의학석사 학위논문

Relationship between pericyte and endothelial cell in retinal neovascularization

- a histological and immunofluorescent study of retinal angiogenesis -

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- 망막 혈관신생의 조직학 및 면역형광법 연구 -

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이 논문을 의학석사 학위논문으로 제출함

2016년 10월

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2016년 12월

위 원 장 이 왕 재 (인)

부위원장 박 규 형 (인)

위 원 유 영 석 (인)

Abstract

Relationship between pericyte and endothelial cell in retinal neovascularization

- a histological and immunofluorescent study of retinal angiogenesis -

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Purpose: To evaluate the relationship between pericyte and endothelial cell in retinal neovascularization by histological and immunofluorescent study.

Methods: Oxygen induced retinopathy (OIR) model was induced using C57BL/6J mice. The cross sections of enucleated eyes were processed with hematoxylin & eosin. Immunofluorescent staining of

pericytes, endothelial cells, and N-cadherin was performed. Microfluidic devices were created and human retinal microvascular endothelial cells, human brain microvascular endothelial cells or human umbirical vein endothelial cells and human placenta pericyte were mixed and co-cultured.

Results: Unlike the tree-layered vascular plexus found in normal mouse retinal angiogenesis, distinguishing neovascular tuft extending into the vitreous is identified in the OIR model. Neovascular tufts and three-layered vascular plexus were both covered with pericyte in the OIR model. In this pathologic vascularization, N-cad, known to be crucial in intercellular contact was also present. Further evaluation using microfluidic in vitro model, successfully developed microvascular network of endothelial cell covered by pericyte, mimicking normal retinal angiogenesis within 6 days.

Conclusions: Not only normal vasculature, but also pathologic neovascularization show pericytes covering endothelial cells. Factors involved in endothelial cell-pericyte interaction can be evaluated as an attractive novel treatment target. These future studies can be performed with microfluidics system, which can shorten the time and provide three-dimensional structural evaluation.

keywords : Retinal neovascularization; Endothelial cell; Pericyte; Oxygen induced retinopathy; Microfluidics;

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Chapter 1. Introduction

1.1. Study Background

Angiogenesis plays an important role not only in embryonic development and cancer development, but also in retinal diseases [1]. Pathological retinal neovascularization is a well-known cause of sight-threatening diseases including diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration [2]. Thus in recent years many efforts have been made to understand the mechanism of angiogenesis [3, 4]. The findings of different participating cell types and molecules have suggested multiple attractive therapeutic targets [5-7]. One of the first treatment targeting factors associated with retinal angiogenesis, anti-vascular endothelial growth factor (VEGF) is widely used as standard of treatment in proliferative retinal diseases [8]. However, still a number of patients not responding well to anti-VEGF treatment exist and alternative treatment target should be investigated.

Recently pericytes are regaining attention for its role in vascular maintenance through endothelial cell-pericyte interaction [9]. Microscopically pericytes regulate vessel permeability, endothelial cell proliferation, and vessel diameter through direct contact with endothelial cells and various paracrine signals [10, 11]. Many pathways including platelet-derived growth factor (PDGF) B/PDGFβ -receptor, angiopoietin-1/tie-2, sphingosine-1-phosphate-1 has been reported to be involved in pericyte-endothelial cell signaling [12-14]. In addition, cell adhesion molecules, such as N-cadherin are also known to be crucial in blood-brain barrier and intercellular contact

[15, 16].

Among the various experimental models developed for investigating retinal neovascularization, oxygen-induced retinopathy (OIR) model for retinopathy of prematurity is widely used [17]. Using this model diverse factors including epidermal growth factor receptor, insulin-like growth factor binding protein-3, NADPH oxidase have been reported to be involved in retinal angiogenesis [18-20]. However, the exact mechanism of pathologic neovascularization in OIR model is still poorly understood, especially for the pericyte and endothelial cell relationship.

1.2. Purpose of Research

We underwent the present study to assess the relationship between pericyte and endothelial cell in retinal angiogenesis through histological and immunofluorescent study.

Chapter 2. Materials and Methods

2.1. Animals

C57BL/6J mice, purchased from Central Lab. Animal (Seoul, Korea), were used for the animal experiments in this study. The care, use, and treatment of all animals in this study were handled according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and the guideline established by the Seoul National University Institutional Animal Care and Use Committee. The mice were kept in standard 12-hour dark-light cycle and the cage temperature was maintained at 23℃± 2 ℃. All mice were sacrificed by cervical dislocation, and the eyes were enucleated for the analyses.

2.2. Oxygen-induced Retinopathy

OIR was induced in new-born mice as described by Smith *et al* [21, 22]. Briefly, newborn mice were exposed to hyperoxia (75 ± 0.5% oxygen) from postnatal day (P) 7 to P12, and were returned to room air (21% oxygen) until P17, when the retinas were assessed for maximum neovascularization. Oxygen was checked twice daily with an oxygen analyser (Miniox I; Cremona, Italy).

2.3. Histologic Evaluation

The enucleated eyes were fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin. Then, the paraffin blocks were trimmed and sectioned with rotatory microtome (Leica RM2255, Nussloch, Germany). The sections were floated on the 45℃water bath for 2-3

minutes. After the straightening of folds in the tissue, the sections were taken on a adhesive coated slides and allowed to cool on slide holder. The dried slides were deparaffinized with xylene, dehydrated with alcohol, and stained with hematoxylin and eosin (H&E).

2.4. Immunofluorescent Staining

Phosphate was used for rinsing. After blocking by DAKO® protein block serum-free (X0909, Dako, CA, USA), the samples were incubated with the primary antibodies or conjugated antibodies at 4 °C overnight and some samples with the second antibodies on the next day. The following antibodies were used for cryostat sections: Alexa Fluor® 488 isolectin GS-IB4 conjugate (1:500 dilution, Life Technologies, CA, USA), Anti-neural/glial antigen 2 (NG2) (1:500 dilution, Millipore), anti-human CD235 (N-cadherin) (1:500 dilution, Biolegend, CA, USA), Alexa Fluor® 594 goat anti-rabbit IgG (1:200 dilution, Life technologies) Alexa Fluor® 647 donkey anti-mouse IgG (1:200 dilution, Life technologies). Nuclei were counterstained with 4′,6-diamidino-20 phenylindole (DAPI). The sections were observed with a fluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan).

2.5. Microfluidics

Microfluidic devices were fabricated using the replica molding method [23]. Briefly, A master mold with positive relief patterns of photoresist, SU-8 (MicroChem, USA), on a silicon wafer was prepared by photo-lithography. Microfluidic devices were fabricated out of polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, USA) using soft lithography and replica molding. Human retinal microvascular endothelial cells (HRVECs), human brain microvascular endothelial cells (HBMECs) or human umbirical vein endothelial cells

(HUVECs) were mixed with human placenta pericyte in the ratio of 5:1 then attached on patterned fibrin surface of the microfluidic device. Human dermal fibroblast embedded in fibrin gel were patterned on the lateral micro channel.

Chapter 3. Results

3.1. Development of Retinal Angiogenesis in Mice

Normal retinal structure was evaluated by histological examination with H&E stain and immunofluorescence. During the first week the superficial plexus began to develop by radial pattern from the optic nerve. By P8, the superficial plexus was completely extended into the retinal periphery and the formation of deep plexus started. Vertical vessels ran down from the superficial plexus to the deep plexus in the outer plexiform layer. At P12 the intermediate plexus could be found in the inner plexiform layer and three-layered vascular plexus continued to develop since then (Fig. 1).

3.2. Pericytes Exist in Neovascular Tufts

Immunofluorescence study to determine whether structural differences associated with endothelial cell and pericyte exist between normal mouse retina and OIR model retina was performed. Immunostaining was tested with various available primary antibodies targeting pericyte markers, including NG2, platelet-derived growth factor receptor-β (PDGFRβ)

Unlike well organized layered structure in normal mouse retina, the distinguishing pathologic unorganized vascularization, called neovascular tufts, extending into the vitreous were found in the OIR model (Fig. 2A).

Examination of normal retina revealed pericytes covering endothelial cells in all three layers of vascular plexus (Fig. 2B). In OIR model not only vascular plexus but also neovascular tufts were covered by

pericytes (Fig 2C).

3.3. N-cadherin Expression

Further immunofluorescence study to evaluate interaction between pericytes and endothelial cells was performed targeting N-cadherin, known to be crucial in endothelial-pericyte adhesion. N-cadherin signal was detected both in intraretinal vascular plexus and neovascular tufts (Fig. 3).

3.4. Microfluidics

Microfluidic model mimicking in vivo neovascularization was created. The device is composed of a central blood vessel channel, two adjacent media channels, and the outer fibroblast channel (Fig. 2A). After 6 days, we successfully created vascular networks, covered by pericytes on the abluminal endothelial surface wich was confirmed by immunofluorescence study (Fig. 2B).

Chapter 4. Discussion

Retinal neovascularization is a cardinal feature in many common diseases leading to blindness, including diabetic retinopathy, age-related macular degeneration, and retinal vein occlusion [2, 24]. The patients with these diseases can lose their independence in daily activities, and treating and caring them causes substantial economic burden on society [25]. To prevent many patients from vision-loss, it is important to understand retinal vascularization and find a novel treatment.

Pericytes are known to be important in the formation, maturation, and stabilization of the microvasculature [26, 27]. Microcirculation in the nervous system, where vascular permeability is regulated, have a higher pericytes to endothelial cells ratio than that in higher permeable vessels, thus probably provides greater integrity to the retinal vasculature [28, 29]. The physical contact of pericytes with endothelial cells includes the adhesion protein N-cadherin [15].

In our study, unlike well-organized three-layered vascular plexus in the normal mouse retina, OIR model showed multiple neovascular tufts extending into the vitreous. Proliferating vessels in human retina have been suggested to have barrier disruption and to be more permeable than normal retinal vasculature [30]. Against our expectations, not only intraretinal vasculature, but also the tufts consisted of endothelial cells surrounded by adjacent pericytes.

Although the pathologic microvessels are found to be covered and stabilized by the pericyte, barrier function attributed by endothelial cell and pericyte contact seems not as good as in normal retina,

leading to leakage [21]. To find the difference in intercellular junction between the normal and permeable vessels, we further evaluated N-cadherin. According to previous reports, N-cadherin was diffusely expressed on the surface, while VE-cadherin was localized at cell junctions [31]. This nature of N-cadherin, not making clusters, made the examination of immunofluorescence signal by microscopy difficult. Although weak, we could find N-cadherin signal both in intraretinal and neovascular tufts. Extended study of other intercellular contact molecules or paracrine factors between endothelial cell and pericyte which may attribute to vessel permeability is needed.

Existing but not immaculate pericyte and endothelial cell contact in retinal new vessels may also be an attractive new target for the treatment.

Valuable as OIR model is, preparing and staining is time consuming. Less than a week, we could successfully mimic retinal angiogenesis from the HRVECs and HUVECs by microfluidics system, a novel method of cell culture in vitro [32, 33]. This model has an advantage over conventional cell culture because it can generate three-dimension structure, much closer to in vivo structure [34]. With microfluidics system, we anticipate to evaluate the role of N-cadherin in vascularization more precisely, by co-culturing N-cadherin knock-out pericyte with normal endothelial cell or normal pericyte with N-cadherin knock-out endothelial cell.

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Figures

Figure 1. (A) The retinas of normal mice at P4-P26 were examined by Hematoxylin and Eosin (H&E) staining and were photographed under a microscope. G: ganglion cell layer, I: inner nuclear layer, O: outer nuclear layer. (B) The retinas of normal mice at P4-P26 were stained for endothelial cells with isolectin B4 (red) and for cell nuclei with DAPI (blue).

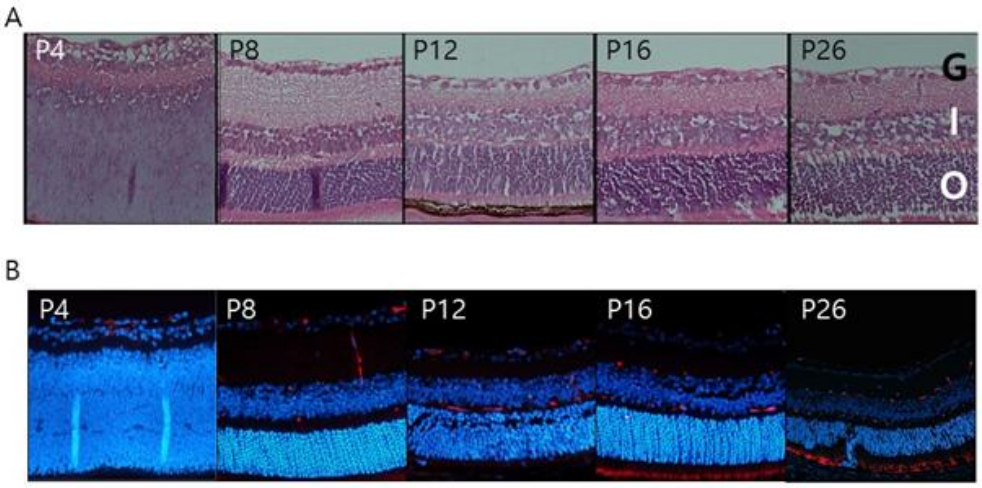
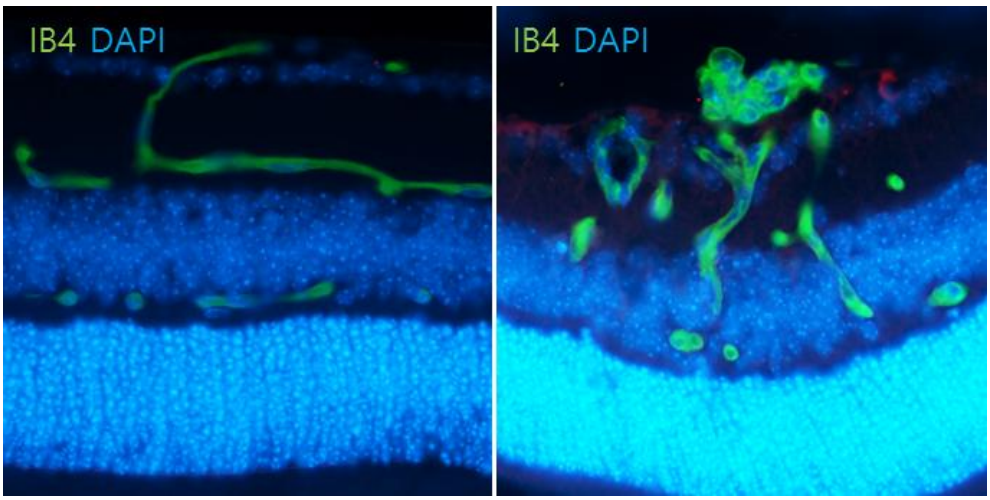


Figure 2. (A) The retinas of normal mice (left) and OIR model (right) were stained for endothelial cell with isolectin B4 (green) and for cell nuclei with DAPI (blue). Neovascular tuft extending into the vitreous is shown in OIR model. The retinas of (B) normal mice (C) and OIR model were stained for endothelial cell with isolectin B4 (green), for cell nuclei with DAPI (blue) and for pericyte with NG2 (red). Endothelial cell and pericyte both existed in neovascular tufts.

(A)



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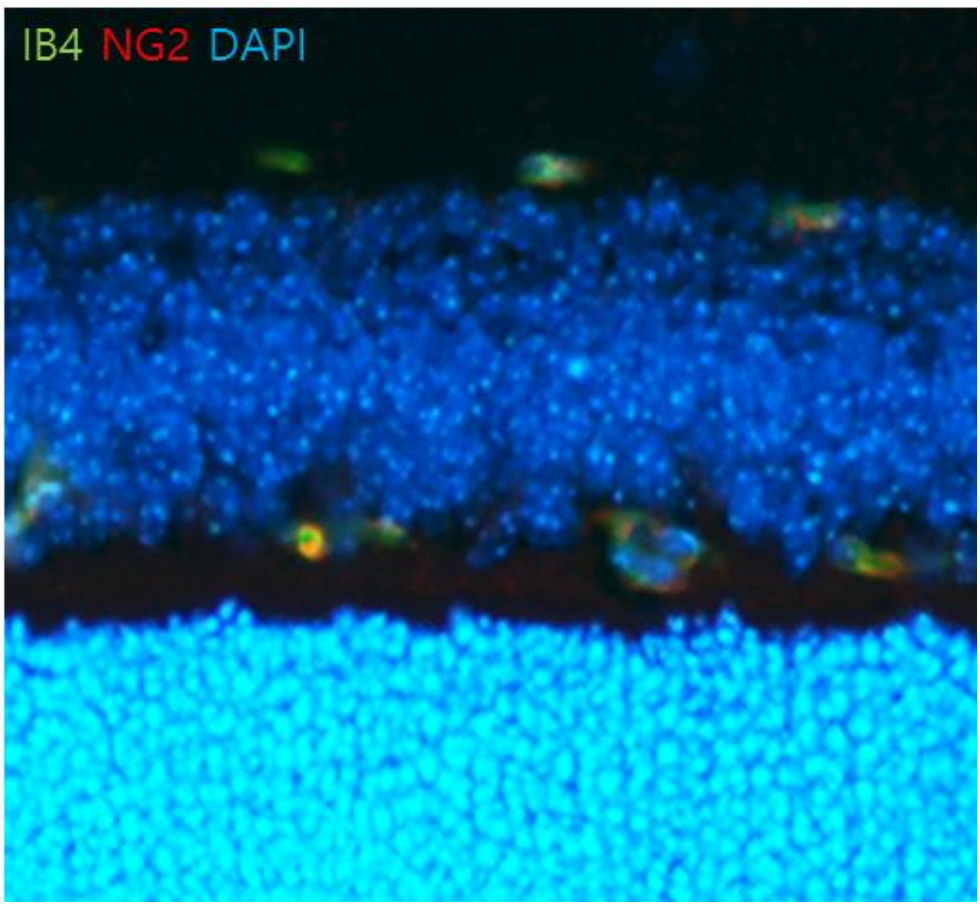

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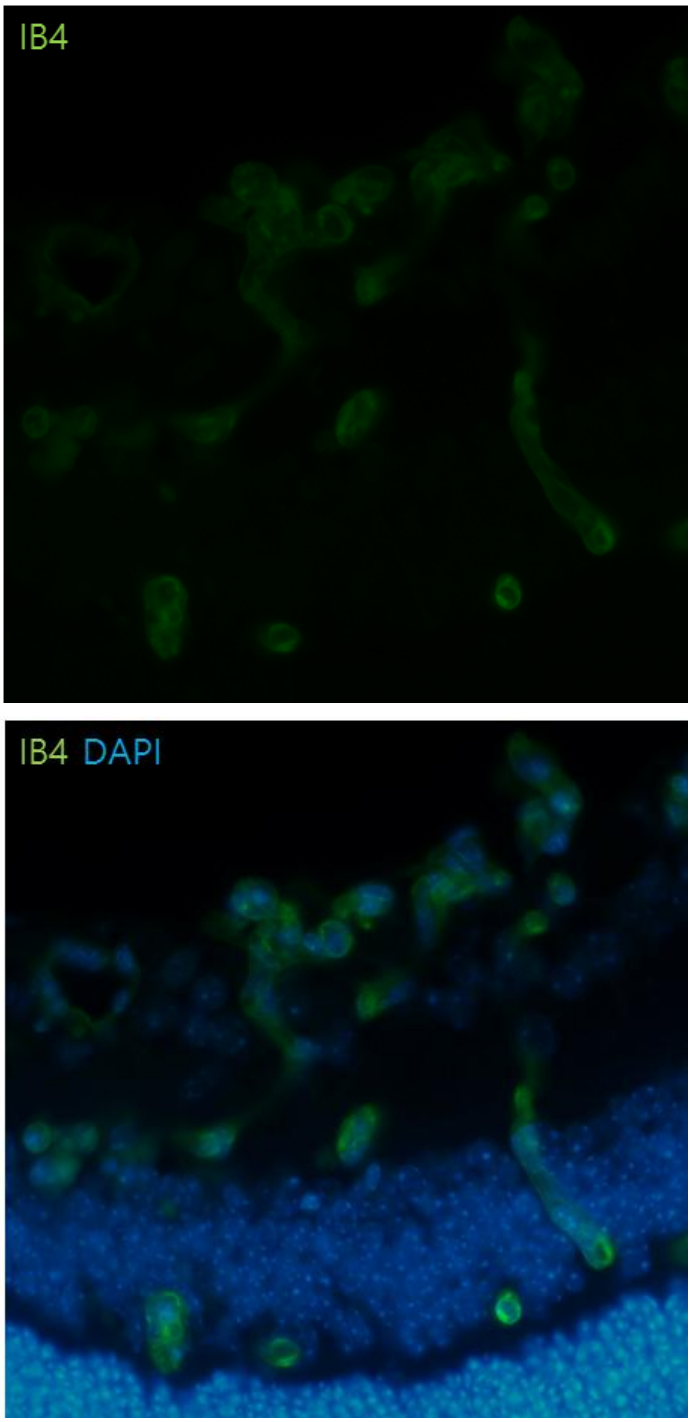


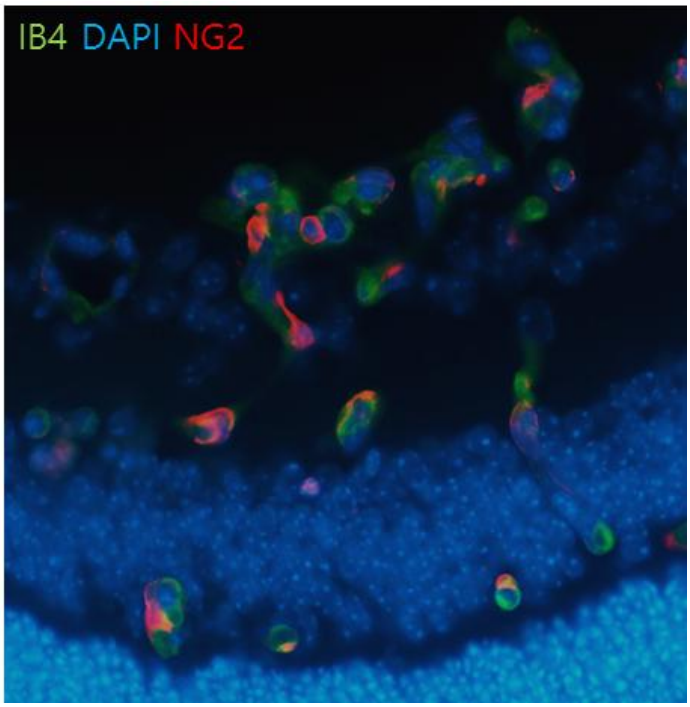
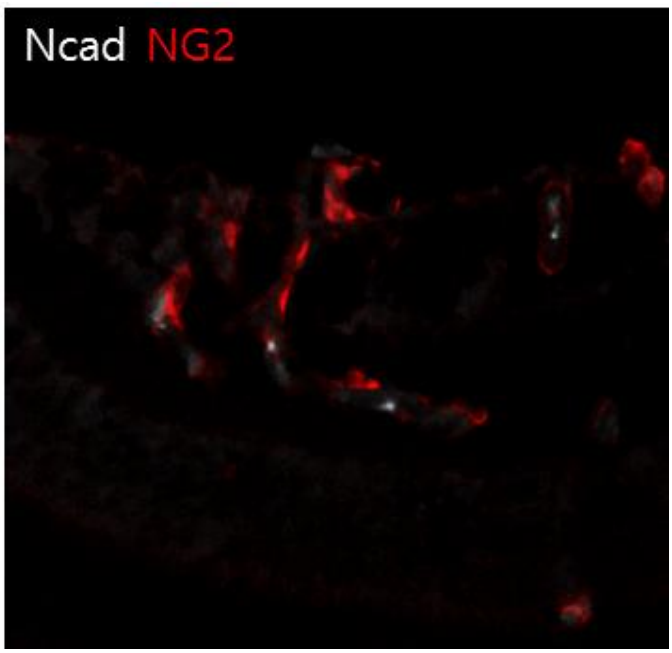
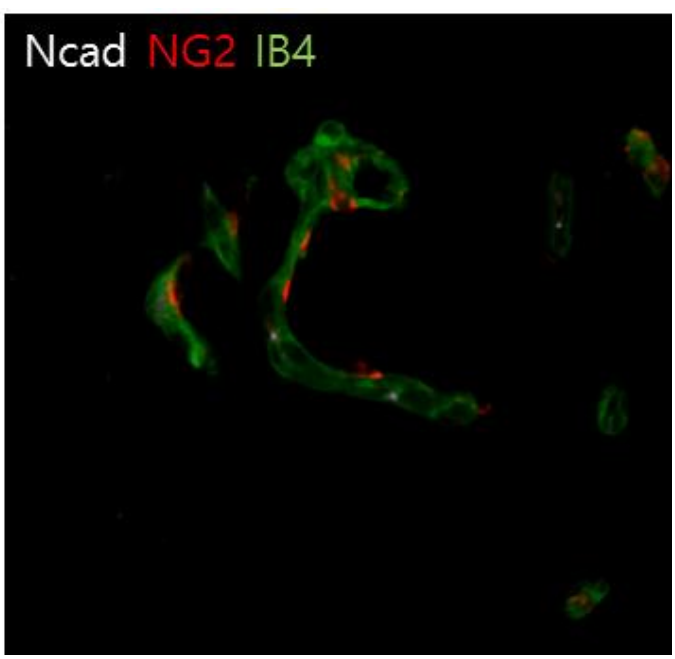

IB4 DAPI NG2

Figure 3. The retina of OIR model was stained for endothelial cell with isolectin B4 (green), for cell nuclei with DAPI (blue), for pericyte with NG2 (red) and N-cadherin (white).





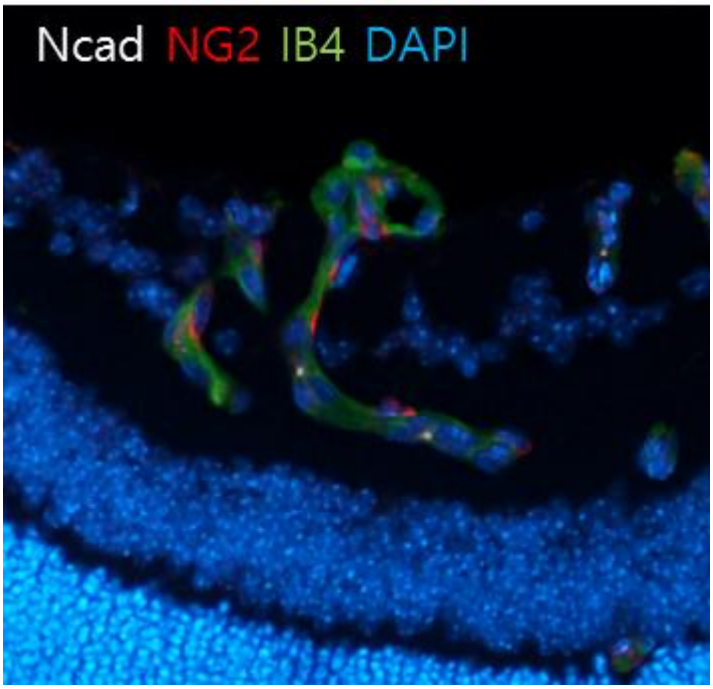
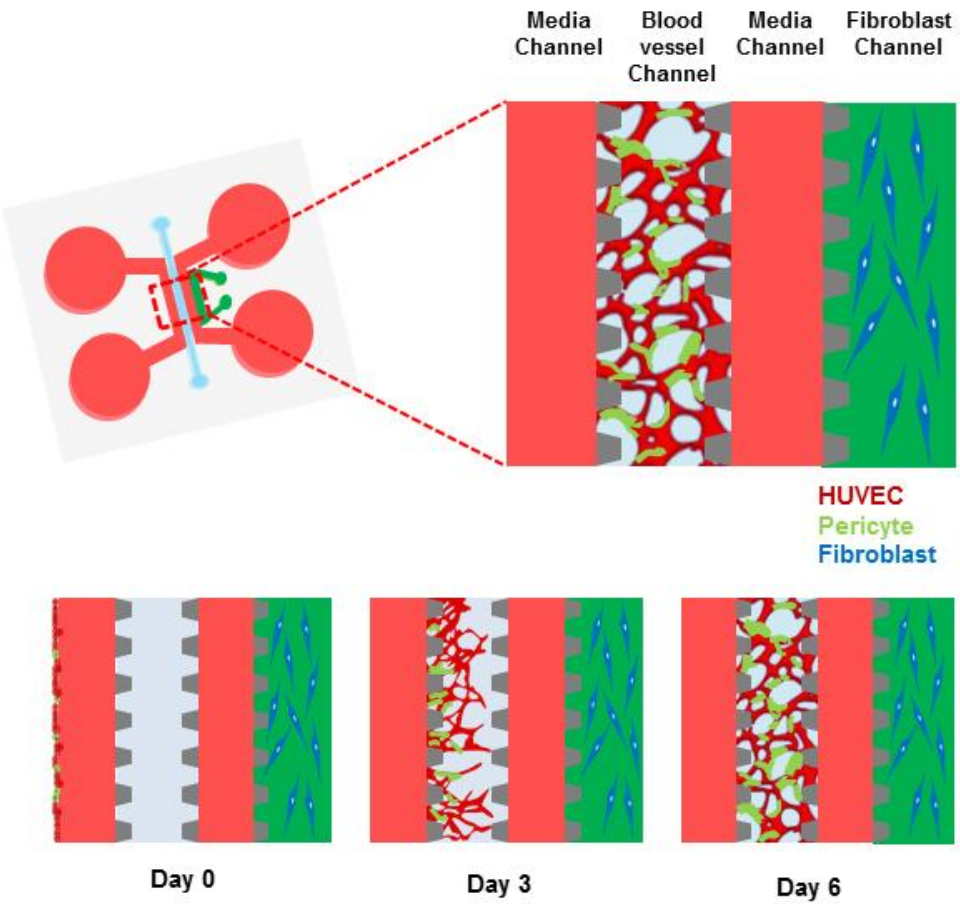
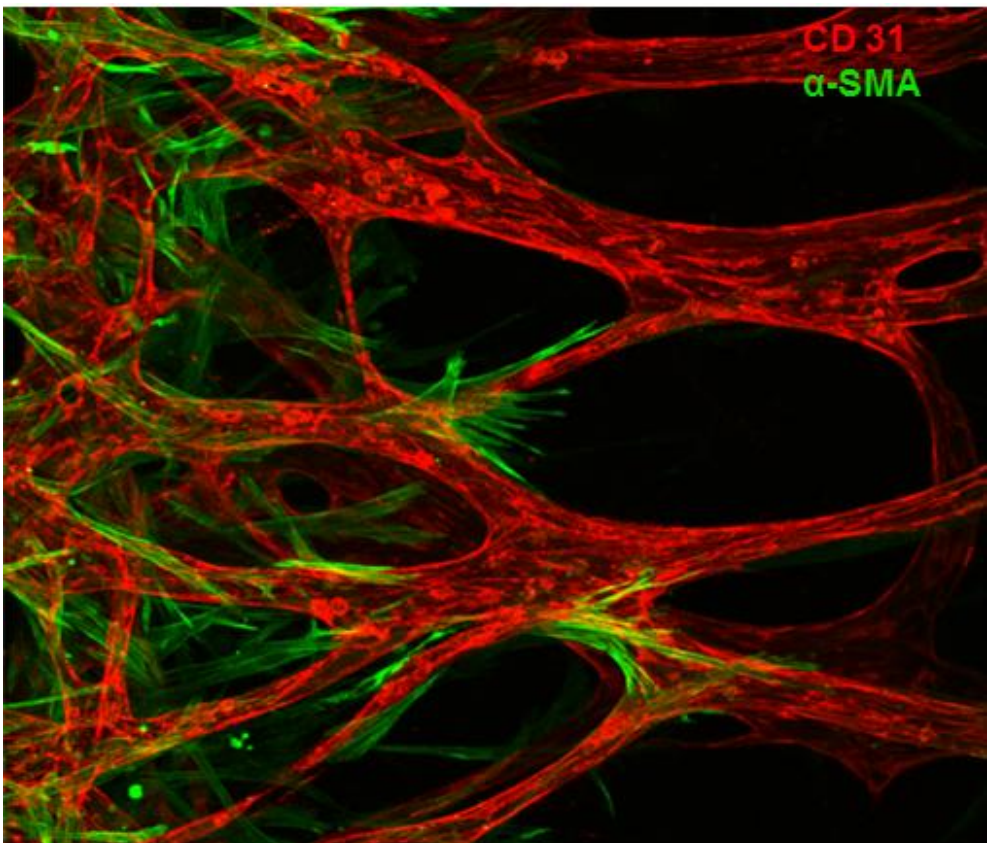

Ncad NG2 IB4 DAPI

Figure 4. (A) Scheme of microfluidic in vitro model that mimic developmental retinal angiogenesis. (B) The angiogenesis model form pericyte (green) covered microvascular network (red) within 6 days. (C) Collagen IV (red) is deposited between endothelial wall (white) and pericyte (green).

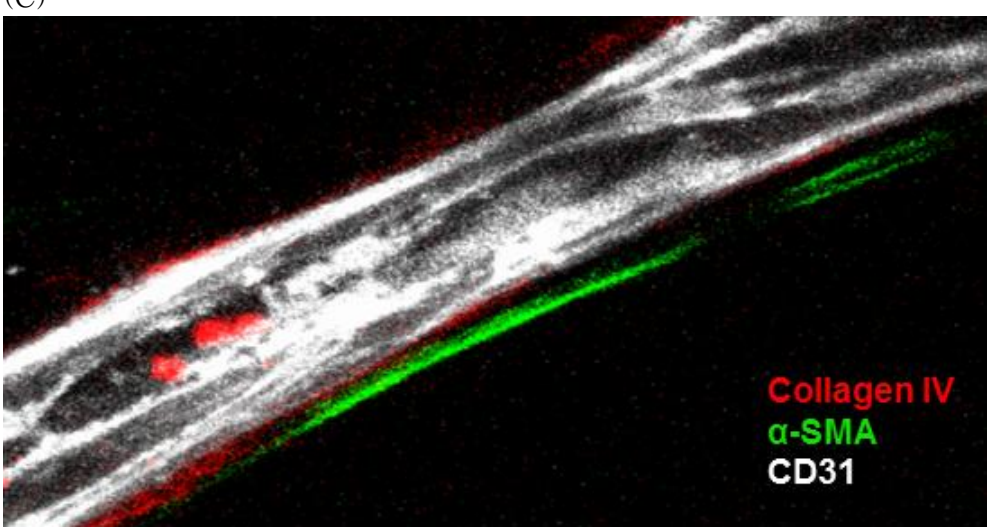
(A)



(B)



(C)



국문초록

망막 혈관신생에 있어서 혈관내피세포 및 혈관주위세포의 관계

-망막 혈관신생의 조직학 및 면역형광법 연구-

최세현

의학과 안과학

서울대학교 대학원

배경: 망막 혈관신생은 실명을 유발하는 주요 질환의 원인으로 잘 알려져 있다. 혈관신생에 관여하는 다양한 기전 및 인자를 대상으로 하는 치료법들이 연구 중에 있으며, 이러한 혈관신생에 있어서 혈관주위세포가 주요한 역할을 하는 것으로 알려지면서 새로운 치료 대상으로 주목 받고 있다. 기존의 망막 혈관신생 연구에서 산소 유도 망막증 생쥐 모델이 널리 사용되어 왔으며, 최근 미세유체 시스템이 개발되어 생체외 세포배양으로의 도입이 시도되고 있다.

목적: 이러한 생체내 및 생체외 모델에서 조직학 및 면역형광법 연구기법을 이용하여 혈관주위세포와 혈관내피세포의 관계에 대해 알아보고자 한다.

결과: 얕은 혈관 얼기, 깊은 혈관 얼기 및 중간 혈관 얼기의 삼층 구조

로 발달하는 정상 생쥐에서와 달리, 산소 유도 망막증 모델에서는 혈관 얼기에서 뻗어 나가는 형태의 신생혈관 다발이 망막 표면에서 관찰되었다. 이러한 신생혈관 다발은 누출이 쉽게 일어나기 때문에 혈관내피세포와 혈관주위세포를 통한 장벽 기능에 이상이 있을 것으로 예측하였으나, 본 연구에서는 혈관내피세포 주위로 혈관주위세포가 관찰되었다. 또한 두 세포의 상호작용에 관여한다고 알려진 N-카데린이 발현되는 양상을 보였다. 생체외 모델인 미세유체 시스템을 만들어 혈관내피세포를 혈관주위세포와 혼합하여 배양하였을 때 배양 6일 째 혈관주위세포가 혈관내피세포를 둘러싸는 양상의 정상혈관과 유사한 구조가 형성됨을 면역형광법으로 확인할 수 있었다.

결론: 망막의 병적 신생혈관에서도 혈관내피세포와 혈관주위세포가 함께 존재하는 것이 관찰되었다. 향후 이들 세포들간의 상호작용에 관여하는 인자들을 신생혈관 치료의 새로운 표적으로 연구할 수 있겠고, 그 과정에서 미세유체 시스템이 간편하고 효과적인 새로운 혈관신생 모델로 이용될 수 있겠다.

주요어: 망막혈관신생, 혈관내피세포, 혈관주위세포
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