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RESEARCH ARTICLE

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Expression of Keratin 10 in Rat Organ Surface Primo-vascular Tissues

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Abstract
The primo-vascular system is described as the anatomical structure corresponding to acupuncture meridians and has been identified in several tissues in the body, but its detailed anatomy and physiology are not well understood. Recently, the presence of keratin 10 (Krt10) in primo-vascular tissue was reported, but this finding has not yet been confirmed. In this study, we compared Krt10 expression in primo-vascular tissues located on the surface of rat abdominal organs with Krt10 expression on blood and lymphatic vessels. Krt10 protein (approximately 56.5 kDa) was evaluated by western blot analysis and immunohistochemistry. Krt10 (IR) in the primo-node was visualized as patchy spots around each cell or as a follicle-like structure containing a group of cells. Krt10 IR was also identified in vascular and lymphatic tissues, but its distribution was diffuse over the extracellular matrix of the vessels. Thus Krt10 protein was expressed in all three tissues tested, but the expression pattern of Krt10 in primo-vascular tissue differed from those of blood and lymphatic vascular tissues, suggesting that structural and the regulatory roles of Krt10 in primo-vascular system are different from those in blood and lymphatic vessels.

1. Introduction

Bonghan Kim claimed that the Bonghan tissues, which consist of Bonghan ducts and Bonghan corpuscles, are anatomical structures corresponding to the meridians of acupuncture [1], because they were initially found beneath the acupressure points. However, Kim's results were not reproduced by other researchers for more than 40 years, because the techniques used by Kim's group were not described in sufficient detail in their reports. Recently, Soh's group identified these tissues on the surface of internal organs [2], skin [3], lymphatic system [4], blood vessels [5,6], omentum [7], central nervous system [8], and adipose tissues [9] using dyes such as Trypan blue, Alcian blue, and Acridine orange (see Reference 10 for review). The Bonghan tissues have since been renamed the primo-vascular system, the Bonghan duct is now the primo-vessel, and the Bonghan corpuscle is the primo-node [3,11].

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Primo-vascular tissue has also been identified on the fascia surrounding tumor tissues, which is thought to be a potential pathway for tumor metastasis [12,13]. Primo-vascular tissue has tree-like branches and fine terminal arborizations [14].

A recent proteomics study reported that the following three keratins are expressed in the primo-vascular tissues: keratin 3 (Krt3), keratin 10 (Krt10), and keratin 12 (Krt12) [15]. Keratins are cytoskeleton proteins of epithelial cells important for the integrity and stability of epithelial cells and tissue [16]. In tumor tissues keratins are widely used to diagnose squamous carcinomas and to classify and characterize metastasis [17]. Krt10, which is the most abundant of the three keratins detected in primo-vascular tissue [15], is paired with keratin 1 (Krt1), a type II keratin, and forms dense bundles that are features of suprabasal epidermal keratinocytes in humans. These Krt10/Krt1 pairs provide mechanical integrity to the epidermis and cells. Krt10 is normally expressed in post-mitotic keratinocytes [18], inhibits keratinocyte proliferation and cell-cycle progression, and reduces skin tumorigenesis [19]. In addition, Krt10 is a duct-specific marker in normal eccrine sweat glands [20]; the latter finding is particularly interesting because primo-vascular tissue is also a duct system [10]. However, few details of Krt10 expression and its role in the primo-vascular tissue are known. In this study we evaluated Krt10 protein expression and distribution in primo-vascular tissue, blood vessels, and lymphatic vessels by western blotting and immunohistochemistry.

2. Materials and Methods

2.1. Animals

Adult Sprague-Dawley rats (female, $n=11$; male, $n=23$; 320.6 ± 9.8 g body weight; Orient Bio, Seongnam, Korea) were housed in the laboratory animal facility at 23°C and 55% relative humidity under a 12-hour light/dark cycle. Water and regular rodent chow were provided *ad libitum*. Procedures involving the animals and their care were performed in accordance with the guidelines of the Laboratory Animal Care Advisory Committee of Seoul National University. All surgical procedures were performed under general anesthesia (25 mg/kg tiletamine-zolazepam and 10 mg/kg xylazine administered by intramuscular injection). The abdominal cavity of the rat was incised, and primo-vascular tissue samples were obtained from the surfaces of the intestine, bladder, liver, and spleen. The organ surface primo-vascular tissues were identified with Trypan blue staining [7] and/or by the typical structures of the primo-vessel and the primo-node [2]. Blood vessel tissues were obtained from the abdominal aorta or mesenteric artery, and lymphatic vessel tissues were obtained from the large branches of the lumbar aortic lymph nodes.

2.2. Antibodies

We used cytokeratin 10 mouse monoclonal IgG antibodies (SPM262, Santa Cruz Biotechnology, CA, USA) as the primary antibody. The secondary antibodies used were Alexa fluor 488 or 555 donkey anti-mouse IgG (Molecular Probes, Oregon, USA) for immunohistochemistry and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, CA, USA) for Western blots.

2.3. Western blot

The primo-vascular tissue specimens rats were washed with ice-cold phosphate buffered saline (PBS) solution, lysed with $2.5\times$ passive lysis buffer (Promega, Madison, WI, USA), and sonicated for 15 minutes at 4°C. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY, USA). The blots were blocked in $1\times$ Tris-buffered saline with Tween-20 containing 5% nonfat milk (Difco, Sparks, MD, USA) and incubated with Krt10 antibodies (1:1000 dilution). The blots were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:6000 dilution), and blots were visualized using an enhanced chemiluminescent detection kit (Intron Biotechnology, GyeongGi, Korea). We used rat corneal tissues as positive controls for Krt10 expression [21].

2.4. Immunohistochemistry

The primo-vascular tissue samples were fixed with 4% paraformaldehyde and then embedded in the optimal cutting temperature compound (Tissue-Tek, CA, USA). The tissue specimens were cut into 10-μm sections on a cryostat, mounted on glass slides, and then incubated with PBS for 30 minutes, 0.1% Triton X-100 for 30 minutes, and 10% normal donkey serum for 30 minutes. All incubations, except for the primary antibody reaction, which was performed at 4°C, were performed at room temperature in a humidity chamber. Primary antibodies were diluted (1:50) in PBS with normal donkey serum (5%). After a 2-hour incubation, the slides were washed three times in PBS and incubated with donkey anti-mouse IgG (1:500) labeled with Alexa 488 or 555 for 1 hour. Finally, slides were washed, dried, mounted with ProLong Gold anti-fade reagent with

4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes), and observed through a Carl Zeiss-LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

3. Results

All primo-vascular tissue specimens used in this study were taken from the surface of abdominal organs (Figure 1) [2]. The primo-vascular tissue appeared as a semi-transparent milky or red thread-like structure. A total of 73 primo-vascular tissue samples were collected from 34 rats: 61 (84.7%) in the intestine, five (6.9%) in the bladder, three (4.2%) each in the spleen and the liver, and one (1.4%) in the genitalia. The average thickness of a primo-vessel, as determined from the images, was $90\pm10\,\mu m$ ($n=72$), and the major and minor axes of a primo-node were 1.2 ± 0.1 mm and 0.5 ± 0.02 mm ($n=72$), respectively.

The Krt10 protein was detected in the primo-vascular tissue ($n=5$), blood vessels ($n=2$), and lymphatic vessels ($n=5$) by western blotting as a 56.6-kDa band, as shown in Figure 2. As a positive control, we detected Krt10 protein in a rat cornea (data not shown) [21].

To further characterize the distribution of Krt10 protein in primo-vascular tissue and blood and lymphatic vessels, we carried out immunohistochemical analysis using the same antibody used for western blot analysis. We confirmed that the antibody could identify Krt10 on the plasma membrane of HeLa cells, a keratin-forming tumor cell line; Krt10 was visualized as patchy spots on the surface and border of the cell body (data not shown). We found that Krt10 was expressed primarily in follicle-like structures containing a group of cells (stained with DAPI) inside the primo-vascular tissue, as shown in Figures 3A and 3B. In contrast, the Krt10 immunoreactivity (IR) was more diffuse in the blood vessels (Figures 3C and 3D) and lymphatic vessels (Figures 3E and 3F). Follicle-like structures showing Krt10 IR were not observed in blood or lymphatic vessels.

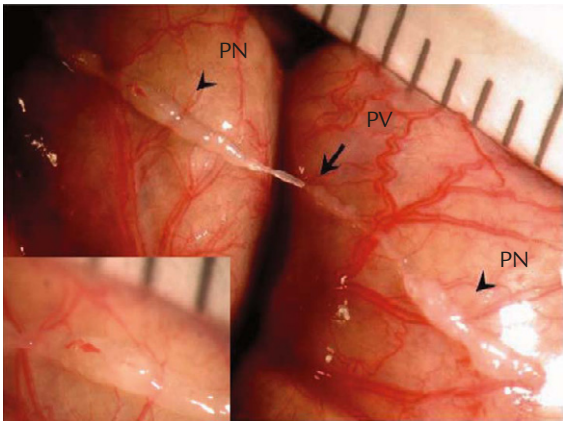

Figure 1 Stereomicroscopic image of primo-nodes (PN) and a primo-vessel (PV) on the surface of the large intestine of a rat. Semitransparent primo-nodes (arrow heads) and a primo-vessel (arrow) are seen on the surface of the large intestine (×100). The inset shows an enlargement of a red spot in the primo-node (upper left). The distance between two marks on the scale is 1 mm.

4. Discussion

In this study, we demonstrated that Krt10 is expressed in primo-vascular tissues on the surface of abdominal organs in the rat. Krt10 IR was visualized as patchy spots around single cells or as follicle-like structures containing a group of cells, but was not detected on the external membranes of primo-nodes. Krt10 IR was also identified in blood and lymphatic vessels, but its distribution was diffuse. To our knowledge, this is the first detailed report of the presence of Krt10 in primo-vascular tissues and further extends the previous findings on Krt10 in these tissues [15].

Krt10 IR was found primarily in structures that wrap around a group of cells within the primo-vascular tissue. This observation is consistent with that of a previous study, which reported that cells inside a primo-node were present as a group within a follicle-like cluster separate from other cells [22]. This follicle-like structure appeared to be larger than the primo-vascular ductule identified inside the primo-vessel [1,22], because ductules appeared to contain only one cell [22]. If the structures marked by Krt10 were to form a duct-like structure, our result would also be consistent with previous findings that Krt10 is a specific marker for straight eccrine ducts [23] and is selectively expressed in the middle ductal cell in the normal eccrine structure and syringomas [24]. Krt10, a type-I keratin, forms dense bundles with Krt1, a type-II keratin, to impart mechanical integrity to cells and the whole epidermis in suprabasal epidermal keratinocytes [16], and structural integrity to the epithelial sheet essential

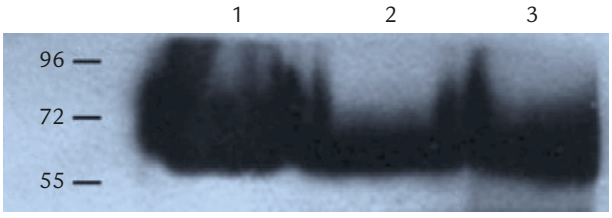

Figure 2 Western blot analysis of Krt10 protein expression in primo-vascular tissues on the surfaces of the rat abdominal organs. Bands 1, 2, and 3 represent primo-vascular tissue, blood vessel, lymphatic vessel, respectively. Antibodies against Krt10 recognized the same 56.5-kDa band protein in all three tissues.

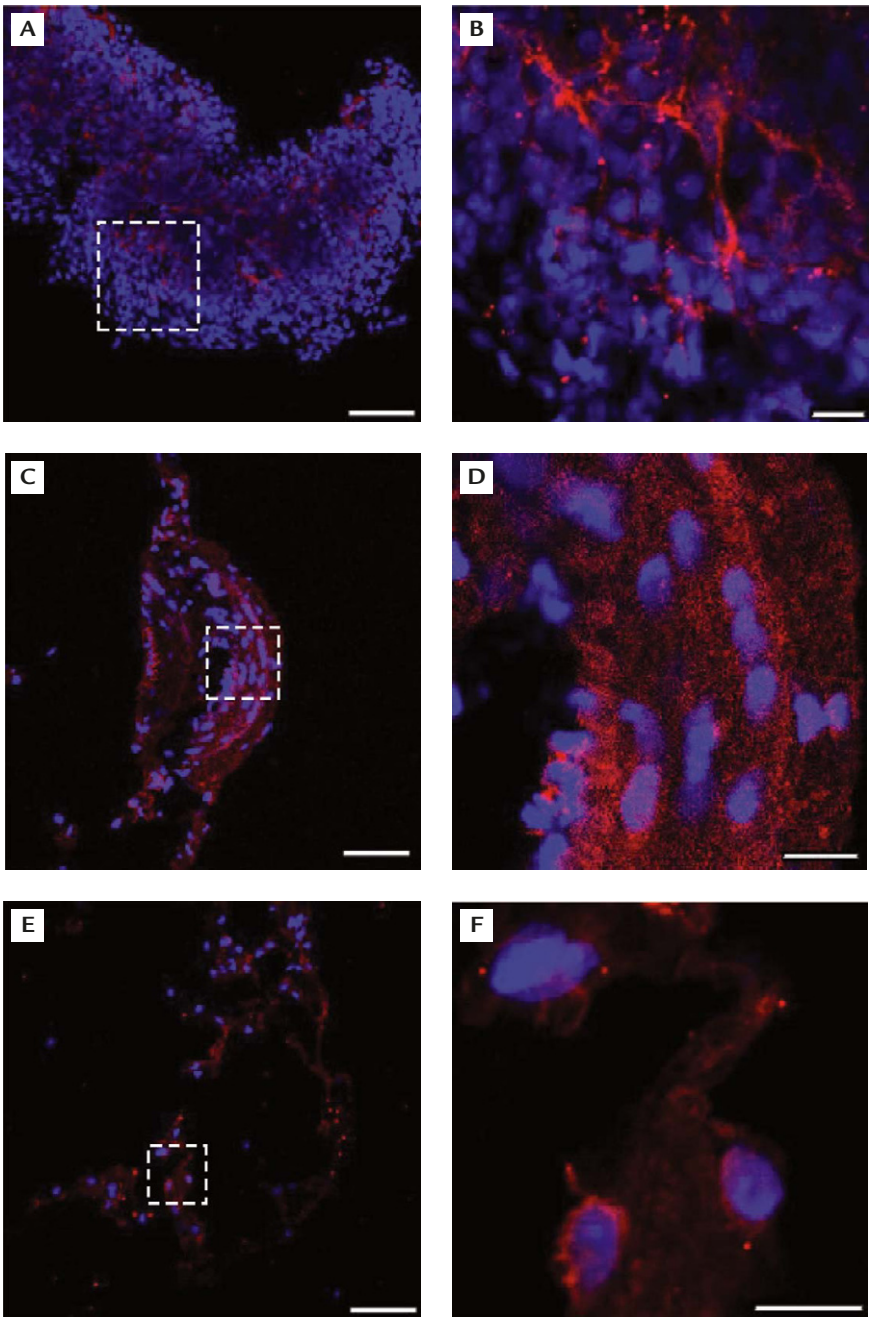

Figure 3 Immunoreactivity of Krt10 in primo-vascular tissues, blood vessels, and lymphatic vessels. (A) Distribution of Krt10 IR in a primo-node. Note the follicle-like structures (red) that cover several nuclei (blue) in the primo-vascular tissue. (B) The dotted rectangle of A is magnified. (C) Krt10 IR in the mesenteric artery. (B) The dotted rectangle in C is magnified. (E) Krt10 IR in a lymphatic vessel of the lumbar aortic lymph node. Krt10 and nuclei were stained red (Alexa Fluor 555) and blue (DAPI), respectively. (F) The dotted rectangle in E is magnified. The scale bar is 50 μm (A, C, E) or 10 μm (B, D, F).

for gastric secretion [25]. Krt10 most likely does not form intracellular keratin filaments as in other epithelial cells [26], because Krt10 IR is not obvious in the cytoplasm of primo-vascular tissue cells. Our results also revealed for the first time that Krt10 is expressed in blood and lymphatic vessels, but the expression pattern of Krt10 differs from that of primo-vascular tissues (discrete *vs.* diffuse).

Krt10 provides mechanical integrity to the internal extracellular matrix and performs regulatory functions [17]. In an early study, Kim [27] reported the primo-vascular tissue (Bonghan tissue) to be a regenerative organ. In support of this, primo-vascular tissues are known to be associated with tumor tissues [12,13] and stem cells [9,15]. Krt10 is also involved in cell proliferation. For example, Krt10 inhibits cell-cycle progression, keratinocyte proliferation [26,28], and keratinocyte turnover [17,29]. The regulatory functions of Krt10 in these three tissues require further study.

Until now, Krt10 has been identified in numerous epithelial cells and tissues, such as the cornea [21] and skin [30]. Krt10 is normally expressed in postmitotic keratinocytes [18] and can be used as a duct-specific marker in normal eccrine sweat glands [20]. The expression of Krt10 in primo-vascular tissues indicates that the tissues are closely associated with epidermal tissues such as the skin, which originates from the ectoderm. Further study is needed to better understand the embryologic origin of primo-vascular tissues.

Taken together, these results indicate the presence of Krt10 in primo-vascular tissues and in blood and lymphatic vessels. However, Krt10 is expressed in follicle-like structures inside the primo-vascular tissues, indicating that the structural and regulatory roles of Krt10 in the primo-vascular system differ from those in blood and lymphatic vessels.

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