N^{ε}-(Carboxymethyl)Lysine-Induced Choroidal Angiogenic Potential Facilitates Retinal Neovascularization in Advanced-Diabetic Rat *In Vitro*

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Abstract: N^{ε}-(Carboxymethyl)lysine (CML) over-stimulates choroidal neovascularization *in vitro* in streptozotocin (STZ)-diabetic rat. In this study, we investigated the effects of CML-induced choroidal angiogenic potential on retinal neovascularization during the course of STZ-induced diabetes in rats. Retinal and choroidal explants were isolated from the same eyeball of early-diabetic, advanced-diabetic and age-matched normal rats. When retinal explant was co-cultured with early-diabetic choroidal explant, the number of retinal microvessels was significantly decreased. When retinal explant was co-cultured with advanced-diabetic choroidal explant, the number of retinal microvessels was significantly increased. Anti-CML antibody blocked the retinal changes caused by co-culture with both early-diabetic and advanced-diabetic choroidal explant. Antibodies against TNF α and VEGF reduced the number of retinal microvessels in the co-culture with advanced-diabetic choroidal explant. These results indicate that the CML-induced choroidal activity is associated with the angiogenic actions of TNF α and VEGF on retinal capillaries in advanced diabetes. During the course of diabetic retinopathy, different actions on retinal neovascularization may operate.

Keywords: Proliferative diabetic retinopathy, retinal neovascularization, N^{ε}-(carboxymethyl)lysine, choroidal neovascularization, tumor necrosis factor α, vascular endothelial growth factor.

INTRODUCTION

Proliferative diabetic retinopathy is major complication of diabetes and a leading cause of blindness [1, 2]. Neovascularization of the retina plays a key role in visual impairment in proliferative diabetic retinopathy. The presence of persistently high blood glucose levels in patients and animal models with diabetes mellitus has been implicated in the development of degenerative microvascular changes during diabetic retinopathy [3-7]. One mechanism linking hyperglycemia with tissue damage in diabetic retinopathy is the formation of early products, such as Schiff base and Amadori rearrangement products, leading to the formation and accumulation of advanced glycation end products (AGEs) [5-8]. Vascular endothelial growth factor (VEGF) is upregulated in diabetic retinopathy and promotes blood retinal barrier breakdown and neovascularization [9]. Vascular lesions in the early stage of diabetic retinopathy in patients and animals are characterized by the presence of saccular capillary microaneurysms, pericyte-deficient capillaries, and obliterated and degenerated capillaries. Tumor necrosis factor (TNF) α is a proinflammatory cytokine that has been implicated in the pathogenesis of diabetic retinopathy [10, 11]. In addition, choroidal capillaries penetrate through Bruch's membrane and retinal pigment epithelium into the retina in patients with age-related macular degeneration and diabetic macular diseases such as retinal edema. These patients are characterized by increased permeability, thickening of basement membranes, capillary occlusion, microaneurysm formation, retinal detachment, and the appearance of neovascularization [12]. We have reported that N^{ε}-(carboxymethyl)lysine (CML) adduct, one of the major structures of AGEs [1], stimulates the release of angiogenic factors, such as TNF α, VEGF and platelet-derived growth factor (PDGF)-B from choroidal tissues of streptozotocin (STZ)-diabetic rats in the advanced stage [6, 13-15]. These CML-produced angiogenic factors cause overproduction of microvessels in cultured choroidal capillaries from early-diabetic, advanced-diabetic and aged-matched aged rats, compared with normal young controls. Onset times of budding in these choroidal capillaries were all similar in culture [1, 2]. Onset time of budding in retinal capillaries of the same eyeball causes more delay than that of choroidal capillaries [16, 17].

In this study, we investigated the interaction of newly formed choroidal and retinal microvessels in a co-culture system of retinal and choroidal explants from the same eyeball of early-diabetic and advanced-diabetic rats. This co-culture system may be useful as a model of retinal detachment in early and advanced diabetic retinopathy. The effects

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of CML, TNF α and VEGF on neovascularization of choroidal and retinal capillaries in advanced diabetes were investigated using the appropriate antibodies in the co-culture system to study participation of these factors for retinal neovascularization in the advanced diabetes.

MATERIALS AND METHODS

Animals

A single dose (0.1 mL/100 g body weight) of STZ-saline solution (60 mg/mL) was injected into Wistar strain male rats through the tail vein at 6 weeks of age (Kiwa Laboratory Animal Science Co., Wakayama, Japan). Blood samples (0.1-0.2 mL) were obtained from the orbital vein plexus of fed rats. Blood glucose levels were measured in these animals by the glucose oxidase method. We used early-stage STZ-diabetic rats at 9-14 weeks of age (body weight, 245-550 g; blood glucose level under fed condition, 18.6 - 37.6 mM) and age-matched control normal rats at 9-13 weeks of age (body weight, 338-529 g; blood glucose level under fed condition, 5.8 mM – 10.0 mM). We also used advanced-stage STZ-diabetic rats at 9 months of age (body weight, 335-480 g; blood glucose level under fed condition, 23.2 mM - 32.1 mM) and age-matched aged controls at 9 months of age (body weight, 730-775 g; blood glucose level under fed condition, 5.9 mM - 7.2 mM). Blood glucose levels of advanced-stage STZ-diabetic rats were similar to those of early-stage STZ-diabetic rats. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Faculty of Pharmaceutical Sciences, Hokuriku University.

Preparation of CML-Modified Proteins and Monoclonal Anti-AGE Antibody

Monoclonal anti-AGE antibody and CML-modified proteins were prepared and characterized as reported previously [18-20]. Briefly, splenic lymphocytes from Balb/c mice immunized with AGE-bovine serum albumin (BSA) were fused to myeloma P3U1 cells. The hybrid cells were screened, and two cell lines that had a positive reaction to AGE-BSA, but a negative reaction to BSA were selected through successive subcloning. Each antibody was produced in ascitic fluid of Balb/c mice and further purified by protein A Sepharose column chromatography. One of these antibodies, designated as 6D12, was used in the present study.

Preparation of Retinal and Choroidal Explants

Explants of retinal and choroidal capillaries of STZ-diabetic rats and age-matched normal rats were prepared as previously reported [1, 2, 16, 17, 21, 22]. Blood vessels, connective and fatty tissues outside the sclera were removed in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) containing 10% heat-inactive fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA), 160 U/mL benzylpenicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/mL streptomycin sulfate (Meiji Seika, Tokyo). After removal of the cornea, lens and corpus vitreum from the eyeballs, explants of retinal and choroidal capillaries were isolated from the same eyeball in 10% FBS-DMEM in the presence of antibiotics under an optical dissection microscope. The sizes of the isolated retinal and choroidal explants were approximately 0.64 mm^2 and 0.16 mm^2, respectively.

Culture of Explants of Retinal and Choroidal Capillaries

Retinal and choroidal explants were plated on fibrin gels prepared by mixing 3 mg fibrinogen (0.3 mL, Sigma), and 1 U thrombin (Sigma) per mL DMEM containing antibiotics in a 16-mm dish (Corning, Corning, NY, USA), by pipetting [1, 2, 16, 17, 21, 22]. In some experiments, a retinal explant and a choroidal explant isolated from the same eyeball were juxtaposed at a distance of approximately 5 mm on the fibrin gels in a 16-mm dish by pipetting; the number of microvessels budded from both explants could be easily counted under an inverted microscope. The same volume of a mixture of the above concentrations of fibrinogen and thrombin solution was overlaid carefully and allowed to solidify. The retinal and choroidal explants were cultured with 5% FBS-DMEM (0.5 mL) containing antibiotics and 300 μg/mL ε-aminocaproic acid in the presence or absence of anti-CML antibody (1 μg/mL), polyclonal goat anti-mouse VEGF neutralizing antibody (0.3 μg/mL, R & D Systems, Minneapolis, MN, USA), and/or polyclonal rabbit anti-mouse TNF α antibody (1:1000, Genzyme) at 37°C under 5% CO_2 and 95% air. The culture medium was replaced every other day. Numbers of microvessels newly budded from cultured choroidal explant and from cultured retinal explant were counted in images taken with an Olympus camera on a CKS inverted microscope (Olympus, Tokyo) at x 40 magnification. These numbers of budded microvessels from choroidal explant and from retinal explant were used as an index of *in vitro* choroidal and retinal neovascularization, respectively [1, 2, 16, 21].

Statistical Analysis

All values were expressed as means ± S.E.M. Differences between group data were evaluated by one-way analysis of variance followed by the multiple range test of Scheffé or by unpaired *t*-test at $P = 0.05$ or 0.01. A value of $P < 0.05$ was considered statistically significant.

RESULTS

1. Effects of Co-Cultured Retinal Explant Isolated from Eyeball of Diabetic Rat on Neovascularization in Choroidal Explant Isolated from the Same Eyeball (Fig. 1)

The onset times of buds of choroidal explant in co-culture with retinal explant isolated from eyeball of early-diabetic and age-matched normal rats were both at the second day in culture. The number of microvessels budded from choroidal explant of the early-diabetic eyeball was significantly greater than that of age-matched young normal control in the co-culture system (Fig. **1A**). The onset time of buds and the number of budding microvessels of choroidal explant from advanced-diabetic eyeball in co-culture with retinal explant from the same eyeball were similar to those of age-matched aged normal control (Fig. **1B**). The number of microvessels of choroidal explant from the advanced-diabetic eyeball in co-culture with retinal explant from the same eyeball was greater than that of the normal young control on 6^{th} day in culture (Fig. **1**).

2. Effects of Co-Cultured Choroidal Explant on Neovascularization of Retinal Explant Isolated from the Same Eyeball of Diabetic Rat (Fig. 2)

The onset time of buds of retinal explant from eyeball of early-diabetic rat in co-culture with choroidal explant from

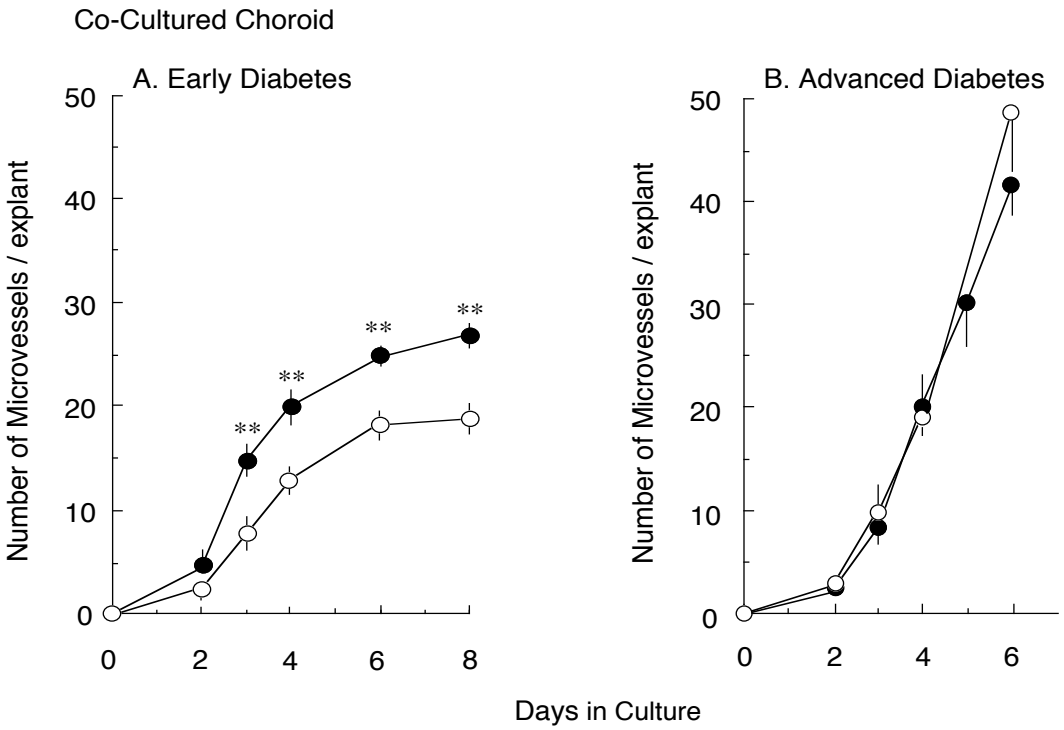

Fig. (1). Neovascularization of choroidal explant co-cultured with retinal explant from the same eyeball of early-stage (**A**: closed circle) and advanced-stage (**B**: closed circle) STZ-diabetic rat, age-matched young rat (**A**: open circle) and age-matched aged rat (**B**: open circle). Values represent means ± S. E. M. of 13-15 (**A**) and 13-16 (**B**) data. ** P < 0.01: Significantly different from the corresponding control value.

the same eyeball was delayed compared with that from eyeball of age-matched young normal rat, and was in the eighth day in culture. The number of budding microvessels in retinal explant of early-diabetic rat was significantly decreased compared with that of age-matched young control (Fig. **2A**). However, the onset time of buds of retinal explant from advanced-diabetic eyeball in co-culture with choroidal explant from the same eyeball was much earlier than that of age-matched aged control. The number of budding microvessels of retinal explant from the advanced-diabetic eyeball was greater than that of age-matched aged control in the co-culture system (Fig. **2B**).

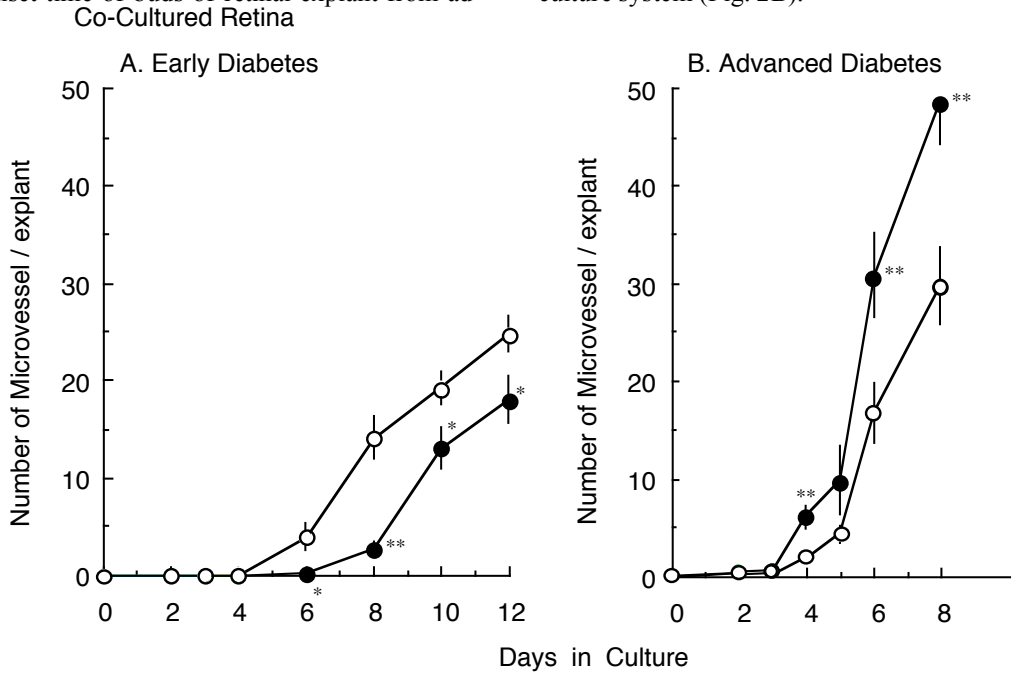

Fig. (2). Neovascularization of retinal explant co-cultured with choroidal explant from the same eyeball of early-stage (**A**: closed circle) and advanced-stage (**B**: closed circle) STZ-diabetic rat, age-matched young rat (**A**: open circle) and age-matched aged rat (**B**: open circle). Values represent means ± S.E.M. of 10-15 (**A**) and 8-12 (**B**) data. * P < 0.05, ** P < 0.01: Significantly different from the corresponding control value.

3. Effects of Anti-CML Antibody on Neovascularization of Retinal Explant from Eyeball of Early-Diabetic Rat in Co-Culture with Choroidal Explant from the Same Eyeball (Fig. 3)

Anti-CML antibody was administered to the culture media of retinal explant from early-diabetic eyeball (Fig. **3A**) and retinal explant from early-diabetic eyeball co-cultured with choroidal explant from the same eyeball (Fig. **3B**). Anti-CML antibody (1 μg/mL) significantly increased the number of budding microvessels of retinal explant from the early-diabetic eyeball on only the sixth day in culture (Fig. **3A**). Anti-CML antibody significantly reversed not only the delayed onset time of budding of retinal microvessels from the early-diabetic eyeball but also the decreased number of microvessels of retinal explant in the co-culture with choroidal explant from early-diabetic eyeball (Fig. **3B**).

4. Effects of Anti-CML Antibody on Neovascularization of Retinal Explant from Eyeball of Advanced-Diabetic Rat in Co-Culture with Choroidal Explant from the Same Eyeball (Fig. 4)

Anti-CML antibody was added to the culture media of retinal explant from advanced-diabetic eyeball (Fig. **4A**) and retinal explant from advanced-diabetic eyeball co-cultured with choroidal explant from the same eyeball (Fig. **4B**). The anti-CML antibody (1 μg/mL) did not affect microvessels in retinal explant from advanced-diabetic eyeball (Fig. **4A**). However, the anti-CML antibody (1 μg/mL) caused a delay of onset of buds and significantly decreased the number of budding microvessels of retinal explant of advanced-diabetic eyeball in co-culture with choroidal explant from the same eyeball (Fig. **4B**).

5. Effects of Anti-TNF α Antibody on Neovascularization of Retinal Explant from Eyeball of Advanced-Diabetic Rat in Co-Culture with Choroidal Explant from the Same Eyeball (Fig. 5)

Anti-TNF α antibody was added to the culture media of retinal explant from advanced-diabetic eyeball and retinal explant from advanced-diabetic eyeball co-cultured with choroidal explant from the same eyeball (Fig. **5**). Anti-TNF α antibody (1:1000) significantly decreased the number of buds of microvessels in retinal explant from advanced-diabetic eyeball from the 5th day in culture (Fig. **5A**). In addition, the TNF α antibody (1:1000) significantly decreased the number of buds of microvessels in retinal explant in co-culture with choroidal explant from the same eyeball from the 4th day in culture (Fig. **5B**). The inhibitory capacity of anti-TNF α antibody in retinal explant in co-culture with choroidal explant was greater than that in retinal explant alone.

6. Effects of Anti-VEGF Antibody on Neovascularization of Retinal Explant from Eyeball of Advanced-Diabetic Rat in Co-Culture with Choroidal Explant from the Same Eyeball (Fig. 6)

Anti-VEGF antibody was administered to the culture media of retinal explant from advanced-diabetic eyeball and retinal explant from advanced-diabetic eyeball co-cultured with choroidal explant from the same eyeball (Fig. **6**). Anti-VEGF antibody (0.3 μg/mL) did not affect budding of microvessels in retinal explant alone (Fig. **6A**), but caused a significant decrease in the number of buds of microvessels in retinal explant in co-culture with the corresponding choroidal explant (Fig. **6B**) on the 8th day in culture. The inhibitory action of anti-VEGF antibody on retinal neovascularization in the co-culture appeared more slowly than those of anti-CML antibody and anti-TNF α antibody (Figs. **4-6**).

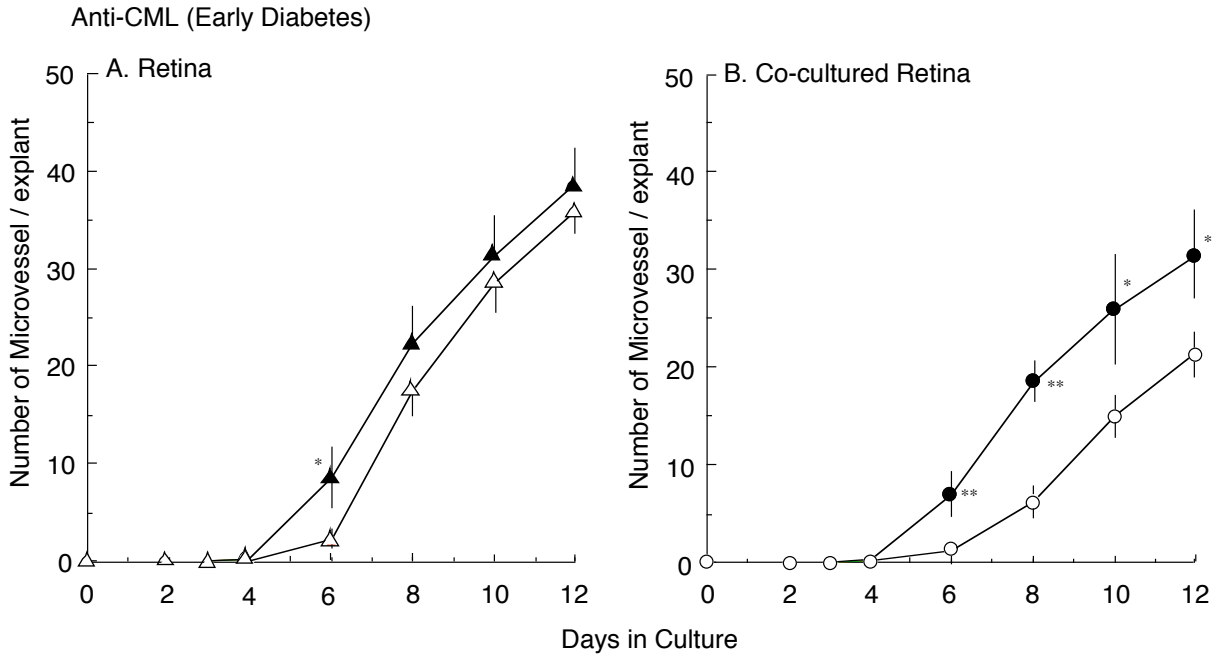

Fig. (3). Effects of anti-CML antibody on the neovascularization of retinal explant in early-diabetic rat in single culture (**A**: triangle) and in co-culture with choroidal explant from the same eyeball of early-diabetic rat (**B**: circle). Anti-CML antibody (1 μg/mL: closed symbol) or saline (open symbol) was added to the culture medium. Values represent means ± S.E.M. of 9-18 (**A**) and 11-31 (**B**) data. * $P < 0.05$, ** $P < 0.01$: Significantly different from the corresponding control value without antibody.

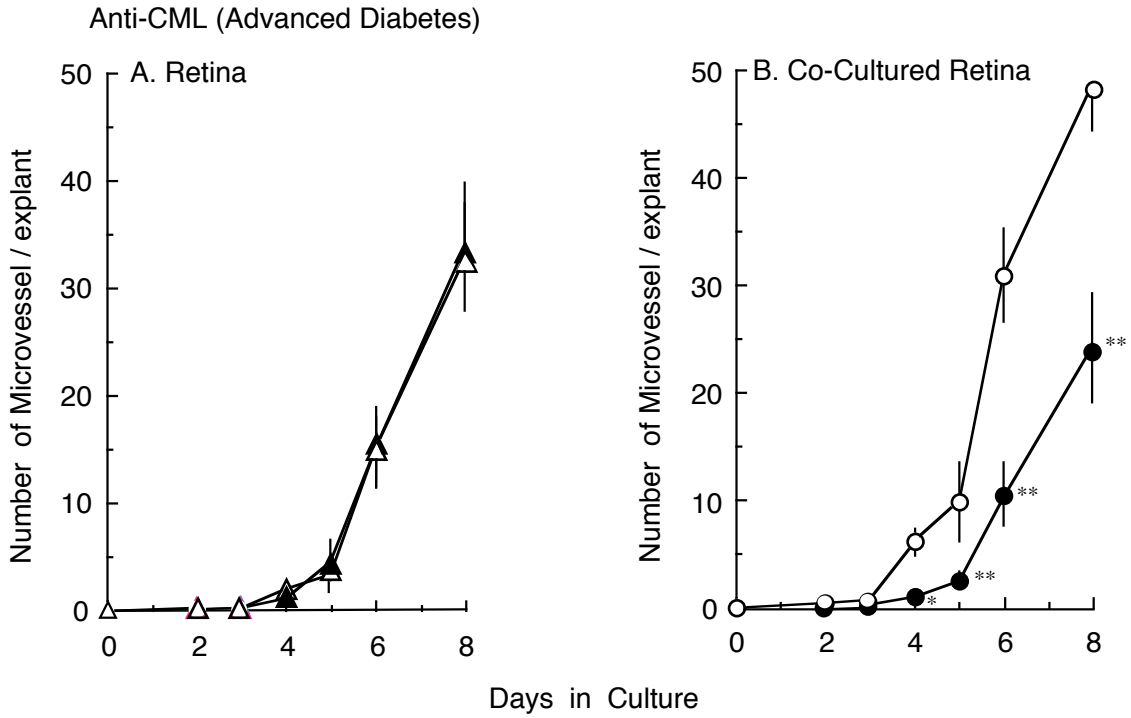

Fig. (4). Effects of anti-CML antibody on the neovascularization of retinal explant in advanced-diabetic rat in single culture (**A**: triangle) and in co-culture with choroidal explant from the same eyeball of advanced-diabetic rat (**B**: circle). Anti-CML antibody (1 μg/mL: closed symbol) or saline (open symbol) was added to the culture medium. Values represent means ± S.E.M. of 11-27 (A) and 12-26 (B) data. * $P < 0.05$, ** $P < 0.01$: Significantly different from the corresponding control value without the antibody.

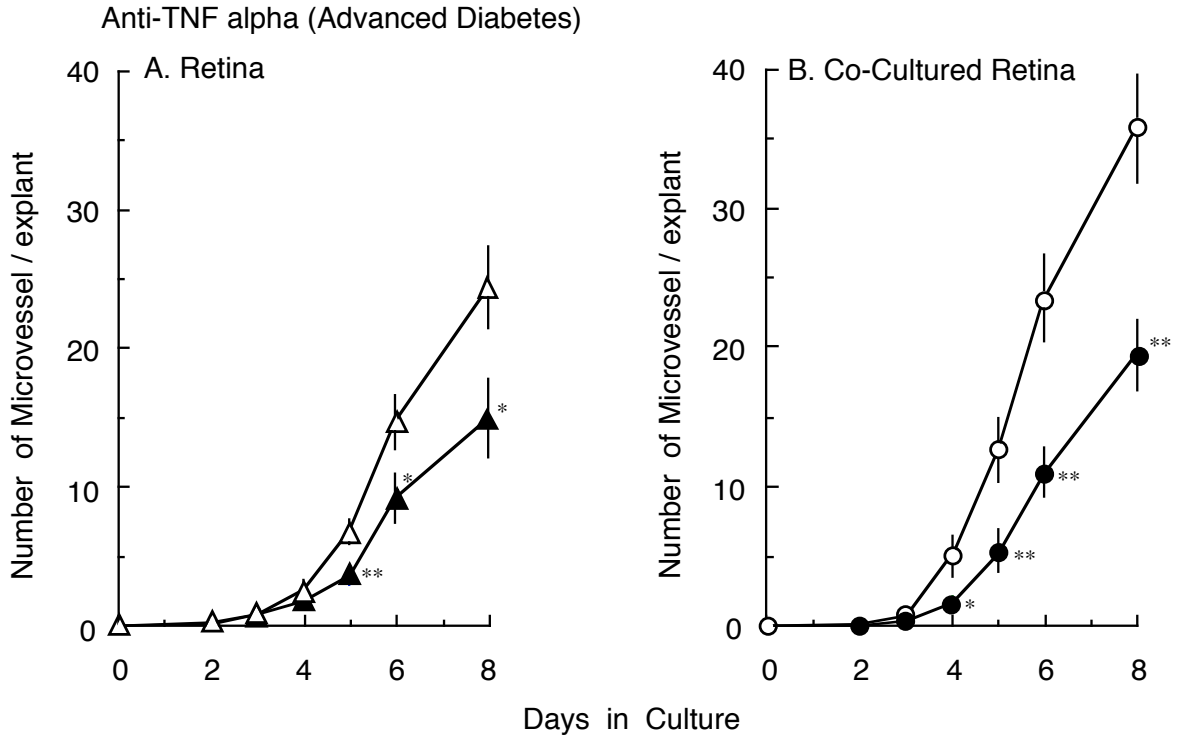

Fig. (5). Effects of anti-TNF α antibody on the neovascularization of retinal explant in advanced-diabetic rat in single culture (**A**: triangle) and in co-culture with choroidal explant from the same eyeball of advanced-diabetic rat (**B**: circle). Anti-TNF α antibody (1:1000: closed symbol) or saline (open symbol) was added to the culture medium. Values represent means ± S.E.M. of 19-27 (**A**) and 13-20 (**B**) data. * $P < 0.05$, ** $P < 0.01$: Significantly different from the corresponding value without the antibody.

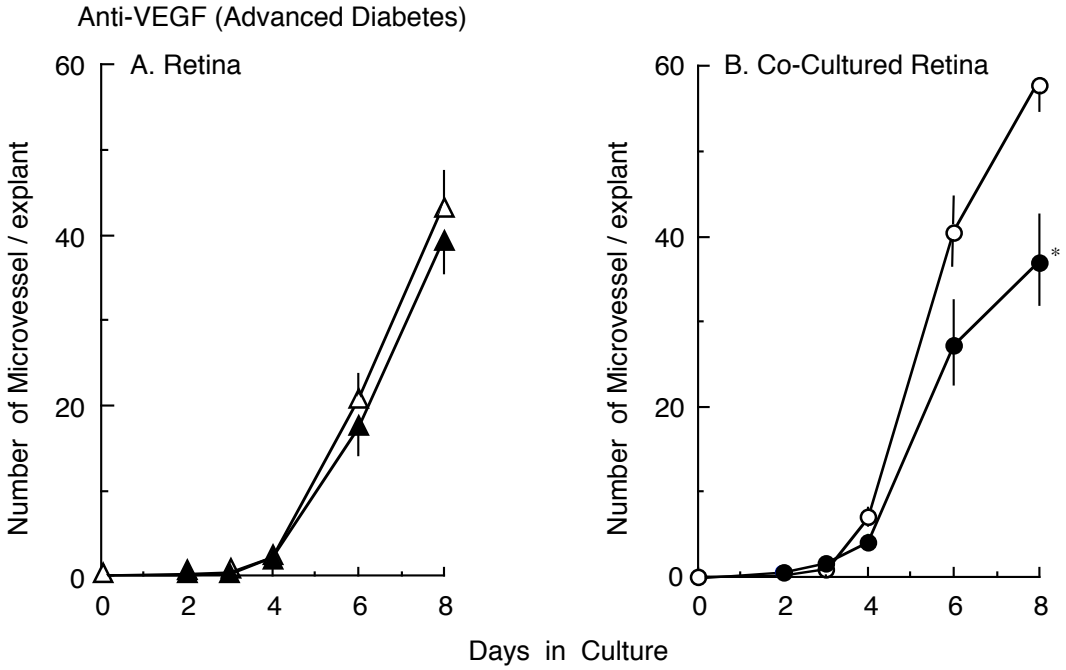

Fig. (6). Effects of anti-VEGF antibody on the neovascularization of retinal explant in advanced-diabetic rat in single culture (**A**: triangle) and in co-culture with choroidal explant from the same eyeball of advanced-diabetic rat (**B**: circle). Anti-VEGF antibody (0.3 μg/mL: closed symbol) or saline (open symbol) was added to the culture medium. Values represent means ± S.E.M. of 8-13 (**A**) and 6-13 (**B**) data. * $P < 0.05$: Significantly different from the corresponding normal value without antibody.

DISCUSSION

In the present study, we compared angiogenic activity of choroidal explant co-cultured with retinal explant between in early-diabetic rat and advanced-diabetic rat at first. The number of budding microvessels of choroidal explant from early-diabetic eyeball in the co-culture with retinal explant from the same eyeball was significantly greater than that of age-matched young control (Fig. **1A**). The onset time of buds and the number of budding microvessels of choroidal explant from advanced-diabetic eyeball in co-culture with retinal explant from the same eyeball were similar to those of age-matched normal aged control (Fig. **1B**). The number of microvessels of choroidal explant from the advanced-diabetic eyeball in co-culture with retinal explant from the same eyeball was greater than that of the normal young control on the 6th day in culture (Fig. **1B**). Since the activity of choroidal explants in the co-culture system is similar to those of choroidal explant singly without retinal explant in culture [1], co-cultured retinal explants do not affect the angiogenic activity of choroidal explants. These results of choroidal explant may relate to the delayed onset time of bud in the retinal explant.

Table **1** summarized activity of retinal neovascularization in the single culture or co-culture with choroidal explant of early-diabetic and advanced-diabetic rats by using possible model of retinal detachment. Co-cultured choroidal explants increased number of choroidal microvessels (Fig. **1A**), but suppressed budding and number of retinal microvessels of the same eyeball from the early-diabetic rat (Fig. **2A**). Anti-CML antibody reversed the choroidal capillary-suppressed neovascularization of retinal explants of the same eyeball in early-diabetic rat (Fig. **3B**). Since the anti-CML antibody

Table 1. Summary of Effects of Anti-CML, Anti-TNF Alpha and Anti-VEGF Antibodies on Retinal Neovascularization Under Single Culture or Co-Culture with Choroid in Early-Diabetes (ED) and Advanced-Diabetes (AD) of Rats

	Retinal Neovascularization	
	Single Culture	**Co-Culture with Choroid**
ED	no effect	decrease
ED + Anti-CML	no effect	increase
AD	no effect	increase
AD + Anti-CML	no effect	decrease
AD + Anti-TNF alpha	decrease	decrease
AD + Anti-VEGF	no effect	decrease

decreases the choroidal neovascularization in the early-diabetic rat [1], but did not directly affect retinal neovascularization in the early-diabetic rat (Fig. **3A**), CML adduct may suppress retinal neovascularization through the activation of choroidal angiogenesis in the early-diabetic stage, at least in the co-culture system. In contrast, co-cultured choroidal explant significantly increased budding of microvessels and neovasculation of retinal capillary isolated from the same eyeball of advanced-diabetic rat (Fig. **2B**). The anti-CML antibody significantly suppressed the increased neovascularization in retinal capillary co-cultured with choroidal explant in the same eyeball of advanced-diabetic rat (Fig. **4B**). Since anti-CML antibody did not directly affect the budding microvessels of retinal capillaries of advanced-diabetic eyeball (Fig. **4A**), the action of anti-CML antibody

on retinal neovascularization is mediated through the inhibitory action on choroidal neovascularization in the advanced-diabetes (Fig. **4B**). These results indicate that CML-induced angiogenic activity in choroidal capillaries has different effects on neovascularization of retinal capillaries in the early-diabetic and the advanced-diabetic rat. These different actions may depend on differences of the retinal capillaries in early-diabetic and advanced-diabetic states, such as different composition of pericytes in the retinal endothelial cells [9]. Pericytes might facilitate the release of anti-angiogenic factors, such as transforming growth factor (TGF)-β or pigment epithelium-derived factor [23, 24], and thus delay the onset of budding of microvessels in retinal capillaries of early-diabetic rat. Retinal capillaries may have a higher ratio of pericytes to vascular endothelial cells, and this may play a role in vascular endothelial cell quiescence in early diabetes [25].

The characteristics at the stage of proliferative diabetic retinopathy are venous dilation, abnormal microvasculatures and neovascularization of retinal vessels. New microvessels are sometimes accompanied by a fibrovascular ridge extending into the vitreous cavity or along the surface of the retina [26]. In the proliferative diabetic retinopathy, the action of VEGF is upregulated and promotes blood retinal barrier breakdown and neovascularization to produce diabetic macular edema [9]. Diabetic macular edema displays three different patterns, which may be combined: retinal thickening with sponge-like retinal swelling, cystoid macular edema, and serous retinal detachment [12]. Choroids of patients with diabetes mellitus also show vascular changes, including thickening of the basement membrane and narrow vessels with neovascularization [27-31]. Choriovitreal neovascularization in the peripheral fundus is suggested to be one of the features of diabetic retinopathy [32]. We have reported that anti-CML, anti-TNF α and anti-VEGF antibodies significantly reduce the choroidal neovascularization *in vitro* in the early-diabetic rat (1-2 months after STZ administration) and the advanced-diabetic rat (ca. 7.5 months after STZ administration) [1, 2, 33], indicating that CML, TNF α and VEGF have a concomitant role in overproduction of choroidal neovascularization of early-diabetic and advanced-diabetic rats. The present study demonstrates that anti-TNF α antibody inhibited neovascularization of retinal capillaries both in single culture and in co-culture with choroidal capillaries of the same eyeball in advanced-diabetes (Table **1**, Fig. **5**). Anti-VEGF antibody inhibited neovasculalization of retinal capillaries in co-culture with choroidal capillaries, but not in single culture (Table **1**, Fig. **6**). The time-course of action of anti-TNF α antibody on retinal neovascularization was faster than that of anti-VEGF antibody in the co-culture system, indicating that TNF α may trigger release of VEGF from choroidal capillaries of the advanced-diabetic eyeball [33]. We have unpublished data that TNF α facilitates to produce mRNA of VEGF and to release VEGF protein in choroidal explant of rat. VEGF mRNA is also expressed by ganglion cells, glial cells such as astrocytes and Muller cells, smooth muscle cells and pericytes in diabetic retina [34]. Moreover, VEGF immunoreactivity is located in the retinal optic nerve layer, retinal pigment epithelium and choroid in spontaneously diabetic rat [35]. These results suggest that VEGF releases from choroidal capillaries and facilitate retinal neovascularization in the advanced-diabetic rat, at least in culture.

The early stage of diabetic retinopathy is suggest to be similar to chronic inflammatory reaction. TNF α, a pro-inflammatory cytokine, plays a role in the pathogenesis of proliferative diabetic retinopathy [36, 37]. Diabetic retinopathy is generally classified into early stage, non-proliferative stage and proliferative stage. Vascular lesions in the early stage of diabetic retinopathy in patients and animal models are characterized by the presence of saccular capillary microaneurysms, pericyte-deficient capillaries, and obliterated and degenerated capillaries. Inflammation typically has beneficial effects on an acute basis, but can have undesirable effects if present chronically. The increased expression of many inflammatory proteins, such as TNF α, interleukin-1 and cyclooxygenase 2, is regulated at the level of gene transcription through the activation of proinflammatory transcription factors, including nuclear factor kappa B (NF-κB) [10]. TNF α can induce the expression of many important immune- and angiogenesis-related genes through two different TNF α receptors: TNF αR1 and TNF αR2. TNF α signaling through TNF αR2 (p75 receptor) is required for vessel development in ischemia-induced neovascularization [11]. Therefore we are planning in the next study to examine role of TNF αR2 for production of mRNA and protein of VEGF in choroidal capillary.

In conclusion, CML-induced angiogenic activity of choroidal capillaries interacts with the neovascularization of retinal capillaries in different ways in early-diabetic and advanced-diabetic rats. CML-induced angiogenic activity of choroidal capillaries reduced the retinal neovascularization in early-diabetes, but facilitated it in advanced-diabetes. The angiogenic actions of TNF α and VEGF influence retinal neovascularization induced by CML-mediated angiogenic activity of choroidal capillaries in advanced diabetes.

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