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Isolation and culture of porcine cerebrocortical microvessel endothelial cells (CMVEC) *in vitro*

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Abstract

The physiological role of cerebral microvasculature is to establish the blood-brain barrier (BBB). Cerebral microvascualr abnormalities have been implicated in some diseases such as diabetic complications in the central nervous system (CNS) and cognitive dysfunction. At present, the pathology of cerebral microvessel in diabetes mellitus is still uncertain. In this study, We investigated the effect of serum from STZ-induced diabetic rat and the several substrates including N^{ε}-(carboxymethyl)lysine (CML) and leukemia inhibitory factor (LIF) on the proliferation of the porcine microvessel endothelial cells (CMVEC). To do this, astrocytes were isolated from newborn Wister rats and astrocytes-conditioned medium was collected. Then the microvessels fractions were prepared from the porcine cerebral cortex. By treating outgrowing cells from the microvessels with the endotheliant cell trypsin, the porcine CMVEC were obtained. We observed that serum from STZ-induced diabetic rat significantly inhibited the cell proliferation. Neither CML (0.1-10 μg/mL) or LIF (0.01-30 ng/mL) alone produced any inhibitory effect on the proliferation of the porcine CMVEC. The proliferation inhibitory effect of serum from STZ-induced diabetic rat on the porcine CMVEC suggests that the diabetic serum contains some inhibitory factors, although they have not yet been identified. More work is required to elucidate the mechanism underlying the diabetes-induced cerebrovascular complication.

Key Words Cerebrocortical microvessel endothelial cells, Diabetic rat serum, Advanced glycation end product (AGE), N^{ε}-(carboxymethyl)lysine (CML), Leukemia inhibitory factor (LIF)

Introduction:

Cerebrovascular disease is an important complication of diabetes mellitus. Epidemiological studies show that diabetes is a risk factor for ischemic stroke and vascular dementia. The pathogenesis of diabetes-associated cerebrovascualr disease appears to be linked to excessive glycation, oxidation, endothelial cells dysfunction and insulin resistance[1]. It is generally accepted that cerebral microvessel endothelial cells form the so-called blood-brain barrier (BBB) in vivo, which regulates the homeostasis of the central nervous system. The cerebral capillaries display a typical ultrastructure crucial to execute BBB function. Brain-derived microvascular endothelial cells are characterized by minimal pinocytic activity, the absence of fenestrations, and the presence of highly resistant, tight intercellular junctions, which lead to the high resistance values of the brain endothelium. The endothelial cells are surrounded by a 30- to 40-nm-thick basement membrane (BM) which is often a target of investigation due to its frequently observed malformations under pathophysiological conditions (for example

Alzheimer's disease). The extracellular matrix components of the BM, namely the intrinsic collagen, heparan sulfate proteoglycan (HSPG), laminin and the extrinsic fibronectin are known to be produced by the cell types of the capillaries. Those BM constituents are arranged into a trilaminar structure with an endothelial layer (lamina rara interna), an astrocytic layer (lamina rara externa) and a transitory, fused layer in-between the two (lamina densa). Those characteristics endow endothelial cells with the ability to regulate the trans- and paracellular passage of most substances and drugs from blood into the brain[2]. Cerebral microvessel endothelial cells are also relevant to the pathophysiology of many CNS disorders such as diabetic complications, ischemic brain damage and dementia. Moreover, cerebral microvessel endothelial cells are key elements of tissue revascularization and angiogenesis, which may have important consequences for the repair of postischemic brain damage or tumor progression[2-3].

Materials and methods:

Materials

Heparin (Sigma), trypsin TRL (Worthington), collagenase type III (Gibco BRL) , DNAse (Worthington), endothelianl cell trypsin (Sigma), collagen type I (BD Bioscience), fibronectin (Roche), poly-D-Lysine (Sigma)

Collection of astrocyte-conditioned medium [4-5]

Astrocytes were isolated from newborn Wister rats (3 days old). Isolated cortices were fragmented and incubated with trypsin-EDTA in Dulbecco's modified Eagle medium (DMEM) at 37℃. The suspension was filtered through a 120- and 45-μm nylon mesh, respectively, and cultured for 3 days in DMEM containing 10% fetal bovine serum (10% FBS-DMEM) in plastic tissue flasks at 37℃, 10% CO_2. The medium was refreshed every other day. When confluent, the cultures were passaged with trypsin-EDTA in a split ratio of 1:3 to poly-D-lysine-coated flasks and grown to confluent again. Then, astrocytes-conditioned medium was collected every other day for 2 weeks.

Isolation of porcine cerebrocortical microvessel [4-5]

Fresh porcine brain was obtained at a slaughterhouse in Toyama and stored on the ice while transporting to the laboratory. Cerebellum and stem were removed and the hemisphere was separated. Then the hemispheres were carefully washed with cold PBS. In the Petri dish containing cold 10% FBS-DMEM, grey matter was collected, and cerebrocortical microvessel fragments were prepared by manual homogenization using a Wheaton homogenizer and subsequently trapped on 150-μm nylon meshes. Then the blood microvessels were digested in digest complex solution for 1 h at 37℃ and subsequently filtered through a 200-μm nylon meshes. In the end, the cerebrocortical microvessels fractions were resuspented in freeze mix (FBS with 10% DMSO) and stored at -80℃, after overnight, microvessels were transfered into liqid nitrogen.

Primary isolation of cerebrocortical microvessel endothelial cells [4-5]

Cerebrocortical microvessels in a vial were transfered into a tube with 37℃, 8.6% FBS-DMEM. After centrifuge, microvessels were resuspended with 10 mL 37℃, 10% FBS-DMEM, and transfered into collagen type I and fibronectin-coated flask. The flask was kept at 37℃, 10% CO_2 until microvessel attached to the flask. Then the medium was changed with 10 mL growth medium consisting of a 1:1 mixture of 10% FBS-DMEM and the astrocyte-conditioned medium supplemented with 100 U/mL heparin, and cells were cultured at 37℃, 10% CO_2 until primary ECs were confluent.

Passage of cerebrocortical microvessel endothelial cells

Upon reaching confluence, the cerebrocortical microvessel endothelial cells were suspended by exposure to EC trypsin. After centrifuge, CMVEC were resuspended, cells were then passaged in a split ratio of 1:3 to collagen type I - coated flasks.

Preparation of rat serum

Rats were decapitated and blood was acquired. Blood was kept in the ice for 1 h, and centrifuged at the speed of 3000 rpm for 30 min. Then serum was aspirated carefully and kept in -20℃. Before used, rat serum was deactivated in the water bath of 56℃ for 30 minutes, then filtered to sterilize.

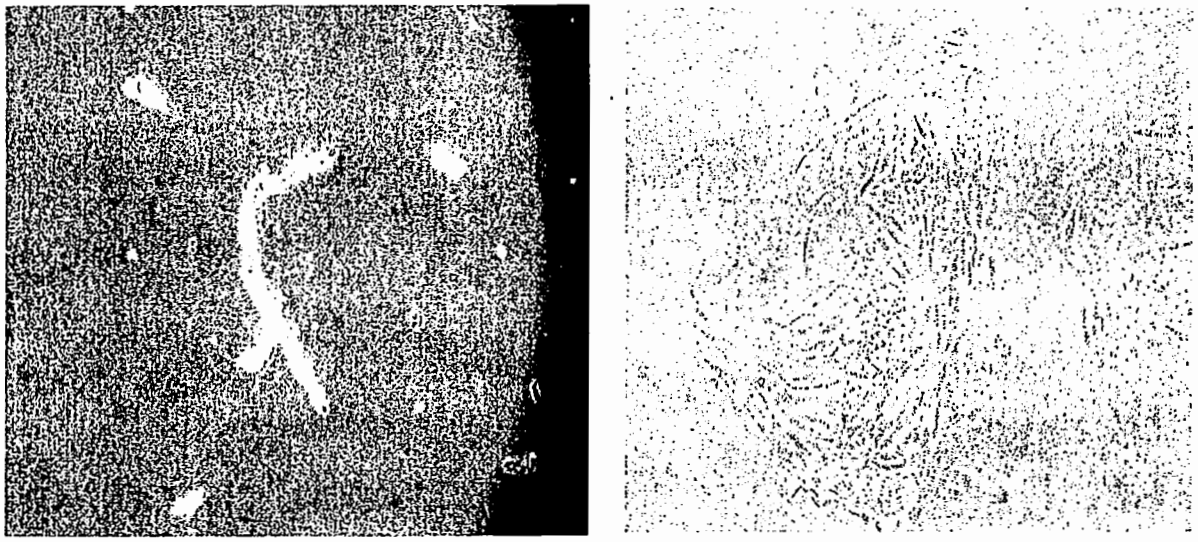
Proliferation assay

For counting the cell numbers, all cells were detached from dishes by treating with endothelial-cell trypsin. Trypan blue was added into the cell suspention to exclude dead cells, and the number of living cells was counted using Fuchs-Rosenthal hemacytometer.

Results:

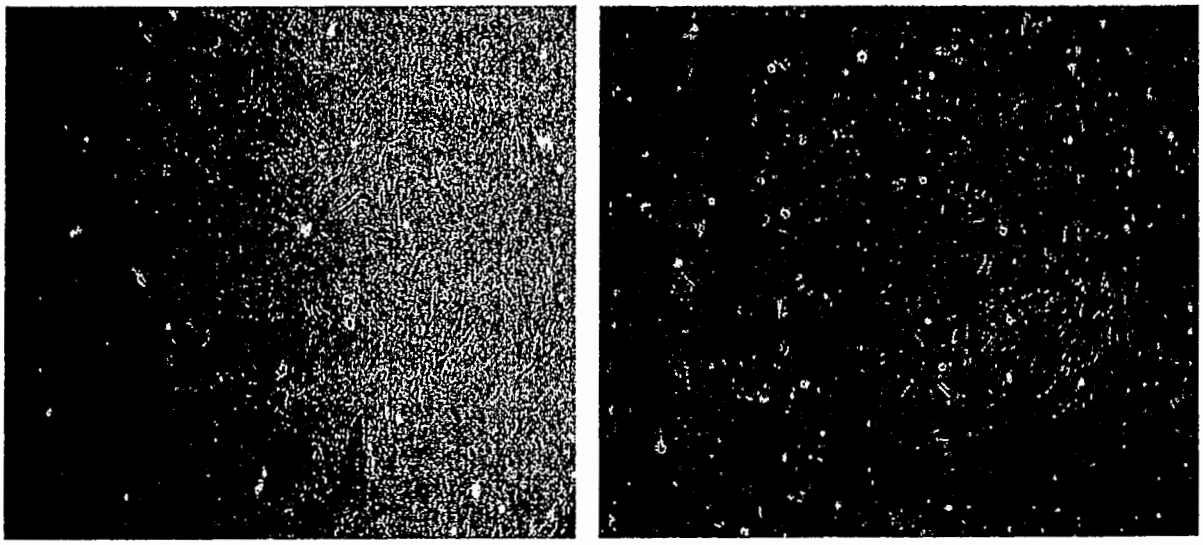
Isolation of porcine cerebrocortical microvessels

Morphology of porcine cerebrocortical microvessels were shown as Fig. 1 a and b. Five h after seeded, CMVEC began to atthach to flask, and cobblestone-shaped endothelial cells were growing from the attached microvessels after 6 day.



a b

Fig. 1 Morphology of the porcine cerebrocortical microvessels at 5 h (a) and 6 day (b) after seeded in flask coated with collagen type Ⅰ and fibronectin.



a b

Fig. 2 Morphology of the porcine cerebrocortical microvessel endothelial cells.

Isolation of porcine cerebrocortical microvessel endothelial cells

Morphology of confluented the porcine cerebrocortical microvessel endothelial cells was shown as Fig. 2. After confluent, the porcine cerebrocortical microvessel endothelial cells presented characteristic morphology, i.e, cobblestone shape, spindle shape and with a centered oval nucleus.

Effect of serum from diabetic rats on the growth of first passage porcine cerebrocortical microvessel endothelial cells

Porcine cerebrocortical microvessel endothelial cells were seeded in 35mm dishes coated with collagen type Ⅰ at the densities of 1×10^5 cells/mL and maintained in growth medium with different concentrations of FBS-DMEM at 37℃, 5% CO_2. And cells were allowed to grow for 2 h. Then serum from STZ-diabetic rat or normal rat was added in the end-concentration of 5%. Cells were allowed to grow for 7 day before subjected to the viability test by trypan blue assay. As showen in Fig. 3, when the porcine CMVEC were incubated with serum from diabetic rat, the growth of cells was significantly inhibited.

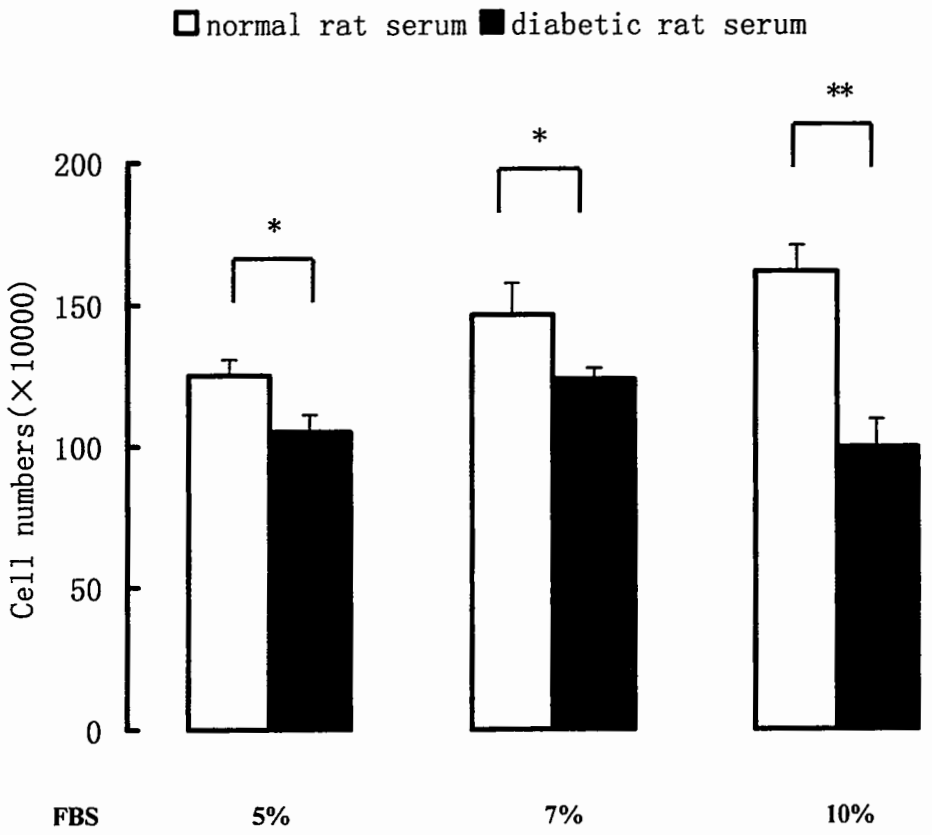


Fig. 3 Effects of serum from STZ-induced diabetic rat (25 weeks old) on the porcine CMVEC. Data are shown as mean±S.E.M (n=5). * $p<0.05$, ** $p<0.01$, difference between two groups was determined by Student's unpaired t-test.

Effect of N^{ε}- (carboxymethyl)lysine-HSA (CML-HAS) on the growth of first passage porcine cerebrocortical microvessel endothelial cells

First passage porcine cerebrocortical microvessel endothelial cells were seeded in 35mm dish coated with collagen type Ⅰ at the densities of 1×10^5 cells/mL. Cells were maintained in growth medium at 37℃, 5% CO_2 and grown for 2 h. Then CML-HSA in the concentrations indicated in the Fig. 4 were added. Cells were grown for 7 d before subjected to the viability test by trypan blue assay. As showen in Fig. 4, CML-HSA produced no obvious effects on the growth of the porcine CMVEC.

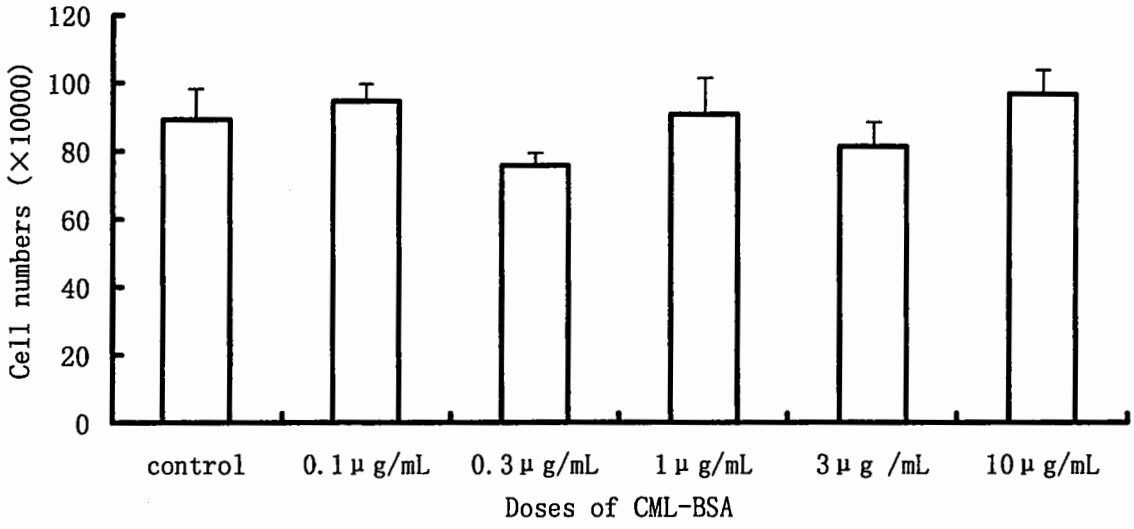


Fig. 4 Effects of N^{ε}-(carboxymethyl) lysine-HSA (CML-HAS) on the growth of the porcine CMVEC. Data are expressed as mean±S.E.M (n=4).

Effect of leukemia inhibitory factor (LIF) on the growth of the third passage porcine cerebrocortical microvessel endothelial cells

Porcine cerebrocortical microvessel endothelial cells (passage 3) were seeded in 35mm dish coated with collagen type I at the densities of 1×10^5 cells/mL. Cells were maintained in growth medium at 37℃, 5% CO_2 and grown for 2 h. Then LIF in the concentrations indicated in the Fig. 5 were added. Cells were grown for 9 d before subjected to the viability test by trypan blue assay. As showen in Fig. 5, LIF produced no obvious effects on the growth of CMVEC.

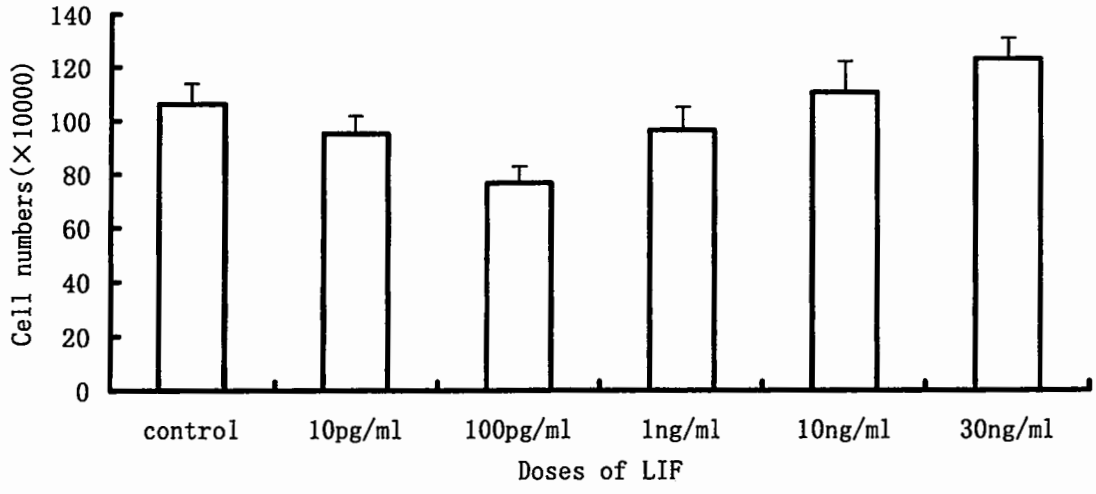


Fig. 5 Effects of leukemia inhibitory factor (LIF) on the third passage porcine CMVEC. Data are shown as mean±S.E.M (n=3-4).

Discussion:

Hyperglycemia is the major factor for development of various diabetic complication, although how hyperglycemia provokes these complications has not been fully elucidated yet. In this report, we have applied diadetic rat serum to treat the first passage CMVEC. As shown in Fig. 3, the diabetic serum inhibited the growth of cells. Because of complicated

component in the serum, it is however difficult to decide which factor acts to produce this effect. Recently, advanced glycation end products (AGE), non-enzymatically glycated protein derivatives, have been recognized as a candidate for some diabetic complication[6-7]. We further investigated the effect of CML-HSA on the porcine CMVES which is AGE oxidative sress product and predominant AGEs in human body in order to explain the roles of CML on microvascular brain diseases induced by diabetes. Yet, CML-HAS produced no obvious effects on the growth of the porcine CMVEC (Fig. 4).

LIF belongs to a family of IL-6-related cytokine and is known to inhibit the growth of macrovessel ECs[8]. Until now, no papers about effects of LIF on the growth of CMVEC was reproted. In our preliminary test, we have observed that LIF produced inhibitory effects on the bovine CMVEC at fourth passage. Here we investigated the effects of LIF on the porcine CMVEC at the third passage. Interestingly, LIF produced no obvious effects on the growth of the porcine CMVEC at the third passage (Fig. 5).

From our study, the inhibitory effect of serum from STZ-induced diabetic rat on the proliferation of CMVEC suggests that the diabetic serum contains some inhibitory factors related to microvesseel pathology in diabetes mellitus. Until now, they have not been identified. More work is required to elucidated the mechanism underlying the diabetes-induced cerebrovascular complication.

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