Inhibitory Effect of Nifedipine on Tumor Necrosis Factor α-Induced Neovascularization in Cultured Choroidal Explants of Streptozotocin-Diabetic Rat

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We have previously reported that the Nε-(carboxymethyl)lysine (CML) adduct, a major structure of an advanced glycation end product, facilitates proliferation of CD34+ endothelial progenitor cells budded from cultured choroidal explants and produces immature vessel-like structures in fibrin gel. The CML adduct is accumulated and facilitates immature neovascularization in cultured choroidal explants of streptozotocin (STZ)-induced diabetic rat. The CML-enhanced neovascularization activity is associated with the actions of tumor necrosis factor (TNF) α, vascular endothelial growth factor and platelet-derived growth factor released from the choroidal explant (Kobayashi et al., Biol. Pharm. Bull., 27, 1382—1387 (2004); 27, 1565—1571 (2004)). The present study was investigated an inhibitory effect of a dihydropyridine calcium antagonist nifedipine on TNF α-induced choroidal neovascularization in the STZ-diabetic rat. TNF α (1–100 ng/ml) increased neovascularization of cultured choroidal explants in the age-matched normal rat but did not increase the neovascularization in the diabetic rat. Anti-TNF α antibody (1:1000) decreased the neovascularization in the diabetic rat but not in the normal rat. Nifedipine (1 μM) inhibited TNF α-induced neovascularization of the normal choroidal explant in a non-competitive manner. Nifedipine (1 μM) also inhibited the diabetic state-induced neovascularization and its inhibitory action was reversed by TNF α (1–10 ng/ml). In conclusion, STZ-diabetic state facilitated choroidal neovascularization through the release of TNF α. Nifedipine inhibited the action of TNF α probably by blocking voltage-dependent Ca2+ channels in the endothelial progenitor cells of the diabetic choroid.

Key words choroidal neovascularization; tumor necrosis factor; nifedipine; diabetes mellitus; streptozotocin

Tumor necrosis factor (TNF) α is a proinflammatory cytokine that has been implicated in the pathogenesis of diabetic retinopathy.1—3) TNF α is found in the extracellular matrix, endothelium and vessel walls of fibrovascular tissue of eyes with proliferative diabetic retinopathy4,5) and vitreous from eyes with this complication.6) This cytokine has been presented as a potent promoting factor in in vivo and in vitro angiogenesis model.7—8) Retinal neovascularization has crucial roles for proliferative diabetic retinopathy. However, there is little evidence that choroidal neovascularization is an important feature of the disease process in the eyes of human diabetic subjects.9) Choroidal neovascularization leads to the cause of rapid severe vision loss in patients with age-related macular degeneration (AMD). Vascular endothelial growth factor (VEGF) could be implicated in the development of choroidal neovascularization found in diseases of AMD and animal models of AMD.10—14) Immunological studies using antibodies against advanced glycation end products (AGEs) have demonstrated accumulation of AGE-modified proteins in several tissues during aging and various disease states including AMD and diabetic retinopathy.15,17)

We have previously reported that Nε-(carboxymethyl)lysine (CML), one of the major structures of AGEs facilitates proliferation of CD34+ endothelial progenitor cells budded from choroidal explant and increases the production of immature microvessels in fibrin gel, suggesting that CML may be one of the factors promoting choroidal neovascularization in AMD and other choroidal neovascular diseases.15) CML adduct increases the release of TNF α, VEGF and platelet-derived growth factor (PDGF)-B from the cultured choroid of streptozotocin (STZ)-diabetic rat. CML-induced neovascularization of the diabetic choroid depends on the actions of these released factors.18,19) Administered VEGF and PDGF-B increase immature neovascularization of normal choroidal explants but depress the neovascularization of diabetic explants in culture.20) The CML adduct may facilitate the affinity of these factors for choroidal neovascularization in the diabetic rats, following with the down-regulation of their activities in the diabetic choroidal capillaries. However, the role of TNF α for the neovascularization in the cultured diabetic choroid is unknown at present.

It is generally accepted that macrovascular endothelial cells are nonexitable and that voltage-dependent channels are functionally not important.21) However, several reports have provided evidence for the existence of voltage-dependent Ca2+ channels in cultured microvascular endothelial cells, which share some similarities with T- and L-type Ca2+ channels.22—24) T-type Ca2+ channels are blocked by the T-type Ca2+ channel blockers Cd2+ and amiloride. L-type Ca2+ channels are blocked by dihydropyridine derivatives such as nifedipine.21) Nifedipine inhibited basic fibroblast growth factor-induced proliferation of endothelial cells by a cell-cycle arrest in G1/G0 through a blocking of the L-type voltage-dependent calcium channel.21,22) There is reported another evidence that nifedipine resulted in the increased release of nitric oxide, that is not due to blocking of the L-type voltage-dependent calcium channels.23,24)

In the present study, we compared actions of TNF α on the neovascularization of choroidal explant in culture in the STZ-induced diabetic and normal rats. The effect of nifedip-
ine on TNF α-induced neovascularization was also investigated to clarify a mechanism underlying the TNF α in the diabetic choroid.

MATERIALS AND METHODS

Animals The animals used were STZ-diabetic Wistar strain male rats (9—14 weeks of age; body weight, 245—550 g; blood glucose level of fed rats, 334—677 mg/dl), and age-matched control normal Wistar rats (9—13 weeks of age; body weight, 338—529 g; blood glucose level of fed rats, 105—181 mg/dl). The STZ-diabetic rats were utilized for the experiments 3—8 weeks after the injection of a single dose (60 mg/kg, i.v.) of STZ into Wistar rats (6 weeks of age, Kiwa Laboratory Animal Science Co., Ltd, Wakayama).

Blood samples were obtained from the orbital vein plexus of fed rats under ether anesthesia. Blood glucose levels of these animals were measured by the glucose oxidase method with a Beckman glucose analyzer (type II, Beckman Coulter, Tokyo). The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Faculty of Pharmaceutical Sciences, Hokuriku University.

Preparation of Choroidal Explants Explants of choroidal capillaries of STZ-diabetic and age-matched control normal Wistar rats were prepared as previously reported.18—20,27) The rats were euthanized by ether anesthesia. Blood vessels, connective and fatty tissues in the outside of the sclera in eye balls isolated from these rats were removed in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) containing 10% heat-inactive fetal bovine serum (FBS, JRH Bioscience, Lanexa, KS, U.S.A.), 160 U/ml benzylpenicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo) containing 10% heat-inactive fetal bovine serum (FBS, JRH Bioscience, Lanexa, KS, U.S.A.), 160 U/ml benzylpenicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo). After removal of the cornea, lens, corpus vitreum and retina from the eye balls, explants of choroidal tissues were isolated from the eye balls, explants of choroidal tissues were overlaid and solidified. The choroidal explants were moved in Dulbecco's modified Eagle's medium (DMEM, Corning, NY, U.S.A.). The same volumes of a mixture of the DMEM containing antibiotics in 16 mm dish (Corning, NY, U.S.A.), and 1 U thrombin (Sigma) per ml

tissue culture of explants of choroidal capillaries in 1% FBS-DMEM or 5% FBS-DMEM in the presence of antibiotics under an optical microscope. The area of isolated explants was approximately 0.16 mm².

Tissue Culture of Explants of Choroidal Capillaries The explants of choroidal capillaries were cultured as reported.18—20,27) The explants were plated on fibrin gels, which were prepared by mixing 3 mg fibrinogen (0.3 ml, Sigma, St. Louis, MO, U.S.A.), and 1 U thrombin (Sigma) per ml DMEM containing antibiotics in 16 mm dish (Corning, Corning, NY, U.S.A.). The same volumes of a mixture of the above concentrations of fibrinogen and thrombin solutions were overlaid and solidified. The choroidal explants were cultured with 1% FBS- or 5% FBS-DMEM (0.5 ml) containing antibiotics and 300 μg/ml e-amino caproic acid at 37 °C under 5% CO₂ and 95% air. In some experiments, we added TNF α (Genzyme, Cambridge, MA, U.S.A.), polyclonal rabbit anti-mouse TNF α antibody (Genzyme) or nifedipine (Sigma), respectively. The cultured media were exchanged every other day.

Measurement of Neovascularization The number of microvessel-like structures newly budded from cultured explants of choroidal tissues was counted with an Olympus camera equipped with a CKS microscope (Olympus, Tokyo) under ×40 magnifications. The number of budded microvessel-like structures per explant was used as an index of in vitro choroidal neovascularization.18,27) Typical photographs of microvessel-like structures have been published previously.18,27) Cells composed in the microvessel-like structures are positive against CD34, which is a marker of endothelial progenitor cells and endothelial cells, and have characteristics of fibroblast-like cells. These sprouted structures have narrow lumina occasionally surrounded by attenuated cellular extension.18)

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 at p=0.05 or 0.01. A value of p<0.05 was considered statistically significant.

RESULTS

Actions of TNF α and Anti-TNF α Antibody on Neovascularization in Cultured Choroidal Explants of STZ-Diabetic and Age-Matched Normal Rats The onset of sprouting and the growth of microvessel-like structures (immature microvessels) in culture with 1% FBS-DMEM were observed for 8 d in choroidal explants of STZ-diabetic (Fig. 1A) and age-matched control normal rats (Fig. 1B). The number of microvessels in the diabetic choroidal explant increased greater than their number in the normal choroidal explant (Fig. 1).19) TNF α (1—100 ng/ml) significantly increased the number of microvessels from 2 d to 8 d in a culture of normal choroid (Fig. 1B). The result indicated that TNF α facilitated the onset time of the spraying and growth of microvessels of normal choroids. However, TNF α (10—100 ng/ml) did not change the number of microvessels in the diabetic choroid (Fig. 1A). Anti-TNF α antibody (1 : 10000—1 : 100) significantly decreased the number of vessel-like structures in the diabetic choroid in a concentration-dependent manner (Fig. 2A). The anti-TNF α antibody at 1 : 1000 in the diabetic choroid showed a similar growth curve to that of the normal control. The TNF α antibody (1 : 1000—1 : 100) did not inhibit the number of microvessels of normal choroid during 8 d in culture but its concentration at 1 : 100 rather increased it on the 8th day in culture (Fig. 2B). The results demonstrate that TNF α was released from these explants in fibrin gel with 1% FBS-DMEM for 8 d in the presence (●) or absence (○) of TNF α (1—100 ng/ml). The numbers of microvessels from choroidal explants were counted on days 0, 2, 3, 4, 6 and 8. Values represent mean±S.E.M. of 9—11 (A) and 12—19 (B) data. *p<0.05, **p<0.01: Significantly different from the value without TNF α on the corresponding day in culture.
These explants were cultured in fibrin gel with 5% FBS-DMEM for 8 d in the presence (●) or absence (○) of nifedipine (0.1—10 μM). The numbers of microvessels from choroidal explants were counted on days 0, 2, 3, 4, 6 and 8. Values represent means ± S.E.M. of 12—20 (A) and 12—20 (B) data. *p < 0.05, **p < 0.01: Significantly different from the value without nifedipine on the corresponding day in culture.

DISCUSSION

We have previously reported that CML adduct, one of the major AGE structures, facilitates the proliferation of CD34+ endothelial progenitor cells budded from cultured choroid in fibrin gel. CML-modified proteins are accumulated and overproduced neovascularization of cultured choroidal explant during STZ-induced diabetes. The CML-induced neovascularization is associated with the actions of VEGF, TNF-α and PDGF-B released from the cultured choroidal explant of diabetes. The present study focused the action of TNF-α in the diabetic choroid and its inhibitory mode was a non-competitive blockade (Fig. 4B). In contrast, these concentrations of TNF-α did not increase but rather appeared to decrease the vessel number in the diabetic choroid (Fig. 4A). In the presence of 1 μM nifedipine, the basal number of microvessels of diabetic choroid was significantly decreased and the administered TNF-α (0.1—100 ng/ml) reversed the vessel number in a concentration-dependent manner. The reversed effect of TNF-α in the diabetic choroid was weaker than that in the normal choroid. In addition, the TNF-α (1—100 ng/ml) had no effect on the vessel number of diabetic choroid in the presence of 10 μM nifedipine (Fig. 4A). These results suggest that nifedipine blocked the neovascularization associated with the actions of TNF-α that exists in the diabetic choroid.
administered TNF α may not increase the neovascularization of diabetic choroid because of a maximal action of released TNF α. The amount of TNF α released from the diabetic explant is estimated to be approximately 0.4 pg/ml, and equivalent to 0.04% of administered 1 ng/ml TNF α. Nevertheless, the action of released TNF α in the diabetic choroid was similar to that of administered TNF α (1 ng/ml) in the normal explant. The activities of administered TNF α and released TNF α were examined in the presence of 1% FBS and 5% FBS-DMEM, respectively. Since the amount of TNF α was not detected significantly in the 5% FBS-DMEM alone (data not shown), influence of different concentrations of FBS in DMEM on the amount of TNF α was ignored. The results suggest that the sensitivity for the activity of TNF α is increased in the diabetic choroid. Alternatively, the relatively high concentration of TNF α administered may down-regulate the activity of TNF α.

It is reported that macrovascular endothelial cells are nonexcitable and voltage-dependent Ca²⁺ channels are functionally not important. However, the present study demonstrated that nifedipine, a blocker of L-type voltage-dependent Ca²⁺ channels, inhibited the choroidal neovascularization of microvascular endothelial progenitor cells in the STZ-induced diabetic rat. Several reports have provided evidences for the existence of voltage-dependent Ca²⁺ channels in cultured microvascular endothelial cells. Amlodipine, a T-type Ca²⁺ channel blocker, inhibits angiogenesis in vitro through blocking proliferation of vascular endothelial cells. Tetrandrine, a blocker of L type and T type voltage-dependent Ca²⁺ channels as well as Ca²⁺ release-activated Ca²⁺ channels, inhibits the neovascularization of the choroidal capillary in the diabetic rat. Nifedipine inhibits basic fibroblast growth factor-induced proliferation of vascular endothelial cells by a cell-cycle arrest in G0/G1 phase. Endotoxin-induced transcription and production of TNF α is regulated by the voltage-dependent Ca²⁺ influx in various kinds of cells. Since there is evidence that the sensitivities of voltage-dependent Ca²⁺ channels increase in vascular smooth muscle cells in the diabetic state, those in the endothelial progenitor cells in the diabetic choroidal capillary might be also increased. Although nifedipine and anti-TNF α antibody decreased the diabetic state-induced neovascularization, it is difficult to demonstrate clearly at present that TNF α is produced and released by the activation of voltage-dependent Ca²⁺ influx from the cultured diabetic choroid. Further investigations need to prove this consideration.

Dual actions of TNF α on neovascularization are reported as follows; TNF α at low concentrations induces the neovascularization in the angiogenesis system whereas TNF α at high concentrations inhibits it. TNF α has immediate effects via an interaction of voltage-dependent Ca²⁺ channels. The present study demonstrates that nifedipine inhibited TNF α-induced choroidal neovascularization in a noncompetitive manner, meaning that nifedipine does not antagonize the receptors of TNF α. Among transcriptional factors activated by TNF α, nuclear factor (NF) kappa B plays a major specific role in the regulation of inflammatory response genes. The activation of NF kappa B participates in the control of the gene expression of many modulators of inflammatory and immune responses, including vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). TNF α increases the production of VEGF, interleukin-8, basic fibroblast growth factor and their receptors, suggesting that these factors play crucial roles as secondary mediators in TNF α-induced neovascularization. The action of NF kappa B is also mediated by voltage-dependent Ca²⁺ channel in various cell types. One report shows, however, that nifedipine inhibits TNF-induced activation of NF-kappa B in human lung carcinoma cell line, which may be independent of its calcium channel blocking activity. On the contrary, NF kappa B does not seem to be activated by any growth factors including VEGF. The action of VEGF on vascular endothelial cells is mediated by the activation of tyrosine receptor kinases and inositol triphosphate-induced activation of membrane calcium-permeable channels. We have previously reported that the action of CML-released TNF α was completely inhibited by the anti-VEGF antibody. Together with the present results, the released TNF α may facilitate the NF-kappa B activity, resulting in the production of VEGF in the endothelial progenitor cells budded from the diabetic choroid.

The high concentration of TNF α exhibits anti-angiogenic activities in an in vitro model with vascular endothelial cells and in an in vivo corneal assay model. TNF α produces plasminogen activator inhibitor-1 and down-regulates integrin aVb5 and tissue-type plasminogen activator to inhibit neovascularization. Nifedipine and N-acetylcysteine blocked TNF α-induced inhibition of the proliferation of endothelial cells. Nifedipine exerts this blocking effect by promoting the release of nitric oxide from endothelial cells, without affecting the activities of voltage-dependent calcium channels. The antioxidative action of nifedipine increases the angiogenesis of endothelial cells. In the present study, we observed that TNF α had no alone effect or rather had a slightly inhibitory effect on the neovascularization in diabetic choroid in cultures (Fig. 4A). We also found that the neovascularization in the diabetic choroid was inhibited by nifedipine, and that the inhibitory effect of nifedipine was reversed by the addition of TNF α. We consider that a large amount of TNF α may be accumulated in a diabetic state and its neovascularization effect may reach the maximal level.

We have previously reported that the cultured choroidal explants of normal rats increase the formation of immature vessel-like structures with CD34⁺ endothelial progenitor cells in fibrin gel. The CML adduct facilitates the formation of the immature vessel-like structures during the period of streptozotocin-induced type I diabetic model rat. It is reported that human bone marrow-derived endothelial progenitor cells from type II diabetic patients were less likely to participate in tubule formation of human microvascular endothelial cells in Matrigel compared with the controls. The diabetic endothelial progenitor cells bind poorly to TNF α-activated human umbilical endothelial cells. Work currently underway in our laboratory demonstrates that TNF α may release VEGF from CML-treated choroidal explants to overproduce the choroidal neovascularization (unpublished data).

Since the results of cultured choroidal explants in the diabetic rat are not mimicked by the results of isolated aortic endothelial cells in the diabetic rat (unpublished data), extracellular matrixes and conditions in the explants may be impor-
tant for the induction of the neovascularization of diabetic choroid in culture.

In conclusion, STZ-diabetic state facilitates choroidal neovascularization through the release of TNF-α. Nifedipine may inhibit the action of TNF-α probably by blocking voltage-dependent Ca²⁺ channels in the endothelial progenitor cells of the diabetic choroid.

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