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Fibrinogen stimulates in vitro angiogenesis by choroidal endothelial cells via autocrine VEGF

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Abstracts *Background:* The purpose of this study is to investigate the effect of fibrinogen on angiogenesis in vitro formed by cultured bovine choroidal endothelial cells (BCECs) and the involvement of vascular endothelial growth factor (VEGF) in this mechanism. *Methods:* For in vitro tube formation assay, BCECs were seeded on collagen gel containing fibrinogen (0–1.5 mg/ml). After 3 days of cultivation, the total length of the tubular structure was measured using Macscope Analyzer. Total RNA and conditioned media were collected after fibrinogen treatment and subjected to Northern and Western blot analyses, respectively. Transcription factor HIF-1α was also analyzed by Western blot analysis using cytosolic and nuclear fraction of BCECs. Involvement of VEGF in fibrinogen-dependent in vitro tube formation was evaluated using anti-VEGF neutralizing antibody or VEGF receptor 2-selective inhibitor (SU5416). *Results:* Formation of the tubular structure was enhanced 20~50 times in fibrinogen-containing gel in a concentration-dependent manner. The treatment of BCECs with fibrinogen resulted in a significant increase in VEGF gene and protein expression. Accumulation of HIF-1α protein in the nuclear fraction was also detected after the treatment with fibrinogen. Finally, fibrinogen-induced tube formation was significantly inhibited in the presence of anti-VEGF-neutralizing antibody (52.0% inhibition at the concentration of 1 μg/ml, $P<0.05$) or SU5416 (54.8% inhibition at the concentration of 3 μM, $P<0.05$). *Conclusions:* Extravasated fibrinogen might play an important role in the development of choroidal neovascularization associated with age-related macular degeneration, at least in part, through the function of VEGF in an autocrine manner. Transcription factor HIF-1 appears to be involved in fibrinogen-induced VEGF expression.

Introduction

Age-related macular degeneration (AMD) is the most common cause of severe visual acuity loss in patients over the age of 60 in developed countries [17]. Subfoveal choroidal neovascularization (CNV) is the major pathological feature of AMD that leads patients to the more severe stage of the disease. However, the precise mechanism of choroidal neovascularization is not fully clarified to date.

Neovascularization, the formation of new blood vessels from pre-existing vessels, plays a critical role during many physiological and pathological processes, such as inflammation, wound repair and tumor growth [11]. Folkman and Haudenschild [10] demonstrated that cultured capillary endothelial cells form tubular structures, and these tubes formed in vitro were also shown to be ultrastructurally similar to capillaries in vivo [31, 21]. It was also revealed that angiogenesis is regulated not only by the angiogenic growth factors such as basic fibroblast

growth factor (b-FGF) and vascular endothelial growth factor (VEGF), but also by the composition of the extracellular matrix (ECM) [12, 20]. Many investigators [16, 28, 36, 44] have shown that the ECM of endothelial cells is capable of influencing several aspects of cellular behavior including cell-to-cell and cell-to-ECM attachment, cell growth and migration.

The Blue Mountain Eye Study [37] found there to be a significant increased risk of AMD associated with increasing plasma fibrinogen levels. Additionally, it is reported that fibrinogen extravasates and deposits around the newly formed choroidal vessels of eyes with AMD [26, 35, 23]. The temporarily deposited fibrin matrix acts as a sealing matrix as well as the scaffolding for invading leukocytes and endothelial cells during the process of angiogenesis [42]. However, the molecular mechanisms underlying the association between extravasated fibrinogen and AMD are incompletely understood.

VEGF is known to be an endothelial cell-specific mitogen and an angiogenesis inducer released from a variety of cells [25]. The heterodimeric protein hypoxia-inducible factor consists of the constitutively expressed HIF-1β and the inducible protein HIF-1α [43]. HIF-1 is activated in response to several stimuli such as thrombin [15], advanced glycation end products [40] and insulin [41] in addition to hypoxic conditions [13]. However, to our knowledge, the association between fibrinogen and HIF-1 is still unknown.

VEGF could also cause vascular leakage, by which fibrinogen extravasates and forms a fibrinous bed providing additional scaffolding for the invasion process. Immunohistochemical analysis of surgically excised CNV membranes from eyes with AMD showed that the CNV area was strongly immunoreactive to VEGF, especially in transdifferentiated retinal pigment epithelial (RPE) cells [27]. We previously demonstrated increased expression of VEGF in CNV membranes in an experimental CNV model by immunohistochemical examination and in situ hybridization [22]. Considering these previous reports, it is reasonable to assume some association between fibrinogen, VEGF and the development of CNV in AMD.

In the present study, we demonstrated the promoting effect of fibrinogen on angiogenesis in vitro formed by choroidal endothelial cells, and possible involvement of VEGF in this effect. We also addressed the possible transcription factor mediating fibrinogen-induced VEGF gene expression. These in vitro studies will contribute to a better understanding of the role of extravasated fibrinogen on the progression of CNV membrane in AMD.

Materials and methods

Reagents

Human fibrinogen (fraction I, type IV) was purchased from Sigma (St. Louis, Mo.), and contaminating plasminogen was removed by lysine-Sepharose 4B column chromatography (Pharmacia Biotech, Uppsala, Sweden). A VEGF receptor-2 (VEGFR2)-selective inhibitor SU5416 was synthesized in our laboratory as previously described [2].

Antibodies

Agarose-conjugated rabbit anti-human VEGF (A-20), mouse anti-human VEGF (C-1) and rabbit anti-human HIF-1α (C19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Goat anti-human VEGF-neutralizing antibody and non-immunized control goat IgG were purchased from R&D Systems (Minneapolis, Minn.). Anti-phospho-specific ERK1(p44)/ERK2(p42) monoclonal antibody was from New England Biolabs (Beverly, Mass.). Non-phosphorylation-specific anti-ERK1 antibody was from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibody was from Amersham Corp. (Arlington Heights, Ill.).

Cell cultures

Primary cultures of bovine choroidal endothelial cells (BCECs) were prepared by homogenization and a series of filtration step as previously described for the isolation of retinal capillary endothelial cells [18]. Cells were maintained in Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), penicillin G (100 IU/ml) and streptomycin sulfate (50 mg/ml). Passage 5–10 cultures of BCECs were used in the experiments. In our research, we conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Capillary tube formation assay

Collagen gel containing 1 mg/ml of type I collagen (Koken Co., Ltd., Tokyo) was prepared in a 24-well plate (Nunc, Roskilde, Denmark) as previously described [18]. Fibrinogen plus collagen gel was prepared by adding various concentrations of fibrinogen to the control gel instead of distilled water. After gelatinization, BCECs (2×10^5 cells/1 ml DMEM) were seeded on each gel. The tubular structure was photographed under a phase-contrast microscope 3 days after seeding. Five fields per gel were randomly selected, recorded and the total length of capillary tubes per unit area (mm/mm^2) was measured using Macscope Analyzer (Mitani Corporation, Fukui, Japan).

Northern blot analysis

Subconfluent monolayers of BCECs in a 9-cm dish were starved with DMEM containing 1% FBS for 6 h and then incubated with various concentrations (0, 0.5, 1 and 1.5 mg/ml) of fibrinogen for 16 h. Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [3] and subjected to Northern blot analysis for VEGF gene expression. Radioactive VEGF cDNA probe [38] was generated using Amersham Multiprime labeling kits and [α-^{32}P]dCTP (NEN)]. The radioactivity of the hybridized signal was detected and quantified using a Fujix BAS2000 Bioimage Analyzer (Fuji Photo Film Co., Tokyo). Lane

loading differences were normalized by rehybridization with a radiolabeled 36B4 cDNA probe [38].

Preparation of conditioned media and cytosolic/nuclear protein

Subconfluent BCECs were starved with DMEM containing 1% FBS for 6 h. After starvation, the medium was changed to fresh DMEM containing 1% FBS with either vehicle or fibrinogen at a concentration of 1 mg/ml. Forty-eight hours after stimulation, supernatants were collected and concentrated five-fold using a centrifugal filter device (Millipore, Bedford, Mass.) with a 10,000-MW cutoff. Cytosolic and nuclear protein was isolated as previously described [19] after 12 h stimulation with fibrinogen or hypoxic condition (1% O_2).

Immunoprecipitation and Western blot analysis

Five hundred microliters of 5-fold concentrated conditioned media were immunoprecipitated with agarose-conjugated rabbit anti-human VEGF antibody (A-20) and subjected to 15% SDS-PAGE after the treatment with 2-mercaptoethanol. Proteins were transferred to nitrocellulose filters (BioRad, Hercules, Calif.). The membrane was incubated with mouse monoclonal anti-human VEGF antibody (C-1), followed by horseradish peroxidase-conjugated secondary antibody (Amersham Corp.). Cytosolic and nuclear protein was subjected to 6% SDS-PAGE and analyzed for HIF-1α protein expression and localization. Visualization was performed using Amersham's enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times with similar findings, and results are expressed as means +SD. The Mann-Whitney U test was used, and a P value of less than 0.05 was considered significant.

Results

In vitro tube formation enhanced by fibrinogen

First, we examined the effect of fibrinogen on angiogenesis in vitro formed by cultured BCECs. The total length of the tubular structure was enhanced 20–50 times in fibrinogen-containing gels compared with that in gels without fibrinogen (Fig. 1). The promoting effect of fibrinogen on the tubular structure was dose dependent (0.5–1.5 mg/ml).

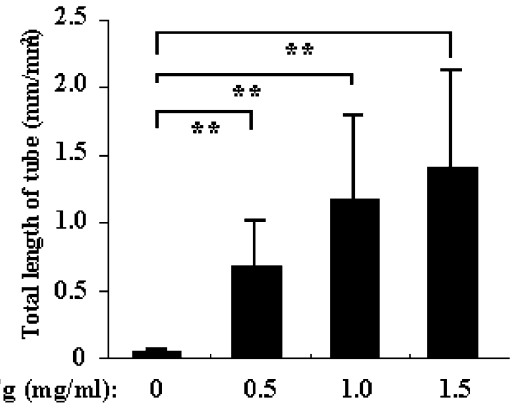

Fig. 1 The effect of fibrinogen on tube formation by BCECs. After 3 days cultivation with various concentrations of fibrinogen (*Fg*), the tubular structure was photographed under a phase-contrast microscope, and the total length of capillary tubes per unit area (mm/mm^2) was measured. The length of the tubular structure was enhanced 20–50 times in fibrinogen-containing gels compared with control. The effect was dose dependent (n=5, $^{**}P$<0.05)

Effect of fibrinogen on VEGF mRNA expression by BCECs

Although the angiogenic effect of fibrinogen has been reported previously, the exact mechanism is not fully defined. Thus, to make certain whether fibrinogen induces VEGF mRNA expression in BCECs, starved BCECs were stimulated with 0, 0.5, 1 or 1.5 mg/ml fibrinogen for 16 h. As shown in Fig. 2, fibrinogen-enhanced VEGF mRNA expression by BCECs increased in a dose-dependent manner (3.9-fold increase at 1 mg/ml fibrinogen, P<0.05; 5.3-fold increase at 1.5 mg/ml fibrinogen, P<0.05). These results suggest that VEGF, at least in part, mediates fibrinogen-dependent angiogenesis.

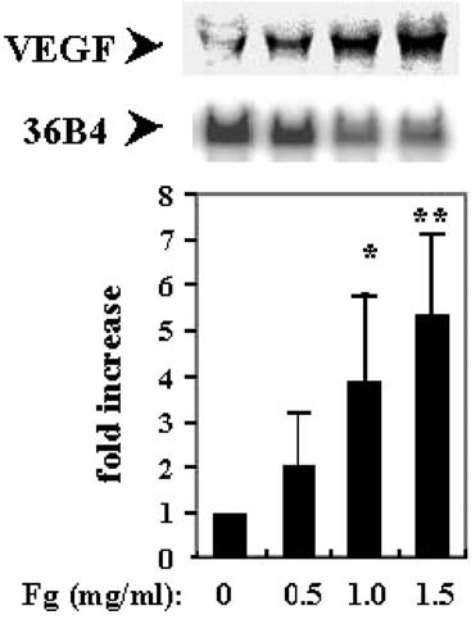

Fig. 2 The effect of fibrinogen on VEGF mRNA expression by BCECs. Subconfluent BCECs in a 9-cm dish were starved with DMEM containing 1% FBS for 6 h and then stimulated with 0, 0.5, 1 or 1.5 mg/ml fibrinogen (*Fg*) for 16 h. Total RNA was isolated and subjected to Northern blot analysis for VEGF mRNA expression. Fibrinogen significantly increased VEGF expression in BCECs in a dose-dependent manner (n=5, $^{*}P$<0.01, $^{**}P$<0.05)

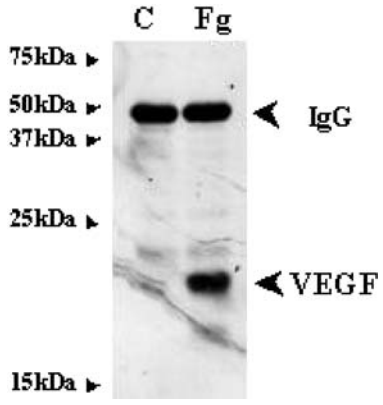

Fig. 3 Fibrinogen-dependent VEGF protein secretion by BCECs. Subconfluent BCECs were stimulated with vehicle (control: *C*) or 1 mg/ml fibrinogen (*Fg*) for 48 h, and then supernatants were collected. Samples were subjected to immunoprecipitation and Western blot analysis after treatment with 2-mercaptoethanol. A single band of approximately 20 kDa, which corresponds to the VEGF monomer, was detected in immunoprecipitates of fibrinogen-stimulated BCECs, but not in unstimulated BCECs

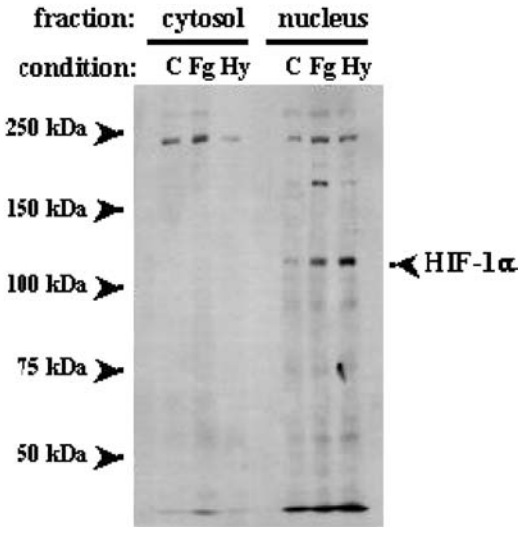

Fig. 4 Fibrinogen-induced nuclear accumulation of HIF-1α. BCECs were stimulated with vehicle (control: *C*), 1 mg/ml fibrinogen (*Fg*) or hypoxic condition (*Hy*) for 12 h. Isolated cytosolic or nuclear faction was subjected to 6% SDS-PAGE. Increased accumulation of transcription factor HIF-1α was detected in samples stimulated with fibrinogen or hypoxia as compared with control

Effect of fibrinogen on VEGF protein secretion by BCECs

To determine whether VEGF mRNA overexpression by BCECs was accompanied by an increase in protein synthesis and secretion into the conditioned media, the supernatants were collected and analyzed by Western blotting. Immunoprecipitates were electrophoresed after the treatment with 2-mercaptoethanol. As a result of immunoblotting, a single band of approximately 20 kDa appearing to correspond to the VEGF monomer was detected in immunoprecipitates collected from fibrinogen-stimulated BCECs, but not those from unstimulated BCECs (Fig. 3).

Nuclear accumulation of transcription factor HIF-1α

Transcription factor HIF-1α is known to be one of the key regulators of VEGF gene expression. We thus examined whether HIF-1α was activated in BCECs in response to fibrinogen treatment. As shown in Fig. 4, fibrinogen treatment resulted in increased accumulation of HIF-1α into the nuclear fraction. As a positive control, we also confirmed the effect of hypoxic cultivation on nuclear accumulation of transcription factor HIF-1α.

Involvement of VEGF in fibrinogen-induced tube formation

To confirm the participation of VEGF in fibrinogen-induced in vitro tube formation, we assessed the effect of anti-VEGF-neutralizing antibody. BCECs were seeded on

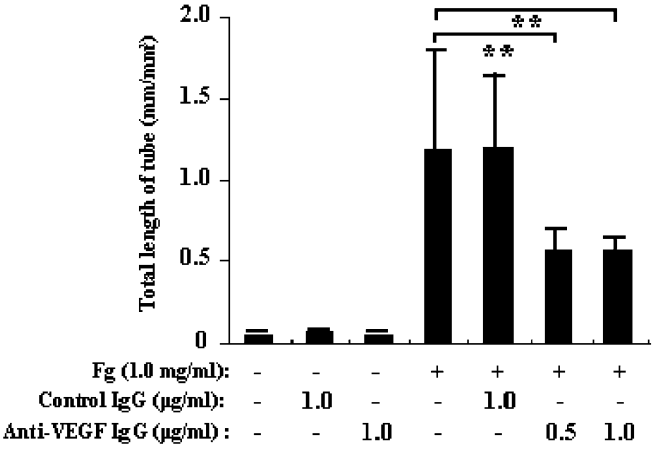

Fig. 5 Involvement of VEGF in fibrinogen-induced tube formation. Anti-human VEGF antibody (1 μg/ml) added to the medium did not have any effect on tube formation by BCECs in the absence of fibrinogen (*Fg*), while anti-human VEGF antibody (0.5, 1 μg/ml) added to the medium of fibrinogen-containing gels significantly inhibited the tube formation of BCECs. Non-immunized control IgG did not show any inhibition (n=5, $^{**}P$<0.05)

the collagen gels (without or with fibrinogen) and cultivated in the absence or presence of anti-VEGF-neutralizing IgG (0.5 and 1 μg/ml) or non-immunized control IgG (1 μg/ml). As shown in Fig. 5, fibrinogen-induced tube formation was significantly prohibited by anti-VEGF neutralizing IgG (51.9% inhibition at a concentration of 0.5 μg/ml, P<0.05; 52.0% inhibition at a concentration of 1 μg/ml, P<0.05). However, the tube formation in colla-

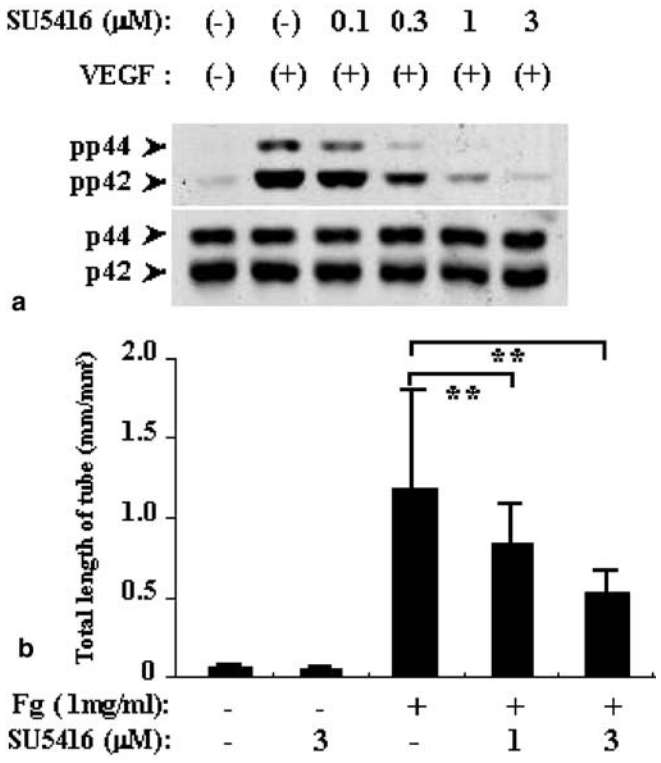

Fig. 6 The effect of SU5416 on tube formation by BCECs. The effect of a potent VEGFR-2 selective inhibitor SU5416 was assessed by its inhibitory effect on VEGF-dependent MAP kinase activation, a downstream target of VEGFR-2 (**A**). BCECs were stimulated with VEGF (25 ng/ml) for 5 min. Total cell lysates were immunoblotted with anti-phospho-p44/p42 MAPK antibody (*top*) and re-probed with anti-non-phospho-specific ERK1 antibody (*bottom*). The inhibitory effect of SU5416 on fibrinogen (*Fg*)-induced tube formation by BCECs was also analyzed (**B**), (n=5, $^{**}P<0.05$)

gen gel only was not influenced by anti-VEGF-neutralizing IgG, suggesting that VEGF protein in the medium was newly secreted VEGF from fibrinogen-stimulated BCECs. Non-immunized control IgG did not show any inhibitory effects on the tube formation in fibrinogen containing gel or in control gel.

Effect of SU5416 on fibrinogen-induced tube formation

Previous studies have shown that SU5416, a potent and a selective inhibitor of VEGFR-2, inhibited VEGF-driven mitogenesis of HUVECs [2]. To determine the receptor responsible for VEGF signaling in fibrinogen-induced tube formation by BCECs, we tested the effect of SU5416. To determine an appropriate concentration of SU5416, the inhibitory effect of VEGF-induced p44/p42 MAP kinase, one of the downstream targets of VEGFR-2, was examined. As shown in Fig. 6A, VEGF caused dramatic phosphorylation of p44/p42 MAP kinase, while SU5416 almost totally abrogated this effect at a concentration of 3 μM. SU5416 treatment also resulted in a dose-dependent inhibition of fibrinogen-induced tube formation in vitro at the corresponding concentrations, which inhibited MAP kinase phosphorylation (Fig. 6B).

Discussion

Fibrinogen has been considered as one of the factors that contribute to neovascularization in the areas of wound healing and tumor growth [4, 7]. An elevated serum fibrinogen level is associated with many diseases, including diabetes mellitus [14, 6], arthritis [24], cardiovascular risk factors [5] and AMD [37]. Fibrinogen deposit around the newly formed choroidal vessels of eyes with AMD has been reported [26, 35, 23].

In the present experiments, we clearly demonstrated that fibrinogen/fibrin enhanced in vitro angiogenesis formed by BCECs at concentrations of 0.5–1.5 mg/ml. Although many authors have shown that fibrinogen/fibrin induces in vitro [32, 33, 39] and in vivo [8] angiogenesis, this is the first report, to our knowledge, demonstrating the angiogenic effect of fibrinogen/fibrin using ocular vascular endothelial cells.

While the detailed mechanism of the angiogenic effect of fibrinogen is not fully clarified to date, angiogenesis is known to be regulated by angiogenic growth factors including b-FGF, VEGF and epidermal growth factor (EGF), as well as by the composition of ECM. Takei et al. [39] showed that the angiogenic effects of fibrin in vitro are mediated by b-FGF released from the endothelial cells in an autocrine manner. In the case of wound healing, it was also reported that platelet-derived growth factor (PDGF) released from platelets and macrophages might contribute to the proliferation of endothelial cells in the fibrin clot [30]. Accumulating studies, however, revealed VEGF as the most critical angiogenic growth factor because of its pattern of spatial and temporal expression that establishes its pivotal role both in physiological and pathological angiogenesis [9]. Our Northern and Western blot analyses demonstrated VEGF overexpression by BCECs under stimulation with fibrinogen, and the addition of anti-VEGF-neutralizing antibody or SU5416 in the media resulted in a strong suppression of fibrinogen-dependent tube formation by BCECs. These findings indicated that the angiogenic effect of fibrinogen on BCECs appeared to be mainly mediated through VEGF in an autocrine manner. Additionally, SU5416 suppressed fibrinogen-induced elongation of tube formation by BCECs as potently as VEGF-neutralizing antibody. SU5416 has an inhibitory effect on the autophosphorylation of receptor tyrosine kinase selectively for VEGFR-2 and is less potent for PDGF receptor β, but does not affect that of the bFGF receptor [2]. So this result is in agreement with the results obtained by other researchers using en-

dothelial cells from non-ocular organ origin, demonstrating that the VEGF-dependent angiogenic property of endothelial cells is mainly mediated through VEGFR-2.

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor involved in normal mammalian development and in the pathogenesis of several disease states. Activated HIF-1 induces the expression of genes involved in angiogenesis, erythropoiesis and glucose metabolism. We investigated the effect of fibrinogen on the nuclear accumulation of HIF-1 protein using BCECs. Although we could not exclude the involvement of transcription factors other than HIF-1, this is the first report demonstrating the possible role of HIF-1 mediating fibrinogen-induced VEGF gene expression.

The pathological features of CNV in AMD also have been studied to date. Considering the accumulating reports, diffuse thickening of Bruch's membrane seems to cause the degeneration of RPE cells and the occurrence of chronic inflammation in the eyes with AMD. As a result of this inflammation, growth factors including VEGF are considered to be produced by RPE cells, macrophages, retinal glial cells and choroidal endothelial cells [1, 29, 34]. This process could be the mainstream of the development of CNV. Additionally, extravasated fibrinogen/fibrin because of the local hyperpermeability would further increase the expression of VEGF by the cells, including choroidal endothelial cells. VEGF secreted from choroidal endothelial cells would act in an autocrine manner, and these phenomena could result in a vicious circle of CNV formation.

In summary, we partly approached the elucidation of the molecular mechanisms underlying the association between extravasated fibrinogen and AMD. Our studies may allow a better understanding of the role of extravasated fibrinogen/fibrin in the development of CNV, at least in part, through the function of VEGF in an autocrine manner.

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