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An efficient and non-enzymatic method for isolation and culture of endothelial cells from the nidus of human cerebral arteriovenous malformations

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HIGHLIGHTS

- Notch-1 signaling pathway in endothelial cells from AVM nidus was activated.
- Hypoxic endothelial cells from AVM nidus had angiogenic potentials.
- We created a non-enzymatic method to isolate endothelial cells from AVM nidus.

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ABSTRACT

In this report, we describe an efficient and non-enzymatic method for isolating and culturing endothelial cells (ECs) from the nidus of surgically resected arteriovenous malformation (AVM) specimens. These cultured cells possessed typical phenotypic markers (i.e. von Willebrand factor and CD34), as well as morphological and ultrastructural characteristics of ECs. However, they had activated Notch-1 signaling, which plays a critical role in the development of AVM. The present study suggests that hypoxic endothelial cells from the nidus of human cerebral arteriovenous malformation (CAVMECs) have angiogenic potentials, as our data showed that VEGF gene expression and cell proliferation were more evident with prolonged hypoxia. In our study, we successfully used the vascular tissue explants adherent method to isolate and culture CAVMECs with high purity. This may prove to be a useful tool for studying the molecular mechanisms that mediate abnormal vessel development and maintenance in AVM.

1. Introduction

Many previous studies [2,6] have reported the successful isolation and culture of endothelial cells (ECs) from human cerebral arteriovenous malformations (CAVM), using the enzyme digestion method. This approach involves trituration, filtration, enzymatic digestion, and gradient centrifugation, which was originally used for the isolation of cerebral microvascular endothelial cells, as previously described [8,9]. Unfortunately, this method was unable to identify the region of the arteriovenous malformation (AVM) (i.e. nidus, feeding arteries, draining veins, or perinidal parenchyma) from which the ECs were derived.

The AVM nidus is an abnormal vascular structure consisting of tortuous arteries and dilated veins, with a macroscopically visible and bigger caliber size compared to perinidal and normal cerebral microvessels. The diameter of the arterioles, venules, and capillaries in the perinidal brain tissue is 30–150 μm (normal arterioles, 15–100 μm), 50–300 μm (normal venules, 10–150 μm), and 30–300 μm (normal capillaries, 3–12 μm), respectively [1,3]. However, after enzyme digestion isolation employing filtration or gradient centrifugation [8,9], the diameter of residual vessels is generally no more than 250 μm. Therefore, cells obtained by this method mainly contain ECs from perinidal or normal microvessels in addition to ECs from the AVM nidus. Furthermore, due to the larger caliber size, it takes a relatively long period of time to homogenize and digest abnormal vessels from the AVM nidus, during which mechanical injury and chemical damage of digestive enzymes may cause marked structural and functional changes to the ECs.

To circumvent the shortcomings of the aforementioned approach, we created a less destructive method with greater tissue

Abbreviations: AVM, arteriovenous malformation; CAVM, cerebral arteriovenous malformation; ECs, endothelial cells; CAVMECs, endothelial cells from the nidus of human cerebral arteriovenous malformation; PBS, phosphate buffered saline; α-SMA, α-smooth muscle actin; vWF, von Willebrand factor; GFAP, glial fibrillary acidic protein; NICD, intracellular domain of Notch.

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purity, the vascular tissue explants adherent method, to isolate and culture ECs from the nidus of surgically resected AVM specimens. Using this technique, vascular tissue explants were obtained from thin-walled deformed vessels from the nidus of AVMs to avoid contamination of nonendothelial cells, ECs of perinidal microvessels, normal cerebral microvascular ECs surrounding the AVM, and ECs of feeding arteries and draining veins. To the best of our knowledge, this study shows for the first time changes in VEGF gene expression and levels of proliferation of cultured CAVMECs with prolonged hypoxia.

2. Materials and methods

2.1. Patient and tissue specimens

From December 2009 to November 2010, surgical resection was performed on 8 patients with AVM in the Neurosurgery Department of The General Hospital of Shenyang Military Region. This prospective study on patients was specifically comprised of 3 males and 5 females, aged 17–42 years (average 34.4 years). The patients commonly presented with headaches, seizures, hemorrhaging, and focal neurological deficits. Two patients had a history of administering anticonvulsant medication for at least one month. One patient had a positive family history. The clinical course of these selected cases ranged from 1 day to 2 years (average length was 15.2 months), and the mean diameter was 5.1 cm (range, 3.1–6.4 cm). Patients who had received gamma knife irradiation or embolization were excluded. The experimental protocol was approved by the Human Subject Review Committee of The General Hospital of Shenyang Military Region (China). All subjects provided written informed consent before participating in the study.

2.2. Immunohistochemistry

Surgically resected AVM specimens were immediately placed in ice-cold Hank's balanced salt solution (Life Technologies) and repeatedly rinsed to remove surrounding gliotic tissues. Adherent blood cells, thrombi, and calcified tissues were removed under a dissecting microscope. Deformed vessels from the nidus of CAVMs were chosen, including those with thin-walled vessels of 0.8–1.5 mm in diameter that had good flexibility and were not affected by burning of the intraoperative bipolar coagulator. Microvessels from fragments of brain tissue adherent to these CAVMs were obtained by the method described by Lamszus et al. [4]. Notch signaling plays a critical role in arteriovenous cell fate determination during vascular development, and is implicated in vascular malformations. Therefore, we performed immunohistochemistry on deformed vessels from the nidus of CAVMs, as well as from microvessels obtained from fragments of brain tissue adherent to these CAVMs. The vessels used for immunohistochemistry were initially fixed in 10% buffered formalin phosphate and embedded in paraffin. Sections were deparafinized and hydrated in PBS, incubated for 30 min in 1% hydrogen peroxide to inhibit endogenous peroxidase, and incubated in 2% goat serum in PBS for 20 min to block nonspecific binding. The primary antibodies used were against NICD (1:500; intracellular domain of Notch, Abcam), which is the activated form of Notch-1 [10]. Sections were then incubated with the primary antibody for 60 min at room temperature. After washing in PBS, sections were then incubated for 30 min with a biotinylated goat anti-rabbit antibody (1:200; Sigma), and were treated with avidin biotin peroxidase complex (Sigma) for 1 h, followed by treatment with diaminobenzidine solution (Sigma). PBS was used instead of the primary antibody as negative control. Samples were counterstained with hematoxylin.

2.3. Isolation and culture of CAVMECs

The deformed vessels from the nidus of CAVMs were cut longitudinally, and vascular intimal tissue explants of about 0.5 mm^2 were stripped off under an operating microscope. The vascular intimal tissue explants were plated in a 25 ml tissue culture dish with the endothelial cell surface facing the bottom of the dish. Cells were cultured in M199 media (Gibco BRL) supplemented with 25 μg/ml heparin, 200 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator (5% CO_2, 95% air). It took about 60 h for ECs to adhere to the dish, when smooth muscle cells, fibrocytes and other tissues were easy to be rinsed off because they had not adhered to the dish. Even though there were small numbers of confluent smooth muscle cells and fibrocytes, they could be removed easily under an inverted microscope according to their morphological characteristics, as described by Thorin et al. [7]. The culture medium was changed every 3 days. Adherent cells formed a single layer, at which point they were suspended at a density of 10^5 cells/ml, inoculated in a 24-well plate, and cultured at 37 °C in the humidified incubator (5% CO_2, 95% air) for 48 h. According to the duration of hypoxia, the cells were cultured at 37 °C in a humidified incubator (5% CO_2 and 95% N_2) for 0, 2, 8, and 24 h.

2.4. Ultrastructure observation

Cells were fixed in 50.0 g L^{-1} glutaraldehyde for 2 h and in 10.0 g L^{-1} osmic acid for 1 h, dehydrated in acetone, and then embedded in an Epon 812 resin. Ultra-thin sections were obtained using an Ultracut LKBV ultramicrotome. The sections were examined under an EM400 ST transmission electron microscope (Philips).

2.5. Immunocytochemistry

Briefly, cytocentrifuged cell preparations were fixed in acetone, blocked in 5% goat serum, and incubated at 4 °C with the following commercially available primary antibodies: rabbit polyclonal anti-NICD (1:500; Abcam), anti-Flk-1 (1:100) and anti-Tie-2 (1:100) (Santa Cruz Biotechnology, Inc.), mouse monoclonal antibody of anti-CD34 (1:100; Santa Cruz), mouse anti-human von Willebrand factor (vWF) (1:100; Creative Diagnostics), glial fibrillary acidic protein (GFAP) (1:100; Dako North America, Inc.), and α-smooth muscle actin (1:100; Sigma) for 48 h. The slides were

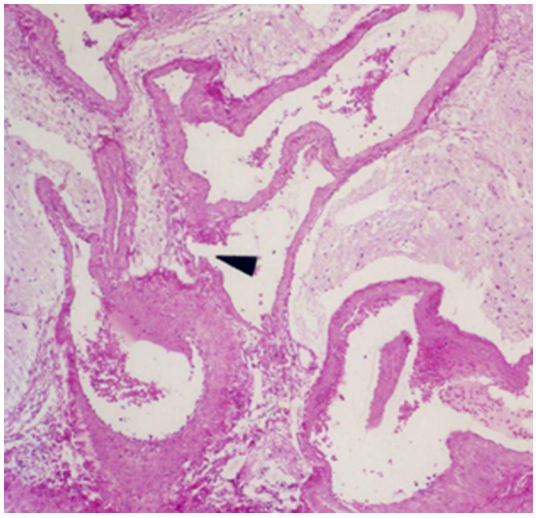

Fig. 1. HE staining of specimens. The deformed vascular wall from the nidus of CAVMs has a lot of collagen fibers, lacks smooth muscles and elastic fibers, and has an incomplete wall (triangle, 40×).