize and release lubricants such as hyaluronan to create a slippery surface on various visceral organs [22,23], as well as a diverse array of mediators regulating inflammatory processes, the immune response, and tissue repair [12,24]. MCs may protect from peritoneal dissemination of tumors until the integrity of the mesothelium is damaged [12]. Furthermore, MCs have the potential to undergo the epithelial-to-mesenchymal transition. They can further transform into myofibroblasts and possibly smooth muscle cells [24]. In view of these functions, the MCs on PVS tissue are likely to be the source of high hyaluronan levels in this tissue [9,10,25] and the origin of the endothelial/mesenchymal properties of PVS tissue [26].

In conclusion, this study showed that squamous MCs tightly covered the surface of the PVS tissue. These results suggest that squamous MCs demarcate subunits of PVS tissue, are the source of hyaluronan in PVS tissue, and are the origin of the stem cell-like properties of PVS tissue. Our findings will help to further elucidate the inner structure and function of the PVS as a novel circulatory channel.

Conflict of interest

Authors declare that they have no conflict of interest and no financial interests related to the material of this manuscript.

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