·Basic Research·

Advanced glycation endproducts enhance proliferation, but not tube formation in choroidal microvascular endothelial cells

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Abstract

• AIM: To investigate the role of advanced glycation endproducts (AGEs) in the pathogenesis of age-related macular degeneration (AMD).

• METHODS: Bovine choroidal endothelial cells (CEC) were isolated by the modified protocol using lycopersicon esculentum agglutinin coated Dynabeads, and identified by immunocytochemical staining with anti-Factor VIII antibody and uptaking of dil-acetylated low-density lipoprotein (dil-ac-LDL). AGEs were prepared by incubating 50g/L bovine serum albumin and 150g/L glucose at 37℃ for 6 weeks, which were characterized by dot blot assay with anti-AGEs antibody. CEC proliferation was evaluated by 3,(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay, and tube formation in CEC was determined by a Vitrogen system.

• RESULTS: More than 90% of the cultured cells were positive to Factor VIII immunostaining and had the ability to uptake dil-ac-LDL, which were the features of endothelial cells. 219AGEs we prepared were affinitive to anti-AGEs antibody. After treatment with AGEs for a time course of 3 days, CEC proliferation was significantly increased in a dose-dependent manner by AGEs at concentrations between 62.5mg/L and 500mg/L. The cytokine, basic fibroblast growth factor (bFGF), enhanced strongly tube-like structure formation in CEC to 124%($P<0.05$) above that of untreated controls. In this condition, AGEs at the concentrations of 500mg/L and 50mg/L showed no effect on CEC tube formation ($P>0.05$).

• CONCLUSION: The present study demonstrated that CEC proliferation was increased by AGEs. However, there was no statistical effect on CEC tube formation. These findings confirm and extend that AGEs could be a potential initiator in the pathogenesis of choroidal neovascularization in exudative AMD, at least in part, through enhancement of CEC proliferation.

• KEYWORDS:advanced glycation endproducts; angiogenesis; choroidal microvascular endothelial cells; proliferation; tube formation; age-related macular degeneration

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INTRODUCTION

The development of choroidal neovascularization (CNV) or angiogenesis is the major contributor to severe vision loss in patients with age-related macular degeneration (AMD) [1]. Although current treatments for CNV, such as laser photocoagulation, photodynamic therapy, pharmacological inhibition, surgical intervention, radiation and transpupillary thermotherapy,have been shown to be therapeutic in some cases, all treatment modalities have limitations [2, 3]. Future treatment advances should base on understanding of the pathogenesis of AMD; however, this remains largely unclear to date.

The prevalence of AMD increases dramatically with advancing age [4]. Among the current hypotheses about aging process, the accumulation of advanced glycation endproducts (AGEs) has been suggested as an important etiological initiator[5,6]. Moreover, it was demonstrated that cell activation in response to AGEs is associated with angiogenesis [5, 7-9]. Recently, immunohistological studies have sh own that AGEs accumulate in human Bruch's membrane and choroid

with age [10, 11], and were present in CNV of AMD [12, 13]. These studies have pointed to a causal link between the accumulation of AGEs and the pathogenesis of AMD. However, the interactions of choroidal microvascular cells with AGEs have not been investigated.

AGEs are a group of heterogeneous undefined structures, which are formed by the nonenzymatic glycation of sugars and proteins through the Maillard reaction [5,8,9,14]. Several studies have been reported about the effects of AGEs on non-choroidal macro- and microvascular cells [15-18]. The relevance of those studies to understand the pathogenesis of CNV is limited because endothelial cells are highly organ-specific and heterogeneous [19, 20]. Angiogenesis, the formation of new blood vessels from preexisting endothelium, is a complex multistep process which includes proliferation, migration and differentiation of endothelial cells, degradation of the extracellular matrix, tube formation and sprouting of new capillary branches [21]. To provide evidence that AGEs are directly implicated in choroidal angiogenesis, we tested the effects of AGEs on proliferation and tube formation in choroidal endothelial cells (CEC), which are crucial components of CNV formation.

MATERIALS AND METHODS

Reagents Lycopersicon esculentum agglutinin (LEA), collagenase/dispase and DNase, rabbit anti-human Factor antibody, fibronectin, bovine serum albumin (BSA, fraction, fatty acid free) and 3, (4,5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma, Saint Louis, USA; M-450 Dynabeads, from Dynal, Oslo, Norway; endothelial basal medium (EBM) and endothelial growth medium (EGM) supplements, from Clonetics, San Diego, California, USA; Dil-acetylated low-density lipoprotein (dil-ac-LDL), from Paesel & Lorei, Hanau,Germany; StreptABC/Horseradish peroxidase complex and diaminobenzidine (DAB) kit, from DAKO, Hamburg, Germany; human recombinant basic fibroblast growth factor (bFGF), from Life Technologies, UK; vitrogen was obtained from Cohesion, Palo Alto, California, USA; the AGEs as positive control and anti-mouse carboxymethyllysine (CML) monoclonal antibody (mAb 6D12) were generous gifts from Dr. HP Hammes, Giessen, Germany.

Cell Culture and Identification CEC were isolated by the modification of previously described method using LEA (a lectin specific for bovine microvessels)-coated Dynabeads [22] from bovine eyes obtained from a local slaughterhouse 12-16 hours after death. In brief, the eye was bisected posterior to the ora serrata and the anterior section was discarded. The vitreous and neural retina were removed from the posterior eyecup, and then retinal pigment epithelial cells (RPE) were gently scraped out. The choroid was dissected and microvessels were collected with the aid of forceps using a dissecting microscope. The vessels were digested with 1g/L collagenase/dispase and 17.3kU/L DNase at 37℃ for 90 minutes. The cell suspension was then filtered through a 70μm mesh filter (Falcon). The LEA-coated beads of 4×10^{11} beads/L were mixed with the filtrated cell suspension at a ratio of 1:3, and then were incubated at 4℃ for 30 minutes with agitation. The cells attached to the beads were harvested with a magnetic particle concentrator (MCP-1, Dynal, Oslo, Norway), and then were incubated in EGM (EBM supplemented with 12mg/L bovine brain extract, 1mg/L hydrocortisone, 20mL/L fetal calf serum (FCS), and 100mg/L gentamycin) in fibronectin-coated six-well culture plates (Falcon) in 50mL/L CO_2/950mL/L air at 37℃. Seven to 10 days later, the contaminating cell colonies were scraped out [23], and this procedure was repeated more than 3 times before CEC became confluent. Cells were confirmed by their positive immunostaining for Factor VIII and by their uptaking of dil-ac-LDL. For identification, cells were seeded at density of 1×10^4 cells/well onto fibronectin-coated chamber-slides (Nunc, Wiesbaden, Germany). One part of cells were fixed with 900mL/L ethanol and processed for FITC-immunocyto chemical staining with anti-human Factor VIII antibody (dilution 1:200) using routine procedures prior to fluorescence microscopic examination. Another part of cells were incubated with dil-ac-LDL at a final concentration of 10mg/L for 4 hours, and then was fixed in 30g/L buffered formaldehyde at room temperature for 20 minutes. After mounted, they were observed with a fluorescence microscope (Zeiss Axioskop HBO 50/AC, Germany) using standard rhodamine excitation. The parallel experiments were performed using bovine RPE and rat Müller cells. All experiments were carried out in the subconfluent cells at cell passage number 3 to 7.

Preparation and Characteristics of AGEs AGEs were prepared as described previously [17,24,25] by incubating 50g/L BSA in phosphate buffered saline(PBS) with 150g/L glucose at 37℃ for 3 days to 6 weeks under sterile conditions. Control unmodified BSA was incubated under the same conditions except for the absence of glucose. After unincorporated sugars were removed by dialysis against PBS, protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad, Muenchen, Germany) according to the manufacturer's instruction. The resultant preparations were characterized by antibody affinity with mAb 6D12 using dot blot analysis as previously described [26]. We loaded 1μL of

positive control AGEs, AGEs preparations and control BSA preparation at protein concentrations of 500, 100 and 50mg/L on nitrocellulose membranes (Biometra, Goettingen, Germany) and dried them at room temperature. Membranes were blocked with 30mL/L skim milk for 30-60 minutes, incubated with 1:5000 mAb 6D12 for 90 minutes, exposed to peroxidase-labelled anti-mouse IgG for 60 minutes, and then with StreptABC/Horseradish peroxidase complex for further 30 minutes. The staining was then developed in DAB solution. Between each step of the procedure, it is necessary to rinse sufficiently the membranes in PBS at room temperature.

Proliferation Assay CEC proliferation was evaluated using MTT colorimetric assay of Mosmann [27]. Briefly, CEC were trypsinized and plated out at a density of 4×10^3 cells in 100μL EGM per well into a fibronectin-coated 96-well plate and allowed to attach overnight. The cells were washed twice with PBS, and then were stimulated with 10μg/L bFGF, 500mg/L BSA control, or AGEs at various concentrations of 500, 250, 125 and 62.5mg/L in EBM with 10mL/L FCS. The cells cultured in EBM with 10mL/L FCS were used as negative control. The cells were incubated at 37℃ for 72 hours, and at the last 4 hours 10μL of 5g/L MTT was added in each well. The formazan crystals formed were dissolved in 100μL dimethyl sulfoxide and the optical density was recorded at 570nm on a Molecular Devices Spectra Max 250 Multiscan (BMG, Offenburg, Germany). The impact of AGEs on CEC proliferation was estimated by the percentage (%) compared to negative controls.

Tube Formation Assay The tube formation assay was performed as described by Sakamoto *et al* [28] with a minor modification. Four hundred μL Vitrogen (2.4g/L) containing 0.001mol/L sodium hydroxide, 200mmol/L HEPES, 5mg/L fibronectin and laminin, and 40μL of 10×DMEM medium was put into each well of 24-well plates and incubated at 37℃ for 2-3 hours to gel. After polymerization of the gels, 1.0×10^5 CEC were seeded onto each gel and incubated with 500μL EGM at 37℃ for 24 hours. The medium was then aspirated, and the remaining cells were overlaid with 120μL gel per well, and incubated for 1 hours at 37℃. Next, 50μg/L bFGF, AGE at concentrations of 50mg/L and 500mg/L, 500mg/L BSA in 500μL EBM with 10mL/L FCS, 100kU/L penicillin and 100mg/L streptomycin was added to each well. The incubation was continued to the 3rd day. To evaluate the lengths of tube-like structures in the gels, 4 photographs in different fields around center in each well were randomly taken at 3 days with a phase-contrast microscope (×5 objective). The negative films were then scanned and digitized. All tube structures in the resulting images were traced manually and the lengths of the tubes were automatically measured by the Image Analyzer System KS300 2.0 (Kontron Elektronic GmbH, Eching, Germany). The total additive length of all tube structures in 4 photos was represented as the tube length of the well. Results were shown as percentages compared with the negative controls.

Statistical Analysis Proliferation and tube formation assays were done in pentagonal and triplicate respectively; and the experiments were carried out on at least 3 different occasions. Results were shown as mean percentage standard deviation of the mean compared with the negative control of 3 experiments. Mean values were compared using One-way ANOVA and $P<0.05$ was considered significant.

RESULTS

Bovine CEC The plated cells became attached and flattened after 3-5 days. In the following 1-3 weeks, cell colonies were formed, and contaminating cells, distinguished from CEC by their appearance, were scraped away. The residual endothelial cells reached confluent in about 3-4 weeks after seeding. At this time, the cultures had the typical 'cobblestone' appearance of endothelium in a confluent monolayer (Figure 1A). Indirect immunocytochemical staining with anti-Factor antibody revealed that positive yellow-green staining was localized in cytoplasm or concentrated in the perinuclear space in the passaged cultures (Figure 1B). The assay for uptaking of dil-ac-LDL showed that cytoplasm was filled with red fluorescent granules in the positive cells (Figure 1C). By counting, more than 90% of the cultured cells were confirmed vascular endothelial cells by their positive immunostaining for Factor and their uptaking of dil-ac-LDL. The parallel experiments performed in bovine RPE and rat Müller cells had no positive staining (data not shown).

Dot Blot Analysis Immuno-dot-blot analysis showed that the highly modified AGEs (positive control) reacted markedly against mAb 6D12 (4-5+). We collected the preparations of BSA and glucose at various time points between 3 days and 6 weeks. Our AGE preparations started to react against this antibody at 2 weeks. The reactivity increased in a time-and concentration-dependent manner, which reached the maximal reactivity (3+) at 6 weeks (Figure 2). No immunoreaction was detected in BSA control at 6 weeks against the CML antibody. CML is a major AGE-epitope [29] and the mAb 6D12 is a medium affinity CML-protein-recognizing antibody [26]. The results indicated the presence of CML in our AGEs preparations, but not in the control BSA preparation. Based on the above results, AGEs and

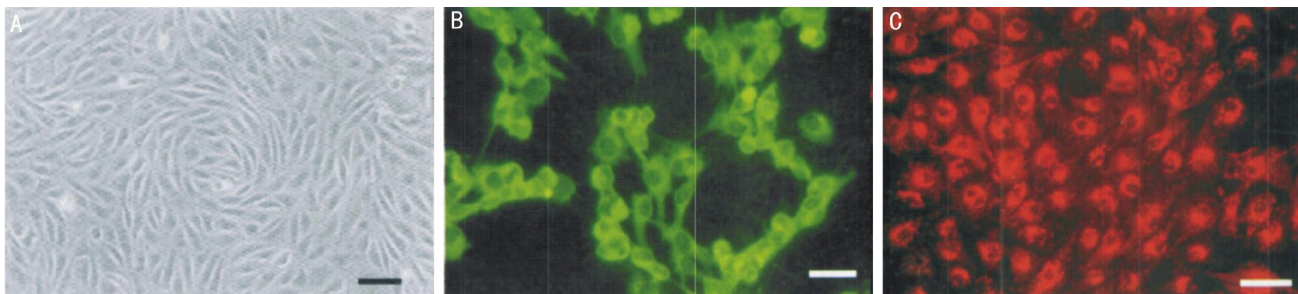


Figure 1 Morphology and identification of CEC A: The appearance of endothelium in a confluent monolayer under phase-contrast microscope. Bar, 80μm; B: Immunocytochemical staining with anti-Factor antibody (FITC). Bar, 35μm; C: Uptaking assay of dil-ac-LDL (Rhodamine fluorescence). Bar, 50μm

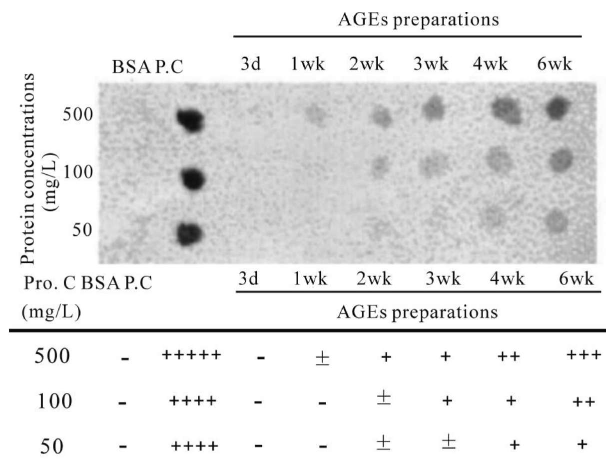
BSA preparations at 6 weeks were used in the following experiments.

CEC Proliferation It was observed that CEC proliferation was increased by 10μg/L bFGF by about 2.5 fold over the basal level (EBM group) (P<0.01). Treatment of CEC with AGEs at various concentrations between 62.5mg/L and 500mg/L resulted in a dose-dependent increase in CEC proliferation (Figure 3). CEC proliferation was significantly increased by about 44% (P<0.01) and 33% (P<0.05) by 500mg/L and 250mg/L AGEs respectively. There was no statistical effect of BSA on CEC proliferation (P>0.05).

CEC Tube Formation Tube formation was evaluated by Vitrogen collagen system. CEC were seeded into solidified Vitrogen and incubated in the presence of 50μg/L bFGF, AGEs or BSA as indicated for 48 hours. Stimulation of CEC with 50μg/L bFGF led significantly to tube-like structure formation (P<0.05). Under the conditions, both AGEs and BSA did not stimulate tube formation in CEC compared with basal level (P>0.05) (Figure 4, 5).

DISCUSSION

Nonenzymatic glycation, a complex series of reactions between sugars and amino groups of proteins, initiates with the reversible formation of Schiff's base, then undergoes a rearrangement to form stable Amadori product, and after that could end with the formation of irreversible AGEs through multiple chemical rearrangements. This reaction, with subsequent formation of AGEs has been implicated in aging process and accelerated in diabetes [5,14]. A lot of age-associated diseases, such as cataract, Alzheimer's disease and arteriosclerosis have been proposed to relate to AGEs accumulation [9]. Recently, Handa's group [10, 11] postulated that the formation and accumulation of AGEs in human Bruch's membrane and choroid could promote aging of the RPE-Bruch's membrane-choroid complex. Ishibashi *et al*[12] found that AGEs (CML) accumulated in soft, macular drusen in aged eyes, and in RPE in surgically excised CNV



Pro. C (mg/L)	BSA	P.C	3d	1wk	2wk	3wk	4wk	6wk
500	-	+++++	-	±	+	+	++	+++
100	-	++++	-	-	±	+	+	++
50	-	++++	-	-	±	±	+	+

Figure 2 Dot blot analysis of AGEs using an anti-CML antibody There were common structures between CML and AGEs preparations. The density of the dots was scored into 7 levels, e.g., negative (-), suspected positive (±), and slightly to strongly positive (1-5+). Pro. C., protein concentrations; P.C., positive control. The numbers and characters, 3 days, 1 week, 2 weeks, 6 weeks, indicate that incubation time for preparation of AGEs are 3 days, 1 week, 2 weeks and 6 weeks, respectively

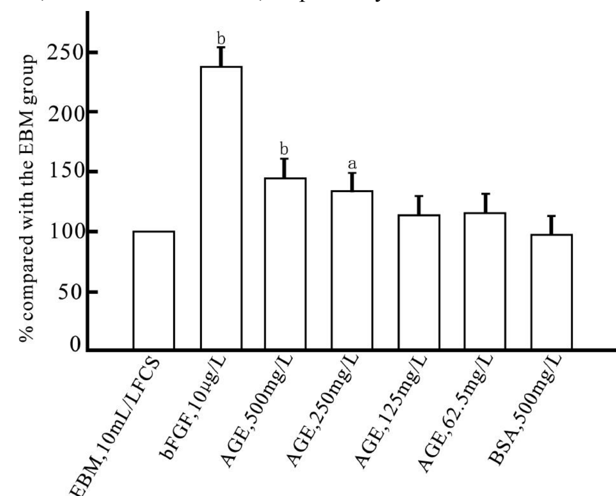


Figure 3 Effects of AGEs on CEC proliferation CEC proliferation was increased by AGEs in a dose-dependent manner. Each column represents mean % compared with EBM group standard deviation of 3 independent experiments. [a]P<0.05, [b]P< 0.01 compared with EBM group

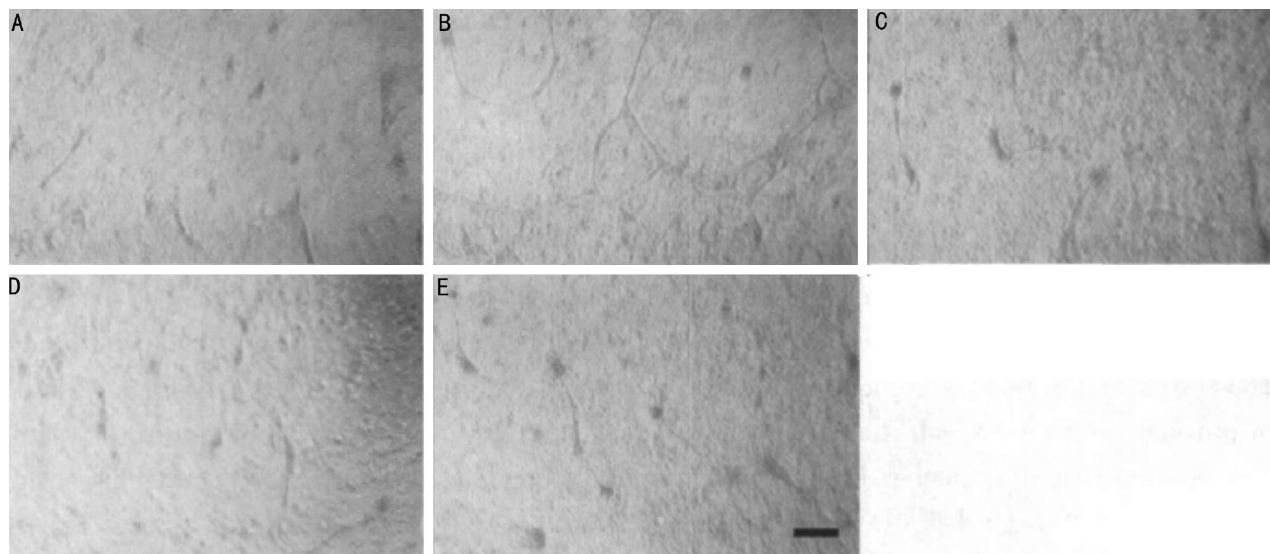


Figure 4 The representative morphology of tube-like structures in CEC The tube-like structures in CEC were actively increased by stimulation of bFGF(B). There were no obvious changes in the groups of 500mg/L AGEs (C), 50mg/L AGEs (D) and 500mg/L BSA group (E) compared with EBM group (A). Bar, 250μm

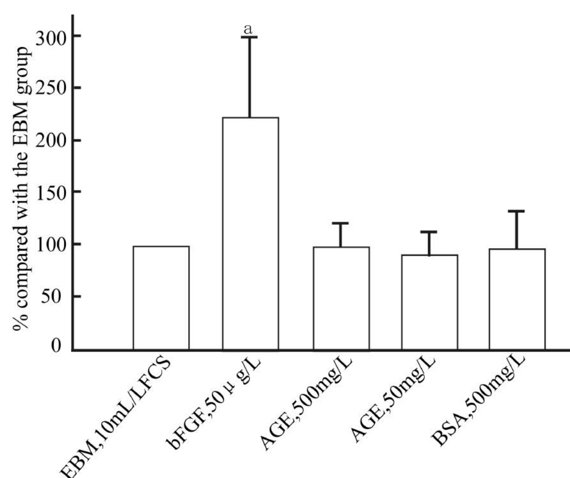


Figure 5 Effects of AGEs on CEC tube formation The tube-like structures in CEC were increased by bFGF, but not by AGEs and BSA. Each column represents mean % compared with EBM Group's standard deviation of 3 independent experiments.[a] $P<0.05$ compared with EBM group

from patients with AMD. Furthermore, Hammes *et al*[13] demonstrated AGEs were adjacent or colocalized with one of the receptors for AGEs (RAGE) in CNV of patients with AMD. Those evidences indicated that AGEs could play a role in the pathogenesis of AMD; however, the precise mechanism should be investigated.

Given that endothelial cells are highly organ specific and heterogeneous [19, 20], it is our interest to evaluate the direct effects of AGEs on the CEC, which is a key cellular component in choroidal angiogenesis. In this study, we showed that AGEs increased CEC proliferation in a dose-dependent manner. Moreover, there was no statistically significant influence of AGEs on tube formation in CEC. However, the tube-like structures were well formed in the bFGF- stimulated group. These findings are suggestive of an implication of AGEs in choroidal angiogenesis, at least through increasing CEC proliferation directly.

The inconsistent effects of AGEs on the substeps of angiogenesis in different endothelial cell systems have been described, although almost all studies indicated that AGEs exert a potent angiogenic effect on endothelial cells. For example, in human umbilical vein endothelial cells, tube formation and migration but not proliferation were stimulated by AGEs [15]. Therefore, the growth (cell number and DNA synthesis) of retinal microvascular endothelial cells treated with AGEs was significantly increased [16]. AGEs were found to induce a significant increase in the number of cells as well as their synthesis of DNA, and to stimulate the tube formation in human skin microvascular endothelial cells [17]. Exceptionally, AGEs at a higher concentration (500mg/L) reduced the cell number because of cytotoxicity to bovine aortic endothelial cells; however, low concentration (62.5mg/L) of AGEs was significantly mitogenic to the cells [18]. One explanation for these discrepant results may be the nature of the cells used in the studies, although other differences in experimental conditions, e.g. AGEs preparation and test methodology, could not be excluded.

Our research was performed using well-characterized cells and AGEs. The CEC we used in this study were isolated by

LEA, a specific lectin for bovine microvessels, and then identified by positive staining to Factor and uptaking of LDL, both distinctive markers for endothelial cells [22]. The AGEs were prepared using the classic protocol as previously described by many researchers [17, 24, 25], and immuno-dot blot analysis has demonstrated that there are common structures between CML (a major AGE-epitope [29]) and our AGEs preparations. The current results on proliferation was consistent with our previous research, which showed that CEC proliferation was enhanced by another kind of AGEs made by incubation at 50℃ for 4 weeks [30]. With regard to the validity and reliability of the methodology for the tube formation assay, it was morphologically and histologically demonstrated that tube-like structures in gel were formed in cytokine-stimulated CEC (the histological data not shown). In our experience, it seems to need more cytokines to stimulate tube formation than to increase proliferation in CEC. Cytokine bFGF of 3μg/L could increase CEC proliferation by about 150%; however, about 20μg/L bFGF may be necessary for reaching the similar level of increasing tube formation in CEC (unpublicated data). Autocrine of cytokines, such as vascular endothelial growth factor (VEGF) by endothelial cells has been considered as a possible angiogenic mechanism of AGEs [17,30]. One possible explanation of our results is that the amount of cytokines in the medium secreted by AGEs-stimulated CEC is sufficient to increase proliferation, but not tube formation in CEC. Of course, we could not exclude other possibilities for explanation of the present results, including the heterogeneity of endothelial cells.

The molecular mechanism used by AGEs to drive angiogeneis is beyond the scope of this paper, but several hypotheses exist. In fact, AGEs have been shown to enhance the following events: ① the expression of VEGF mRNA and/or the production of VEGF protein in cultured Müller cells [24], human RPE and bovine vascular smooth muscle cells (SMC) [31], and microvascular endothelial cells [17,30]; ② the production of platelet-derived growth factor (PDGF)-BB in RPE [32]; and ③ the synthesis of insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) in mesangial cells [33]. It indicates that the effects of AGEs could be mediated by several growth factors and/or cytokines secreted in paracrine and/or autocrine fashions. Another possibility linking AGEs with angiogenesis is that AGEs could induce expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells, which enhanced adhesivity of circulation monocytes [34]. In addition, AGEs could elicit their cellular effects by modi fying the extracellular matrix [8] or the nitric oxide synthase/ nitric oxide (NOS/NO) pathway in endothelial cells [35]. Most of those effects are supposed to be mediated by the interaction of AGEs with cell surface receptors and other AGEs binding proteins, which have been identified on different cell systems including monocytes, SMC and endothelial cells [7, 8]. The binding of AGEs with their receptors or binding proteins has been shown to induce a cellular- oxidant stress activating the transcription factor NF-κB, which further mediates gene expression of cytokines and VCAM-1 [7,8,36].

However, no matter which of mechanisms may be involved in AGEs function, the present results suggested a quite possibility that their direct impact on CEC may take part in the choroidal angiogenesis. We hypothesized that accumulation of AGEs with age changes the biological behavior of CEC through less well-known mechanisms, at least in part, increases CEC proliferation, and then could contribute to the development of AMD. The immunohistochemical studies confirming the presentation of AGEs and their receptor in CNV of AMD [12,13] are significantly consistent with our results. The current and future research efforts will hopefully lead to a better understanding of the pathogenesis of these changes and ultimately to a cure for AMD.

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REFERENCES

1 Bressler SB, Maguire MG, Bressler NM, Fine SL. Relationship of drusen and abnormalities of the retinal pigment epithelium to the prognosis of neovascular macular degeneration. The Macular Photocoagulation Study Group. *Arch Ophthalmol* 1990;108:1442-1447

2 Newsom RS, McAlister JC, Saeed M, McHugh JD. Transpupillary thermotherapy (TTT) for the treatment of choroidal neovascularisation. *Br J Ophthalmol* 2001;85:173-178

3 Ciulla TA, Danis RP, Harris A. Age-related macular degeneration: a review of experimental treatments. *Surv Ophthalmol* 1998;43:134-146

4 Lee P, Wang CC, Adamis AP. Ocular neovascularization: an epidemiologic review. *Surv Ophthalmol* 1998;43:245-269

5 Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 1988;318:1315-1321

6 Schleicher ED, Wagner E, Nerlich AG. Increased accumulation of the glycoxidation product N (epsilon)- (carboxymethyl)lysine in human tissues in diabetes

and aging. *J Clin Invest* 1997;99:457-468

7 Thornalley PJ. Cell activation by glycation proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol (Noisy-le-grand)* 1998;44:1013-1023

8 Chappey O, Dosquet C, Wautier MP, Wautier JL. Advanced glycation end products, oxidant stress and vascular lesions. *Eur J Clin Invest* 1997;27:97-108

9 Stitt AW. Advanced glycation: an important pathological event in diabetic and age related ocular disease. *Br J Ophthalmol* 2001;85:746-753

10 Handa JT, Verzijl N, Matsunaga H, Aotaki-Keen A, Lutty GA, te Koppele JM, Miyata T, Hjelmeland LM. Increase in the advanced glycation end product pentosidine in Bruch's membrane with age. *Invest Ophthalmol Vis Sci* 1999;40: 775-779

11 Farboud B, Aotaki-Keen A, Miyata T, Hjelmeland LM, Handa JT. Development of a polyclonal antibody with broad epitope specificity for advanced glycation endproducts and localization of these epitopes in Bruch's membrane of the aging eye. *Mol Vis* 1999;5:11-16

12 Ishibashi T, Murata T, Hangai M, Nagai R, Horiuchi S, Lopez PF, Hinton DR, Ryan SJ. Advanced glycation end products in age-related macular degeneration. *Arch Ophthalmol* 1998;116:1629-1632

13 Hammes HP, Hoerauf H, Alt A, Schleicher E, Clausen JT, Bretzel RG, Laqua H. N (epsilon)(carboxymethyl)lysin and the AGE receptor RAGE colocalize in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 1999;40:1855-1859

14 Bucala R, Cerami A. Advanced glycosylation: chemistry, biology, and implications for diabetes and aging. *Adv Pharmacol* 1992;23:1-34

15 Tezuka M, Koyama N, Morisaki N, Saito Y, Yoshida S, Araki N, Horiuchi S. Angiogenic effects of advanced glycation end products of the Maillard reaction on cultured human umbilical cord vein endothelial cells. *Biochem Biophys Res Commun* 1993;193:674-680

16 Ruggiero-Lopez D, Rellier N, Lecomte M, Lagarde M, Wiernsperger N. Growth modulation of retinal microvascular cells by early and advanced glycation products. *Diabetes Res Clin Pract* 1997;34:135-142

17 Yamagishi S, Yonekura H, Yamamoto Y, Katsuno K, Sato F, Mita I, Ooka H, Satozawa N, Kawakami T, Nomura M, Yamamoto H. Advanced glycation end products-driven angiogenesis *in vitro* Induction of the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *J Biol Chem* 1997;272:8723-8730

18 Chibber R, Molinatti PA, Rosatto N, Lambourne B, Kohner EM. Toxic action of advanced glycation end products on cultured retinal capillary pericytes and endothelial cells: relevance to diabetic retinopathy. *Diabetologia* 1997;40:156- 164

19 Fajardo LF. The complexity of endothelial cells. A review. *Am J Clin Pathol* 1989;92:241-250

20 Lelkes PI. Conference report: endothelial cell heterogeneity and organ specificity. *Endothelium* 1993;1:69-70

21 Beck L Jr, D'Amore PA. Vascular development: cellular and molecular regulation. *FASEB J* 1997;11:365-373

22 Hoffmann S, Spee C, Murata T, Cui JZ, Ryan SJ, Hinton DR. Rapid isolation of choriocapillary endothelial cells by Lycopersicon esculentum-coated Dynabeads. *Graefes Arch Clin Exp Ophthalmol* 1998;236:779-784

23 Folkman J, Haudenschild CC, Zetter BR. Long-term culture of capillary endothelial cells. *Proc Natl Acad Sci USA* 1979;76:5217-5221

24 Hirata C, Nakano K, Nakamura N, Kitagawa Y, Shigeta H, Hasegawa G, Ogata M, Ikeda T, Sawa H, Nakamura K, Ienaga K, Obayashi H, Kondo M. Advanced glycation end products induce expression of vascular endothelial growth factor by retinal Müller cells. *Biochem Biophys Res Commun* 1997;236: 712-715

25 Kunt T, Forst T, Wilhelm A, Zschabitz A, Gopfert A, Kann P, Pfuetzner A, Harzer O, Engelbach M, Beyer J, Stofft E. Establishment of a quantitative RT-PCR for detection of vascular cell adhesion molecule-1 transcripts in endothelial cells after stimulation with advanced glycation endproducts. *Int J Mol Med* 1998;2:455-460

26 Hammes HP, Alt A, Niwa T, Clausen JT, Bretzel RG, Brownlee M, Schleicher ED. Differential accumulation of advanced glycation end products in the course of diabetic retinopathy. *Diabetologia* 1999;42:728-736

27 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65: 55- 63

28 Sakamoto T, Ishibashi T, Kimura H, Yoshikawa H, Spee C, Harris MS, Hinton DR, Ryan SJ. Effect of tecogalan sodium on angiogenesis *in vitro* by choroidal endothelial cells. *Invest Ophthalmol Vis Sci* 1995;36:1076-1083

29 Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW. N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 1995;34:10872-10878

30 Hoffmann S, Friedrichs U, Eichler W, Rosenthal A, Wiedemann P. Advanced glycation end products induce choroidal endothelial cell proliferation, matrix metalloproteinase-2 and VEGF upregulation *in vitro*. *Graefe's Arch Clin Exp Ophthalmol* 2002;240:996-1002

31 Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I, Bucala R, Adamis AP. Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest* 1998;101:1219-1224

32 Handa JT, Reiser KM, Matsunaga H, Hjelmeland LM. The advanced glycation endproduct pentosidine induces the expression of PDGF-B in human retinal pigment epithelial cells. *Exp Eye Res* 1998;66:411-419

33 Pugliese G, Pricci F, Romeo G, Pugliese F, Mene P, Giannini S, Cresci B, Galli G, Rotella CM, Vlassara H, Di Mario U. Upregulation of mesangial growth factor and extracellular matrix synthesis by advanced glycation end products via a receptor-mediated mechanism. *Diabetes* 1997;46:1881-1887

34 Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 1995;96:1395-1403

35 Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB. Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* 1998;47:945-952

36 Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Berentshtein E, Tritschler H, Muller M, Wahl P, Ziegler R, Nawroth PP. Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. *Diabetes* 1997;46: 1481-1490