A Quantitative Assay for Angiogenesis of Cultured Choroidal Tissues in Streptozotocin-Diabetic Wistar and Spontaneously Diabetic GK Rats

Shinjiro Kobayashi¹, Mizuki Fukuta¹, Hitoshi Kontani¹, Sumino Yanagita² and Ikuko Kimura²

¹Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokuriku University, 3-Ho Kanagawa-machi, Kanazawa 920-1181, Japan
²Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

Received July 6, 1998 Accepted September 18, 1998

ABSTRACT—Angiogenesis of cultured choroids was quantitatively assayed in spontaneously diabetic GK and a bolus-treated streptozotocin (STZ)-diabetic Wistar rats. The number and total length of microvessels budded from cultured choroidal explants were measured to use as angiogenic indices. Both indices in 10-week-old Wistar rats were increased in parallel by 5% fetal bovine serum (FBS) from days 2 to 7 in culture. These indices in STZ-rats (10 weeks of age) were increased by 5% FBS to a greater extent than those in age-matched normal rats. These enhanced actions of FBS were concentration-dependent. The explants of 16-week-old GK rats also increased these indices to a greater extent than those of age-matched Wistar rats. Aging to 18 weeks of age also increased choroidal angiogenesis in the normal rats. In conclusion, the assay model of choroidal angiogenesis was established by determining the number and length of microvessels in cultured choroidal explants. The diabetic states of STZ-Wistar and GK rats enhanced FBS-induced choroidal angiogenesis. This assay model is useful for determining angiogenic activity of growth factors and effective drugs in diabetic choroidopathy and retinopathy.

Keywords: In vitro assay for angiogenesis, Choroidal angiogenesis, Streptozotocin-diabetic rat, Diabetic GK rat

Proliferative retinopathy in the late developing lesions in the complications of diabetes mellitus (DM) includes extensive neovascularization, the intrusion of vessels into the vitreous cavity and severe visual loss (1–3). The majority of cases of severe visual loss is associated with disorders that result in ocular angiogenesis including corneal and choroidal neovascularization secondary to age-related macular degeneration. Choroids from patients with DM show vascular changes including narrowed vessels with neovascularization (4–6). Since the outer retinal layers are largely dependent on the choroid for their nutrients and oxygenation, choroidal vasculopathy is suggested to have a crucial role in the pathogenesis of diabetic retinopathy. However, there has been little emphasis on the diabetic choroidal vasculature. Therefore, the present study focused on choroidal angiogenesis by establishing an in vitro assay model of angiogenesis.

Spontaneously diabetic GK rats that are produced by selective breeding of normal Wistar rats (7) and streptozotocin (STZ)-diabetic Wistar rats (8) were used as the diabetic models. The GK rats show a mild hyperglycemia accompanied by glucose intolerance, impaired pancreatic function and insulin resistance (9). The rats also have several other abnormalities including a thickening of the glomerular capillary basement membranes (10) and enhanced proliferation of primary cultured aortic smooth muscle cells (11). Peritoneal macrophages in the GK rats release a greater amount of immunoreactive platelet-derived growth factor-BB (PDGF-BB) and interleukin (IL)-1α through the action of advanced glycation endproducts (AGEs) in diabetic serum on the macrophages (12). These growth factors enhance tube formation of vascular endothelial cells (EC) in the angiogenic process (12, 13), suggesting that the PDGF-BB and IL-1α released from the diabetic macrophages have key roles in the tube-forming activity of EC in angiogenesis (12, 14).

Nicosia and Ottinetti have reported an assay model of angiogenesis produced by culturing explants of rat aorta (15). The model is useful for screening actions of effective factors and drugs on a series of angiogenic process, including the production and release of growth factors, degradation of basement membranes, migration,
proliferation and tube formation of vascular EC (16). In the present study, we investigated a quantitative assay model for choroidal angiogenesis produced by culturing explants of choroidal tissues in STZ-diabetic Wistar and spontaneously diabetic GK rats. Effects of fetal bovine serum (FBS) on choroidal angiogenesis in these diabetic rats were determined by using this in vitro assay model.

MATERIALS AND METHODS

Animals
STZ-diabetic Wistar male rats (10–11 weeks of age; body weight (BW), 288–460 g; blood glucose level (BGL), 287–374 mg/dl) and spontaneously diabetic male GK rats (16 week of age; BW, 297–355 g; BGL, 206–252 mg/dl) were used. The STZ-rats were utilized for the experiments 3–4 weeks after injection of a single dose (60 mg/kg, i.v.) of STZ into tail veins of Wistar male rats (7 weeks of age; Shizuoka Laboratory Center, Hamamatsu or Kiwa Laboratory Animal Science Co., Ltd., Wakayama). Wistar strain male rats (9–18 weeks of age; BW, 270–425 g; BGL, 114–122 mg/dl) were used as age-matched controls. Blood samples were obtained from the orbital vein plexus of fed animals. BGLs of these animals were measured by the glucose oxidase method with a glucose analyzer (Type II; Beckman Japan, Tokyo) or glucose B-test (Wako, Osaka).

Preparation of choroidal tissues
Diabetic GK, STZ-diabetic Wistar and normal Wistar rats were anesthetized and killed with diethyl ether and their eye balls were rapidly isolated under aseptic conditions. Blood vessels, connective tissues and fatty tissues were removed from the outside of the eye balls in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo) containing 10% heat-inactive FBS (JRH Bioscience, Lanexa, KS, USA), 160 U/ml benzylpenicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo). After removing cornea, lens, corpus vitreum and retina from the inside of eye balls, explants of choroidal tissues were isolated in 10% FBS-DMEM containing antibiotics with forceps under an optical microscope. The sizes of cultured explants were 0.2- to 1.0-mm-long and 0.2- to 1.0-mm-wide. In some experiments, the explants were isolated in DMEM containing 0.5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and antibiotics. Many capillary blood vessels were recognized in the choroidal tissues of Wistar rats by staining hematoxylin-eosin (our unpublished data).

Tissue culture of explants of choroidal tissues
The explants of choroidal tissues were cultured by the method of Nicosia and Ottinetti (15). The isolated explants were plated by pipetting them on fibrin gels that were prepared by mixing 3 mg/ml fibrinogen (0.3 ml, Sigma) and 1 U thrombin (Sigma) per ml DMEM in a 16-mm dish (Corning, Corning, NY, USA). The same volume of the fibrinogen and thrombin solutions was overlaid and solidified. The choroidal explants in the fibrin gel were cultured with 0, 0.2%, 1% and 5% FBS-DMEM (0.5 ml) containing antibiotics and 300 µg/ml ε-aminocaproic acid at 37°C under 5% CO₂ and 95% air. The media were exchanged every other day. Rabbit antisem to human factor VIII-related antigen was indirectly stained in microvessel-like structures budded from choroidal tissues cultured for 8 days by the method of Nicosia and Ottinetti (15), confirming that the structures consist of vascular EC (our unpublished data).

Measurement of angiogenesis
Microvessels newly budded from a cultured explant of choroidal tissues were photographed with a Leitz Diavert camera equipped with a Wild Photoautomat MPS45 (Leitz, Germany) or with a Olympus camera equipped with a CKS microscope (Olympus, Tokyo). Typical photographs of microvessels extended from the explants of tissues on days 2 to 7 in culture are shown in Fig. 1. The number of new microvessels budded from an explant of choroidal tissues as seen in photographs (×40 magnification) taken at various focal distances was counted. The length of all branched microvessels on the photograph was also measured with Software MEAS I (Graphitec Corp., Tokyo) and summed to estimate the total length of microvessels (mm/explant).

Statistical analyses
Significant difference of data was evaluated by one-way analysis of variance followed by the multiple range tests of Scheffé and Tukey; the criterion for significance was P = 0.05 or P = 0.01, respectively.

RESULTS

Morphological observation of microvessels budded from cultured choroidal explants in normal and STZ-diabetic Wistar rats
Morphological changes of explants of choroidal tissues in normal and STZ-diabetic Wistar rats (10 weeks of age) were compared by culturing them in fibrin gel in the presence of 5% FBS. Microvessel-like structures were budded and developed from the explants in normal Wistar rats on days 2 to 7 (Fig. 1). The number and length of microvessels were increased dependently on the days in culture. Choroidal explants of STZ-diabetic rats increased both the number and length of microvessels to a
Fig. 1. Typical photographs of microvessels budded from cultured explants of choroids in normal Wistar rat (10 weeks of age, left) and streptozotocin (STZ)-diabetic Wistar rat (10 weeks of age, right) on days 2, 3, 4, 5, 6 and 7. These explants were cultured in fibrin gel in the presence of 5% FBS-DMEM. The bar represents 500 μm.
greater extent than the normal rat explants during these culture periods (Fig. 1). The vessel-like structures budded from the normal explants that were cultured for 8 days were indirectly bound with antisera to human factor VIII-related antigen (our unpublished data), confirming that the structures consisted of capillary EC. Relatively larger vessels such as ciliary arteries and vorticose veins did not bud new microvessels under these experimental conditions (our unpublished data).

Quantitative analysis of angiogenesis in cultured choroidal explants in STZ-diabetic Wistar and spontaneously diabetic GK rats

The number and total length of all microvessels in cultured choroidal explants of normal Wistar rats (10 weeks of age) on photographs (40× magnification) were each counted and measured during 5–7 days in culture (Figs. 2 and 3). The number of microvessels in the cultured explants was increased by 5% FBS from days 2 to 6 in a time-dependent manner (Fig. 2, left). The total length of all the budded microvessels was increased in parallel with the microvessel number during these days in culture (Fig. 3, left). These angiogenic parameters were not influenced by the sizes of cultured explants, which were from 0.2×0.2 mm² to 1×1 mm² in size (our unpublished data).

From these results, we chose both parameters as angiogenic indices. Both angiogenic indices in cultured chorioids are useful for evaluating choroidal angiogenesis in vitro.

The angiogenesis of choroidal explants were investigated in STZ-diabetic Wistar and spontaneously diabetic GK rats. The number of microvessels in cultured choroidal explants of STZ-Wistar rats (10 weeks of age) was increased by 5% FBS from days 2 to 7. The increase in number of microvessels in the diabetic choroids was significantly greater than that in the age-matched normal rats (Fig. 2, left) from days 3 to 7. The vessel number in cultured choroids of diabetic GK rats (16 weeks of age) was also increased from days 2 to 4 in a time-dependent manner (Fig. 2, right). The vessel number in explants of GK rats was also significantly greater than that of the age-matched normal rats from days 3 to 7.

Total lengths of microvessels in STZ-diabetic Wistar and diabetic GK rats were compared (Fig. 3). The total length of all microvessels in the cultured explants of STZ-Wistar rats was significantly greater than that of age-matched normal rats on the corresponding day from days 3 to 7 (Fig. 3, left). The total length of microvessels in cultured explants of diabetic GK rats was also increased in a time-dependent manner and significantly

![Graphs showing time-dependent increase in the number of microvessels budded from choroidal explants in streptozotocin (STZ)-diabetic Wistar (left) and spontaneously diabetic GK (right) rats. The choroidal explants of STZ-Wistar rats (10 weeks of age) (left, ○), normal Wistar rats (10 weeks of age) (left, □), GK rats (16 weeks of age) (right, ●) and normal Wistar rats (16 weeks of age) (right, □) were cultured for 7 days in fibrin gel in the presence of 5% FBS-DMEM. Values represent means±S.E. of 7–11 data values. *P < 0.05, **P < 0.01: significantly different from the value in the corresponding control Wistar rats.]

Fig. 2. Time-dependent increase in the number of microvessels budded from choroidal explants in streptozotocin (STZ)-diabetic Wistar (left) and spontaneously diabetic GK (right) rats. The choroidal explants of STZ-Wistar rats (10 weeks of age) (left, ○), normal Wistar rats (10 weeks of age) (left, □), GK rats (16 weeks of age) (right, ●) and normal Wistar rats (16 weeks of age) (right, □) were cultured for 7 days in fibrin gel in the presence of 5% FBS-DMEM. Values represent means±S.E. of 7–11 data values. *P < 0.05, **P < 0.01: significantly different from the value in the corresponding control Wistar rats.
greater than that of age-matched normal rats from days 3 to 4 (Fig. 3, right). These results demonstrate that the diabetic states in STZ-Wistar and GK rats enhance the angiogenic indices of cultured choroidal tissues.

Aging increases angiogenesis of choroidal tissues

The angiogenic action of 5% FBS in cultured choroidal explants of 18-week-old Wistar rats was compared with that of 10-week-old Wistar rats (Fig. 4). The number of
microvessels in explants of 18-week-old Wistar rats was increased by 6 days in a time-dependent manner. The vessel number in 18-week-old rats was significantly greater than that in 10-week-old rats on the corresponding day from days 3 to 7 (Fig. 4, left). The total length of vessels in cultured explants of 18-week-old rats was also increased by 5% FBS to a greater extent than that in explants of 10-week-old rats (Fig. 4, right). These results demonstrate that aging to 18 weeks in Wistar rats as well as the diabetic state enhances the angiogenesis of cultured choroidal explants, indicating that this assay model is also useful for studying of aging-induced angiogenesis.

FBS-dependent angiogenic activity of choroidal explants in STZ-diabetic rats

The concentration-dependent effect of FBS on choroidal angiogenesis in STZ-diabetic rats was compared with that in age-matched normal Wistar rats (Fig. 5). FBS (0.2–1%) increased the number of microvessels in explants in 10-week-old Wistar rats on day 6 in culture in a concentration-dependent manner. The action of FBS was maximal at 1% FBS and maintained at a plateau level by 5% FBS (Fig. 5, left). FBS (0.2–5%) enhanced the number of microvessels in explants of STZ-diabetic rats to a greater extent than that of age-matched control rats. FBS (0.2–5%) also increased total length of microvessels of the diabetic choroids in a concentration-dependent manner (Fig. 5, right). The effect of FBS on the total length of microvessels in the diabetic choroids was significantly greater than that in the normal choroids. These results demonstrate that the response of FBS on these angiogenic indices in cultured choroids is enhanced by the diabetic states.

DISCUSSION

Choroidal dysfunction with angiogenesis in diabetes mellitus is suggested to have a role in the induction of pathogenesis of diabetic retinopathy since the dysfunction provides few nutrients and oxygen to the outer layer of retina and foveola (4–6). Various factors are produced and/or released in proliferative lesions in the diabetic choroid and retina (3, 17–22). However, there have been few reports concerning the effects of these factors on choroidal angiogenesis. In the present study, we established a quantitative assay model for in vitro angiogenesis in cultured choroidal explants to determine angiogenic activity of choroids in diabetic rats. Two angiogenic indices, the number and total length of microvessels, in cultured choroidal explants were used in this model. The structures of budded microvessels were confirmed to consist of vascular EC by binding of rabbit antiserum to human factor VIII-related antigen to the microvessels (15) (our unpublished data). The present study provides direct evidence indicating that the diabetic states of STZ-Wistar and spontaneously diabetic GK rats enhance the growth of microvessels in the cultured choroidal explants. This in vitro model is useful for screening the actions of effective

![In Vitro Angiogenesis (6 days)](image)

Fig. 5. Fetal bovine serum (FBS)-enhanced increase in the number (left) and total length (right) of microvessels budded from the explants of choroids in STZ-diabetic (●) and age-matched normal Wistar rats (●) (10–11 weeks of age) on day 6 in culture with 0, 0.2%, 1% and 5% FBS-DMEM. Values represent means ± S.E. of 7–16 data values. C shows the control without FBS. *P < 0.05, **P < 0.01: significantly different from the value of normal choroid at the corresponding concentration of FBS.
drugs and growth factors on the whole angiogenic process, which includes the production and/or release of angiogenic factors, the degradation of vessel basement membranes, migration, proliferation and tube formation of vascular EC (16).

The diabetic states of STZ-Wistar and diabetic GK rats enhanced the response to FBS in the angiogenesis of EC in chorioids. The result suggests two possible actions of FBS in the diabetic choroids: one is the production and/or release of some growth factors, and the other is the enhanced activity of FBS on vascular EC, through the diabetic state-modified receptors of some endogenous growth factors and/or their post-receptor pathway. Cultured peritoneal macrophages in the diabetic GK rats release PDGF-BB and IL-1\textalpha in the presence of 2\% FBS (12). The release of PDGF-BB is selective for the diabetic state. These growth factors accelerate the tube formation of vascular EC in the angiogenic process (12). These results suggest that the angiogenic activity of FBS in the diabetic choroid may be associated with the activities of some angiogenic factors released from chorioidal tissues. In addition, it may not exclude the other possibility that the enhanced activity of FBS on the diabetic choroids is associated with the modification of receptors and/or post-receptor pathway against some endogenous growth factors. However, no direct evidence has yet been forthcoming to support this possibility.

Hyperglycemia is one of the major causes of microvascular dysfunction, but the mechanism of the detrimental effect is not clear. Since glucose and its metabolites are utilized by numerous intracellular pathways, the adverse effects of hyperglycemia no doubt involve multiple mechanisms. Four theories have attempted to link the effects with a mechanism (23). These theoretical mechanisms are: 1) activation of the polyol pathway (24); 2) formation of nonenzymatically glyicated products (25, 26); 3) alteration of redox potential (27, 28); and 4) activation of the diacylglycerol-protein kinase C pathway (29–31). The present study demonstrates that the diabetic choroids showed enhanced angiogenesis under the non-diabetic culture condition including the normal concentration of glucose. The result indicates that characteristics of choroidal tissues are irreversibly modified in the diabetic model rats. We have reported that AGEs of the serum in diabetic GK rats activate peritoneal macrophages to release angiogenic factors including PDGF-BB and IL-1\textalpha (12). Several reports also reveal a close correlation between the AGE-formation of connective tissues and their physiological changes in diabetes mellitus (32–35). On the other hand, pericytes in capillary vessels have been reported to induce the release of active transforming growth factor beta in the culture medium to regulate the function and growth of EC (36). Microvessels newly formed in proliferative retinopathy contain few pericytes and do not form the tight retinal-blood barrier (31). AGEs elevated in the vitreous in diabetic retinopathy have been suggested to cause breakdown of the retinal-blood barrier through the production of vascular endothelial growth factor (37). However, since there is no report that AGEs are elevated in the choroidal tissues in the diabetic models, it is obscure whether AGEs have a role for the enhancement of choroidal angiogenesis in the diabetic model rats.

AGE-formation of connective tissues also correlates with their physiological changes in aging (32–35). The present study suggests that the aging to 18 weeks of normal rats as well as the diabetic state enhances angiogenesis of cultured choroids. The angiogenic activity of aging at 18 weeks on choroidal tissues might be also related with the formation of AGEs in tissues. This in vitro assay model for choroidal angiogenesis is also useful for studying aging-induced activity.

In conclusion, the assay model of choroidal angiogenesis in vitro was established by determining the number and length of microvessels in choroidal explants cultured in fibrin gel. The diabetic states of STZ-Wistar and GK rats enhanced the angiogenic activity of FBS in choroids. This assay model of angiogenesis is useful for studying activities of growth factors and effective drugs in diabetic choroidopathy and retinopathy.

Acknowledgments

This work was supported in part by a Grant-in-Aid (No. 10672069) for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and a Special Research Foundation from Hokuriku University, Kanazawa. The authors thank Ms. E. Matsuda (Department of Chemical Pharmacology, Toyama Medical and Pharmaceutical University) and Mr. E. Hata (Department of Pharmacology, Hokuriku University) for their skillful assistance in performing the experiments and thank Prof. Dr. K. Takaya (Department of Anatomy, Faculty of Medicine, Toyama Medical and Pharmaceutical University) for his kind advice on anatomical identification of the choroidal tissues.

REFERENCES