Journal of Multidisciplinary Dental Research

Original Article

Morphological Study on Development of Vascular Endothelium in Chick Embryo

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ARTICLE INFO

Article history:
Received 05.07.2022
Accepted 28.07.2022
Published 27.12.2022

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https://doi.org/
10.38138/JMDR/v8i2.22.29

ABSTRACT

The vascular endothelium and hematopoietic stem cells are believed to derive from common progenitor cells called hemangioblasts. However, morphological study on the development of vascular endothelial cell has been scarce. The present study is intended to provide more morphological explanation of the differentiation process of the hemangioblast. We performed histological observation, using 10 four-day-old chick embryos. After fixation with 4% concentration paraformaldehyde, hematoxylin eosin staining, toluidine blue staining and immunostaining were performed in that order for observation of morphological changes of the detected cells. Blood corpuscles were aggregated in the dorsal aorta and the heart (that had been observed to beat in its preparation). Some red blood cells showed immunoreactivity to both anti-Flk-1(VEGFR2) and anti-CD31 antibody. Additionally, some blood cells had small vacuoles in the cytoplasm, and they fused to form one larger vacuole ultimately a balloon-like shape that was connected to endothelial cells. These cells were present in close proximity to the vascular endothelium. We reveal that some of the early embryonic blood cells differentiate into vascular endothelial cells.

Keywords: Vasculogenesis; Angiogenesis; Hemangioblasts; Vascular endothelial cells

1 INTRODUCTION

There have been a lot of studies about the development of vascular endothelium [1–3], and vasculogenesis and angiogenesis are two commonly known developmental processes. Angiogeneisis is known to be a process of sprouting of new capillaries from pre-existing blood vessels while vasculogenesis is a process of de novo formation of primitive vascular networks by differentiation, expansion [4,5], and coalescence. Vasculogenesis is also known to be involved in angiogenesis during the progression of periodontal disease [6] and tumor growth [7,8], but their detailed morphological study has been scarce. Both mechanisms might establish the rapid development of the blood circulating system in early embryonic stage.

There is increasing evidence that hematopoietic cells and endothelial cells might be derived from the common precursor cells which is called hemangioblasts [9]. It is thought that the hemangioblasts are derived from mesodermal cells and that they differentiate into blood-cell lineages or the endothelial cells [10]. However, it is morphologically not clear how the hemangioblast differentiates into the vascular endothelial cell.

Our previous histological study [11] reported that some blood corpuscles differentiate into endocardial endothelial cells. The endocardial endothelium is a monolayer of epithelium that is continuous to the vascular endothelium. Therefore, the formation of vascular endothelium and heart endocardium is believed to follow the same developmental process [12,13] and more morphological explanation is needed concerning this differentiation process. Here we report histological analysis of how the red blood cells change their form to vascular endothelial cells.

2 MATERIALS AND METHODS

In this study, twenty fertilized eggs were used. According to Hamburger and Hamilton stages [14], white leghorn chick embryos staged 4 were incubated at 38°C under 80% humidity. The embryos were explanted from yolk sac together with surrounding area vasculosa and whole bodies were fixed with 4% paraformaldehyde solution for both light-microscopic and electron-microscopic observations. Our experiments were approved by the Ethical Committee

of Animal Experiments of the Tsurumi University School of Dental Medicine and conducted in compliance with guidelines for Care and Use of Experimental Animals.

2.1 *Light microscopy*

2.1.1. *HE-Staining*

For light-microscopic observation, the whole embryo was removed and fixed with 0.1M Phosphate-buffered (pH7.3) 4% paraformaldehyde containing 15% saturated picric acid. The fixed samples were embedded in paraffin wax (Palaplast, Leica, Richmond) after dehydration with ethanol. Paraffin sections were cut at a thickness of 6um of the chick embryos. These sections were stained with hematoxylin-eosin (HE).

2.1.2. *Transmission Electron Microscopy (TEM)*

The specimens were fixed with cacodylate-buffered glutaraldehyde solution. After dehydration, the samples were embedded in epoxy resin. Semithin sections were stained with 1% concentration toluidine blue.

2.1.3. *Immunohistochemistry*

For immunohistochemical identification of FLK-1(VEGFR-2) and CD31, the samples were stained by using the streptavidin-biotin-peroxidase complex (SAB) method[15]. Specific cellular staining was employed with mouse monoclonal antibodies IgG1 to FLK1 (Santa Cruz Biotechnology, TX), or CD31 (Dako, Copenhagen, Denmark). Paraffin sections for staining with primary antibodies were deparaffinized and blocked with 20% normal goat serum in phosphate-buffered saline (PBS). The sections were treated with primary antibodies at an appropriate dilution of each antibody for FLK-1 (1:500) or CD31 (1:40) in 1% bovine serum albumin (BSA; SIGMA-Aldrich, St. Louis, MO) in PBS with Triton X-100 (WAKO, Tokyo, Japan) overnight. The sections were rinsed with PBS without Triton X-100. The sections were incubated with secondary antibodies which is in biotinylated goat anti-mouse Ig (1:600: DAKO), diluted in 1%BSA in PBS. They were incubated at room temperature for 30min with peroxidase-conjugated streptavidin (DAKO), dilated 1:300. The reaction was visualized by using 0.025% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and 0.01%hydrogen peroxide in 0.05 M TRIS-HCL buffer (pH 7.3) for 5min. After counterstaining with hematoxylin, the samples were dehydrated and mounted in Permount (Fisher-Scientific, Fair Lawn NJ).

3 RESULTS

The blood-circulation system of the observed chick embryo had developed as early as at the age of four days (Figure 1 a) and at this stage, numerous red blood corpuscles were observed in various places of the chick embryo such as the heart and blood vessels including dorsal aorta, cardial vessels, small vessels and sinusoids in the liver. The nucleated blood corpuscles looking spindle- or oval-shaped (Figure 1b) were observed in some blood vessels. Balloon-shaped cells were in close proximity to the blood vessel wall (Figure 1b).

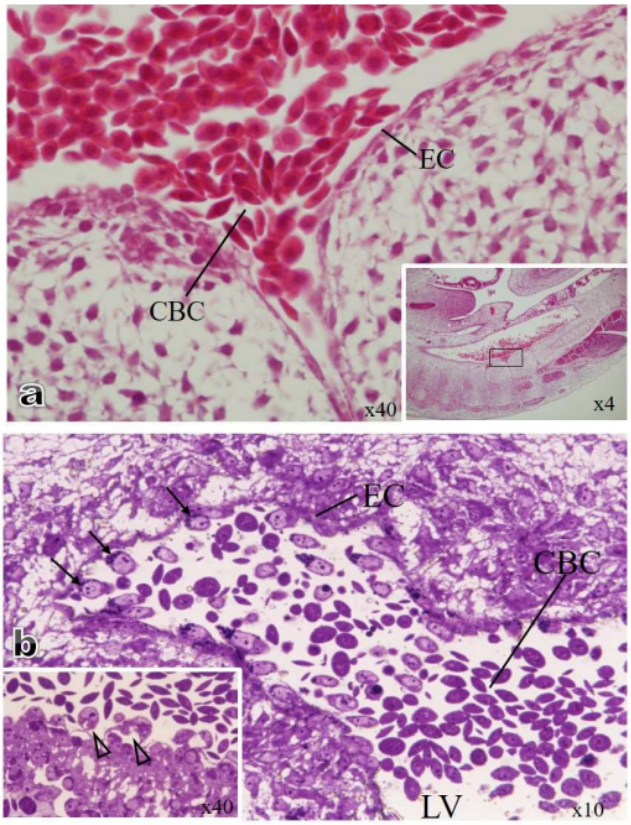

Fig. 1: Four-day-old chick embryo under light microscope. a) Many accumulated red blood cells are observed in the dorsal aorta at a magnification of 40. The rectangular inset shows a lower magnification (x4) of (a), b) In the blood vessels there can be seen many spindle-shaped or roundish undifferentiated vascular endothelial cells with a dark nucleus. The inset shows that crescent-shaped cells (pointed at by the hollow triangles) in close proximity to the vascular endothelium. CBC; clusters of blood corpuscles, EC; endothelial cells, DA; dorsal aorta, LV; liver,

FLK-1(VEGFR-2) positive cells were found in some blood cells of the blood vessels (Figure 2a), and some blood corpuscles were not stained with FLK-1 antibody. In addition, CD31-positive cells were found in some blood cells and CD31 immunoreaction was also identified composing the vascular endothelial cell-chains (Figure 2b).

In the sinusoid of the liver, undifferentiated vascular endothelial cells in which the cytoplasm of some blood cells were deeply stained and changed from spindle-shaped, and cells with vacuoles in the cytoplasm close to the blood vessel wall were observed (Figure 3a-d). The vacuoles gradually appeared in the cytoplasm (Figure 3a, b) and it was observed that some undifferentiated vascular endothelial cells, which had coalesced these vacuoles, were present near the vessel

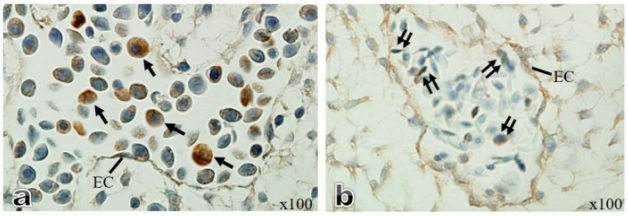

Fig. 2: Immunostaining for FLK-1 (VEGFR2) and CD31
a) The arrows are pointing at Flk-1 (VEGFR2) immunoreactive cells in the blood vessels. These positive cells are either spindle-shaped or roundish, b) The double arrows indicate CD31 positive cells in the blood vessels.

walls (Figure 3b). In addition, dome- and crescent- shaped cells appeared to be continuously gathered so as to cover the inner surface of the blood vessels (Figure 3c, d).

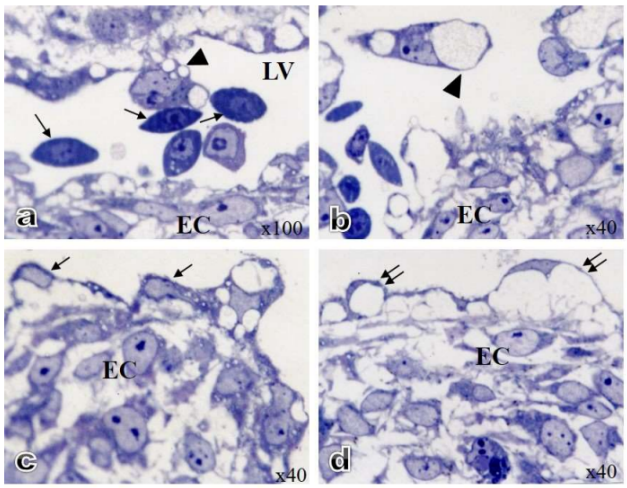

Fig. 3: Morphological differentiation process from precursor cells into endothelial cells
a) Blood cells with a deeply stained cytoplasm (arrows) and cells with small vacuoles in the cytoplasm (black triangle) are observed in the blood vessels of the liver, b) Enlarged vacuoles are seen in the cytoplasm and so is the morphological change of the blood cell, c) Undifferentiated vascular endothelial (arrows) cells have a balloon-like shape and located close to the inner surface of the vascular endothelium, d) Crescent-shaped undifferentiated vascular endothelial cells (double arrows) are seen lining the inner surface of the vascular endothelium.
EC; endothelial cells, LV; liver

4 DISCUSSION

The blood-vessel formation is an important factor in wound healing, tumor development and deterioration of periodontal disease. Although many studies have reported on the development of vascular endothelial cells, morphological evidence of differentiation process of vascular endothelial cells is little available. Hematopoietic cells and endothelial cells are thought to be derived from the same progenitor cell called hemangioblast [9,16]. Hemangioblast differentiates into angioblast and becomes vascular endothelial cells.

It is believed that the hemangioblast is required for vascular formation, and at an early stage, growth factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), that are known to have proliferative and migratory effects on the endothelial cells are of great significance [17]. VEGF is also considered to be involved in the maintenance of periodontal tissue health, progression and healing of periodontal disease [6].

In a previous report of ours, we have reported that some of the blood corpuscles changed their forms from roundish cells to endocardial endothelial cells.

The endocardium is covered with a single layer of vascular endothelial cells and exists spreading like a film. The present study shows that the formation of vascular endothelium is similar to endocardial endothelium. We would call these cells undifferentiated vascular endothelial cells due to their potential to differentiate into vascular endothelial cells. There are many reports on the development of molecular biological endothelial cells [18,19], but few reports are seen on their histological examination.

Flk-1 (VEGFR2) is important for endothelial cell differentiation and CD31 is also crucial to cell adhesion [20]. In the present study, immunopositive reactions of FLK-1 and CD31, which are important for blood vessel formation, were detected. These positive cells are supposed to differentiate into vascular endothelium.

5 CONCLUSION

The present morphological study suggests that some early embryonic blood cells differentiate into vascular endothelial cells.

ACKNOWLEDGMENTS

I am grateful to Dr. Kenjiro Wake for his collaboration in the early stages of this work.

Conflict of interest: None

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