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Original Article

High glucose and AGEs inhibit tube formation of vascular endothelial cells by sustained Ang-2 production

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Abstract: *Objective:* To explicit abnormal expression of angiopoientins and their signal pathway in the mechanism of neovascularization disorder and to provide a theoretical basis for the treatment of diabetic wound with improving neovascularization. *Method:* In the condition of AGEs and high concentrations of glucose interfere (30 mmol/L D-glucose; 150 mg/L AGE-BSA; 30 mmol/L D-glucose+150 mg/L AGE-BSA; 5 mmol/L D-glucose; 30 mmol/L Mannitol), cultured endothelial cell healing rates were observed after injury and the ability to form vascular-like structure in three-dimensional culture system were observed. The protein expression of Ang-2, VEGF and Tie-2 receptor expression from the condition cultured endothelial cells were measured. *Results:* There were no significant difference in the healing rates between high glucose, AGE, glucose plus AGE and normal treated group when the cells were suffered with injury. High glucose and AGE inhibit capillary morphogenesis for cultured endothelial cells in matrigel matrix. Expression of Ang-2 levels in vascular endothelial cells treated by AGEs and high glucose were significantly higher than normal. VEGF is significantly lower than that in the control group; Tie-2 receptors in cultured cells treated by high glucose were expressed in vascular endothelial cytoplasm and nucleus, while Tie-2 expressed only in the nucleus with AGE treatment, and in the membrane with normal control.

Keywords: Diabetes mellitus, impaired wound healing, angiogenesis, endothelial cell

Introduction

Chronic non-healing wound is a common complication of diabetes mellitus. Diabetic wounds exhibit delayed or non-healing wound closure, which shows poor or impaired angiogenesis. Angiogenesis is an essential event of the wound-healing process. The formation of new blood vesselsfrom pre-existing capillaries can ensure the delivery of oxygen, nutrients and cytokines to the wound and carry out wastes. Our previous study found the diabetic rats with chronic wound model show inhibited vascularity in the wound edge.

Advanced glycation end products (AGEs) are derived from nonenyzymatic glycated protein by sugar which is irreversible removed from the protein. AGEs are known to contribute to the pathogenesis of diabetic wound. AGEs have been observed to accumulate around vessels in the skin, which leads to impaired angiogenesis.

Angiogenesis, including endothelial proliferation, migration and capillary tube-like formation, is regulated by many angiogenic factors. Angiopoietins and their cognate receptor play an important role in physiological vessel growth and in pathological angiogenesis. Ang-1 acts via phosphorylation of the Tie-2 receptor, stabilising the endothelium and promoting peri-endothelial cell recruitment to the newly formed vessels. Ang-2 was originally identified as a natural antagonist of Ang-1, inhibiting Ang-1-induced Tie-2 phosphorylation and its subsequent effects. The effect of Ang-2 is VEGF-dependent as Ang-2 in combination with high VEGF levels induces sprouting angiogenesis, whereas, in the absence of VEGF, it results in capillary regression [5]. The building and remodelling of blood vessels is a critical event in the formation of every organ, and the relationship between the blood vessels and the tissues they serve is tightly balanced between stasis and growth, and regression. We have demonstrated

that Ang-2 was sustained increased in diabetic wound.

However, the relationship among AGEs, Ang-2 production and angiogenesis impairment in diabetic wound is not clear. In this study, we investigated the relationship among AGEs, sustained abnormal high expression of Ang-2 and angiogenesis in human microvascular endothelial cells.

Material and methods

Human umbilical endothelial cell (HUVEC) was purchased commercially (Life Technologies, Carlsbad, CA). The cells were cultured and maintained on type I collagen coated tissue culture plastic at 37°C with 5% CO_2 in MCDB-131 (Gibco, Grand Island, NY, USA) medium supplemented with 10 mmol/L L-glutamine (Invitrogen, Grand Island, NY, USA) 10 μg/ml gentamycin (Gibco), 5 pg/ml bFGF (Invitrogen) and 10% fetal bovine serum (Gibco). For all experiments, HUVECs were seeded on study day-2 at 5×10^3 cells/cm^2 in medium 131, which contained 5 mM D-glucose. On day 0, cells were exposed to experimental conditions for 24 hours. According to previous papers and study, we have chosen to add 30 mmol/L D-glucose (Glucose), 150 mg/L AGE-BSA (AGEs), 30 mmol/L mannitol (as control for hyperosmolarity) to medium respectively. We setup normal group (nothing to addition except for MCDB-131 fluid containing 5 mmol/L glucose by itself).

Preparation of AGE-BSA

We modified Tessier et al. method. Briefly as follows: BSA was incubated with 200 mmol/L glucose, 100 μg/ml penicillin, 50 μg/ml gentamycin and 1.5 mmol/L PMSF phosphate buffer (100 mmol/L) at 37°C with 5% CO_2 for 60 days. Meanwhile, the control was the same incubation as above mentioned without glucose addition. The production was dialyzed in sterile phosphate buffer solution (pH7.4) to remove unbound glucose at the end of the incubation. AGE-BSA prepared was identified by the anti-AGEs monoclonal antibody enzyme-linked immunosorbent assay (ELISA) and fluorescence spectrophotometry.

Electrical wound-healing assay for cells in vitro

Electric cell substrate impedance sensing (ECIS) was used to measure endothelial cells migration. Before starting ECIS measurements, 200 μl of MCDB131 medium supplemented with 10% FBS was placed in each well and was allowed to equilibrate in the incubator for 30 min. To inoculate the wells, 200 μl of HUVEC suspension (5×10^5 cells per ml) were added to each well, resulting in a final concentration of 1.25×10^5 cells per cm^2 and a volume of 400 μl of MCDB131 medium supplemented with 10% FBS until fully confluent, after which the media were replaced with different kinds of treatment media for 24 h. To induce wound repair, a uniform circular lesion (250 μm diameter electrode) was produced by lethal electroporation (4 V, 40 kHz, 10 s) of cells in contact with the electrode. Cells were then washed with PBS using a 23-gauge needle to dislodge any remaining electroporated cells from the electrode, and then the treatment media were added. Alternating current (1 μA, 15 kHz) was then continuously applied to the electrode to measure impedance (Z) and track wound repair over a period of 24 h. The time required for complete wound closure was determined from the impedance time courses. At wound closure the impedance plateaus, and the time at which this plateau occurred in each well of ECIS arrays under each treatment condition was reported as the time to wound closure. Experiments were performed in a humidified 5% CO_2 incubator at 37°C.

RT-PCR assay

After 24 hours conditional treatment, total RNA was extracted from the HUVECs using Trizol Reagent (Invitrogen, Carlsbad, CA) followed by purification with the PureLink RNA Mini Kit (Life Technologies, Grand Island, NY), which includes a DNase digestion step. cDNA was then synthesized from 250 ng RNA using a Omniscript RT kit (Qiagen, Valencia, CA). Real time PCR was performed in a ViiA™ 7 instrument (Applied Biosystems, Foster City, CA) using Quantitect SYBR green PCR kit (Qiagen, Valencia, CA). Primer used in this study was designed by our laboratory. The comparative C_t method ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression levels and all the values were normalized to the endogenous housekeeping gene, ACTB; GFA had no significant effect on ACTB cycle threshold in microvascular endothelial cell.

The primers' sequences were described as follows: Ang-2, left primer: 5'-tccggcgaggagtcta-

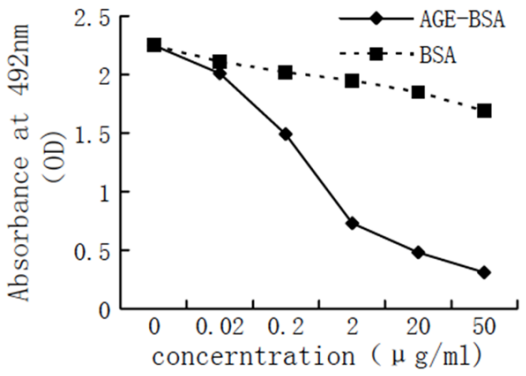

Figure 1. Detection of AGEs by ELISA.

acta-3'; right primer: 5'-aagttggaaggaccacatgc-3'; VEGF, left primer: 5'-caatgatgaagccctggagt-3', right primer: 5'-tttcttgcgctttcgttttt-3'.

siRNA-mediated Inhibition of Ang-2 expression in HUVECs

A siRNA approach similar to that described by Elbashir et al. (27) was used to evaluate the consequences of decreased expression of Ang-2 in the HUVECs. Cells were seeding at a 6 well tissue culture plate, seed 2×10^5 cells per well in 2 ml antibiotic-free normal growth medium supplemented with FBS. When the cells were 60-70% confluent, human Ang-2 siRNA (Santa Cruz) was introduced into HUVECs as following the protocol. siRNAs were formed at room temperature using 4 μl optimal solutioin of siRNA duplex. The cells were incubated 6 h at 37°C. 1 ml of serum-enriched medium was then added, and the cells were cultured for an additional 24-48 h. The transiently transfected cells were harvested, and the levels of Ang-2 protein were measured using the above SDS-PAGE immunoblot approach.

Protein analyses

HUVECs were seeded onto type I coated 6-well plates at a density of 4.0×10^4 cells/well. Cells were treated as indicated condition medium as mentioned above in 37°C, 5% CO_2 incubator for 24 hours. Colleting cells and homogenized in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 5 mM EDTA and 50 mM Tris supplemented with protease and phosphatase) using a homogenizer (Brinkmann Instruments, Inc., Westbury, NY). extracts were separated on 10% sodium dodecyl sulphate-polyacryalamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to polyvinylidene fluoride (PVDF) membrane, and probed with anti-Ang-2 (1:400 dilution, Santa Cruz, USA) in Tris-buffered saline containing 3% BSA (3% bovine serum albumin) and 0.1% (v/v) Tween-20 for 1 h. The membrane was washed three times in Tris-buffered saline containing 0.1% (v/v) Tween-20, followed by incubation with horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h. Immunoreactive bands were detected by NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt). The same immunblots were incubated with the anti-β-actin antibody (Sigma) after blot stripping. Gel loading was normalized for equal β-actin.

HUVECs tube formation assay

HUVECs were seeded onto cultured growth factor-reduced Matrigel in wells of 24-well plates at a density of 4.0×10^4 cells/well. Cells with or without siRNA-Ang-2 transfection was treated as indicated condition medium as mentioned above. At same time, HUVECs were seeded into 24-well plates without Matrigel as blanked control. Twenty-four hours later, Tube formation was examined under a phase-contrast microscopic observation. To quantify the length of capillaries, three different phase-contrast photomicrographs (10 objectives) per well were taken, and the length of each capillary was measured using NIH Image software (version 1.64). The tube formation was expressed as -fold increase relative to those cells cultured in blanked control.

Tie-2 expression in HUVECs

HUVECs were treated as indicated condition in 37°C, 5% CO_2 incubator for 24 hours, then abandoning nutrient solution, washing with PBS twice. Cells werefixed by PBS (0.2% TritonX-100) for 5 min. With PBS containing 4% paraformaldehyde fixed 10 min, washing by PBS 3 times, each time 5 min. Add methanol, fixed -20°C for 5 min, PBS wash 3 times, each time 5 min, 1% BSA enclosed 1 h at room temperature. Join the rabbit source resistance to Tie-2 antibodies (1:200 dilution), 4°C for the night. PBS wash 3 times, each time 5 min. Add an rabbit FITC fluorescent antibody (1:200 dilution), avoid light incubation for 1 h at room temperature. PBS

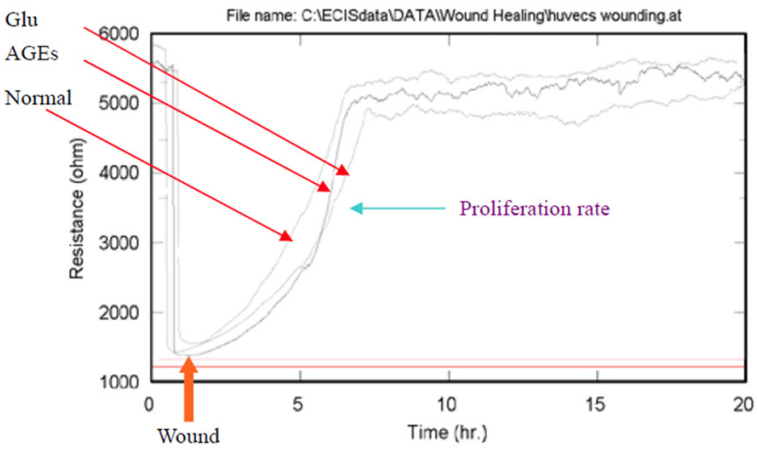

Figure 2. Effects of AGEs exposure on HUVECs migration.

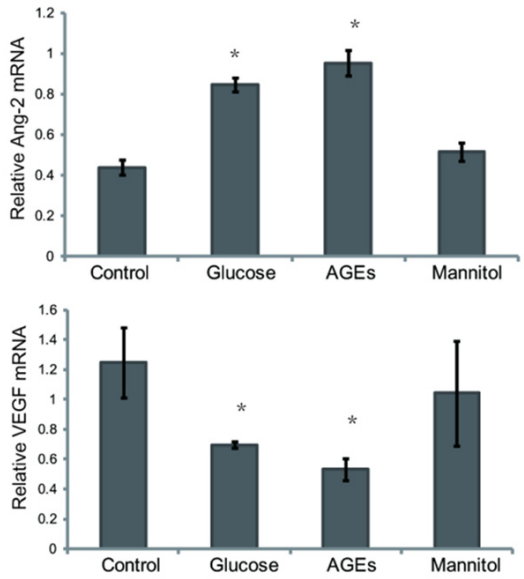

Figure 3. Effect of AGEs exposure on Ang-2, VEGF gene expression.

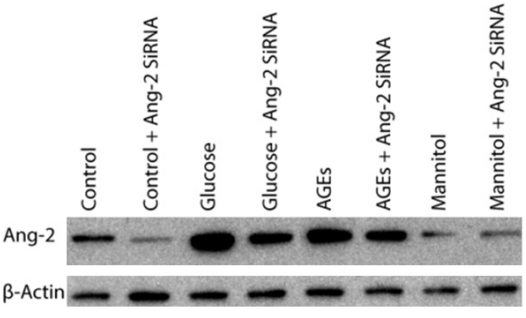

Figure 4. Effect of AGEs exposure on Ang-2 protein production with or without Ang-2 siRNA.

wash 3 times, each time 5 min. 50% glycerol sealing piece, fluorescence microscope.

Statistical analyses

All values are expressed as a mean ± standard deviation. The Student's *t*-test or ANOVA was used to determine statistically significant differences among groups, with *P*-values less than or equal to 0.05 considered significant.

Results

The concentration of the AGE-BSA

The results of fluorescence spectrophotometry showed: Content of AGE between the AGE-BSA group and control group, respectively (122.77-3.24) U/mg protein and protein (37.22-0.47) U/mg, and it can be Statistical analysis ($P<0.05$). AGEs were detected by ELISA (**Figure 1**).

Effects of AGEs exposure on HUVECs migration

We performed ECIS to observe the HUVECs proliferation and migration by monitoring changes in the system impedance. In this case, a 3-V signal at 40 kHz is applied for a duration of 10s. The application of this field results in a very rapid drop in the resistance of cell layers to that associated with the cell-free electrode. In the next few hours, this resistance is observed to increase back to the levels of the cell-covered electrode due to the migration of cells from the perimeter of the electrode inward to replace the injuried cells. We found the rate of cell wounding curve in the high glucose, AGEs and control medium are similar to each other. There is no difference among them (**Figure 2**).

Effect of AGEs exposure on Ang-1, Ang-2, VEGF gene expression

Exposure of HUVECs to high glucose and AGEs resulted in a significant increase in Ang-2 expression; this was accompanied by a dramatic decrease in VEGF expression as compared to low glucose conditions (LG; 5 mmol/L). Ang-1 expression was undetectable in HMVECs under each conditions (data not shown) (**Figure 3**).

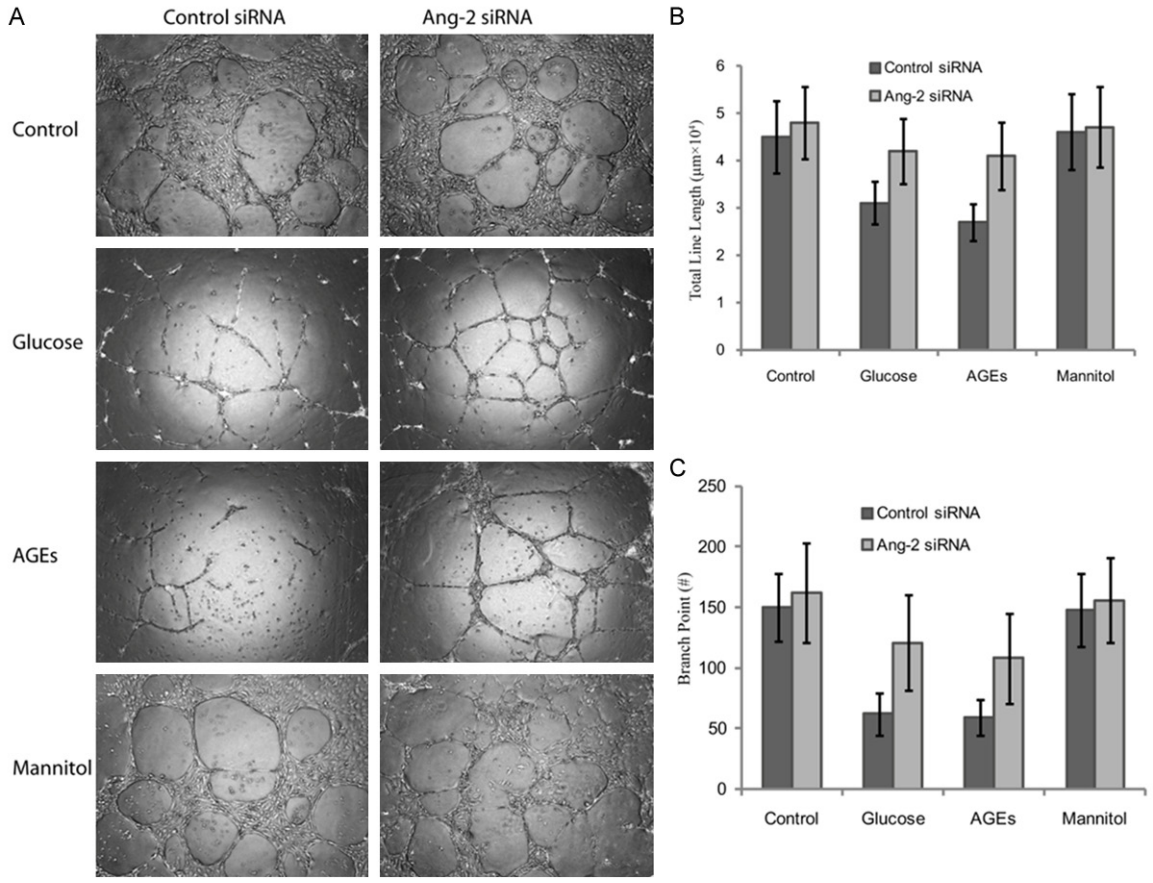

Figure 5. Effect of AGEs exposure on endothelial tube formation with and without Ang-2 siRNA interference.

Ang-2 and VEGF protein production in HUVECs

Ang-2 protein level was increased in 30 mM Glucose and AGE compared with control and mannitol medium. When Ang-2 siRNA was inducted, the protein blot was decreased in 30mM Glucose and AGE condition (P<0.05) (**Figure 4**).

Effect of AGEs exposure on endothelial tube formation with and without Ang-2 siRNA interference

As shown in **Figure 5** HUVECs structures in normal and mannitol medium form capillary-like on matrigel. Cells treated with high glucose showed extended cytoplasm on Matrigel and contacted each other. Between these cells, a lumen-like structure was observed. High glucose and AGEs decreased HUVECs tube formation as evidenced by significantly reduced tube length and total number of branch points compared to control and mannitol condition (P<0.01). When Ang-2 siRNA inducted, the capillary length and branch point was increased significantly compared to before (P<0.05) but still decrease compare to control and mannitol condition (P<0.05). These results suggest that exposure to glucose and AGEs impair angiogenesis via Ang-2 pathway.

Tie-2 receptor immunohistology

The result of fluorescence microscope showed: Tie-2 receptor expression of Mannitol group as same as control group showed in cytoplasm and cytomembrane. The expression of Glu group was detected in cytoplasm and cell nucleus. The expression only exists in cell nucleus of the cells treated with AGE and Glu-AGE as showed in **Figure 6**.

Discussion

In order to further reveal the poor relationship between wound local sugar, AGEs accumulation and new blood vessels, this study setting

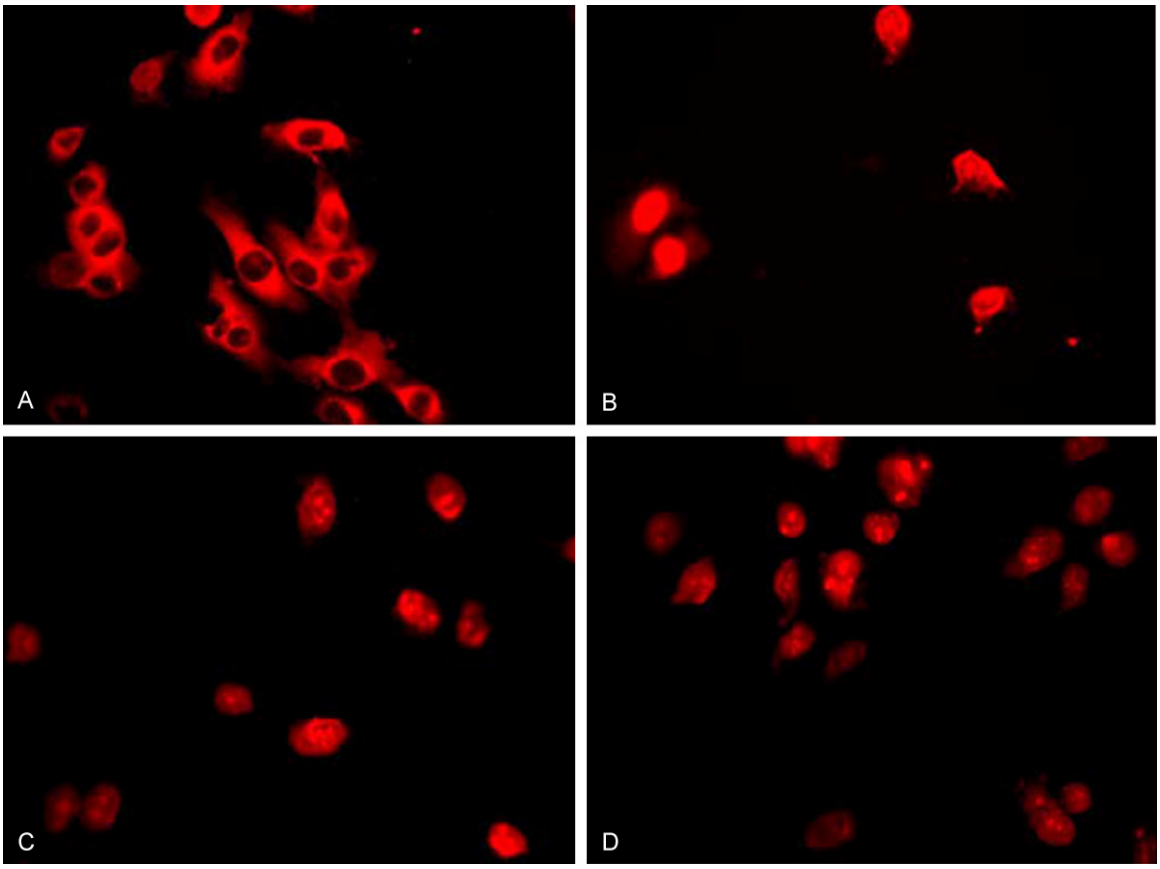

Figure 6. Expression of Tie-2 receptor in HUVEC in vitro with different culture; A: Control; B: High glucose; C: AGE; D: Mannitol.

training system of high glucose and AGEs *in vitro*, to observe high sugar and healing rate of the damaged endothelial cells. At the same time, the same concentration of mannitol set as a comparison group to clarificate the damage of high sugar and AGEs to endothelial cell. The different concentration of glucose has different inhibiting ability to the proliferation of vascular endothelial cell; AGEs can inhibit the proliferation, and promote the apoptosis of vascular endothelial cell [15, 16]. We monitor the Stick wall, cell proliferation and migration of biological behavior by Electrical wound-healing assay for cells *in vitro* [17]. Experimental results show that: Electrical impedance will remain stabilize in fusion state of vascular endothelial cells, but it will be decrease if the cells suffer electric injury; with the injured cells migration, electrical impedance will be rise in the damage area. Related research [12] has confirmed the change of electrical impedance in damage cells or cellular matrix is positively correlated with cell healing ability. Even more, we found the rate of cell wounding curve in the high glucose, AGEs and control medium are similar to each other. There is no difference among them.

Ang-2 mainly secreted by vascular endothelial cells, and the main factor which regulate the gene to organize the vascular endothelial cells becoming lumen. We found AGEs can stimulate high expression of Ang-2 of HUVECs cultured on Matrigel, better than the stimulation caused by High concentrations of glucose. Although regulatory factors Ang-2 bud can stimulate endothelial cells, form a funicular blood vessel structure, but abnormal expression can destroy the stability of blood vessels, causing the disappearance of the sample tube cavity structure. Another major factor participate in vascularization is VEGF. In the absence of VEGF, Ang-2 can lead to endothelial cell apoptosis and vascular fade. The other reason inhibiting vascular endothelial cells in vascular structure is HUVECs cultivated in Glu and AGE medium inhibit the expression of VEGF. Yla-Herttuala et al. [20]

found Adenovirus transfected by plasmid with VEGF could promote the vascular endothelial cells in vascular structure *in vitro*. It indicates VEGF will play an important roleto treat the refractory diabetic wound promote angiogenesis in the future.

As a co-receptor to Ang-1 and Ang-2, Tie-2 can express in different position on HUVECs under the different intervention conditions. Tie-2 receptor expression of Mannitol group as same as control group showed in cytoplasm and cytomembrane. The expression of Glu group was detected in cytoplasm and cell nucleus. Previous studies have found mice lacking the Tie-2 gene often die from vascular developmental disorders in the embryonic stage. When the factors such as Ang-1/2 act on Tie-2 receptor on cell membrane, they induce the vascular endothelial cell conforming vascular structure [4]. In AGEs environment, the strong positive expression of Tie-2 receptor in the nucleus may be one of the reasons why the vascular endothelial cells could not form a tubular structure. On the other hand, in high glucose environment, the Tie-2 receptor was also expressed in the cytoplasm of HUVECs, and the Ang-2 signal transduction was not completely blocked.

The function of vascular endothelial cells in vascular endothelial cells in Matrigel was recovered after siRNA Ang-2 interference was used in high glucose environment. High glucose and AGE environment induced cell can over expression of Ang-2, but by siRNA interference, through the degradation of mRNA template, so that the amount of Ang-2 protein reduced, to achieve the role of down regulation Ang-2. Therefore, the expression level of Ang-2 protein can be restored to normal state, which plays a positive role in the regulation of blood vessel.

In summary, we found HUVECs in high glucose or AGEs culture medium can heal themselves as soon as the HUVECs in normal culture medium; However, the ability of HUVECs to form vascular like structure was inhibited under high glucose and (or) AGEs environment. The inhibition is relative to the factor Ang-2 and VEGF; Tie-2 receptor differential expression in cells also has a certain effect on the regulation of angiogenesis under high glucose and (or) AGEs environment. By means of RNA interference, the expression of inhibitory genes, can promote neovascularization in diabetic environment, provides a feasible strategy for the treatment of diabetes mellitus and impaired wound vascularization; also supports the abnormal activation of Ang-2 pathway may be one of the important mechanism of barriers to angiogenesis in refractory diabetic wound.

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Disclosure of conflict of interest

None.

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References

[1] Qiao L. Translational medicine associated with impaired wound healing study. Chinese Journal of Burns 2010; 26: 472-473.

[2] Qiao L, Wang ZY, Wei J, Yuan B, Jin SW, Hua LN, Liao ZJ, Shi JX, Lu SL. Study of neovaseularization disturbance in deep partial-thickness scald in rats with diabetes mellitus. Zhonghua Shao Shang Za Zhi 2008; 24: 18-21.

[3] Qiao L, Lu SL, Dong JY, Song F. Abnormal regulation of neo-vascularisation in deep partial thickness scalds in rats with diabetes mellitus. Burns 2011; 37: 1015-1022.

[4] Ferand O, Mallet CH, Vilgrain IS. Expressional regulation of the ang-1 and ang-2 and the endothelial-specific receptor tyrosine kinase TIE-2 in adrenalatrophy: a study of adrenocorticotropin-induced repair. Endocrinology 2003; 144: 4607-4615.

[5] Stitt AW, Jenkins AJ, Cooper ME. Advanced glycation end products and diabetic complications. Expert OpinInvestig Durgs 2002; 11: 1205-1223.

[6] Lu SL, Qing C, Xie T, et al. Research on olfactory mechanism of the cutaneous “underlying disorder” in diabetic rats. Chin J Trauma 2004; 209: 468-473.

[7] Xu FR, Qiao L, Li XC, et al. Comparative study on the efficacy of water jet scalpel and tangential excision for debriding medium area deep second degree burn wounds. 2016; 36: 354.

[8] Lu SL, Qiao L, Xie T, Yang YM, Jin SW, Qing C. Effects of the cutaneous and blood contents of

glucose on wound healing in diabetic rats with superficial partial thickness scalding. Zhonghua Yi Xue Za Zhi 2005; 85: 1899-1902.
[9] Tessier FJ, Monnier VM, Sayre LM, Kornfield JA. Triosidines: novel Maillard reaction products and cross-links from the reaction of triose sugars with lysine and arginine residues. Biochem J 2003; 369: 705-719.
[10] Lin WD, Lu SL, Qing C, et al. Expression of and VEGF in two degree burn wounds of diabetic rats and the relationship between the expression of bFGF and the microvessel density. National Medical Journal of China 2003; 83: 1702-1704.
[11] Esposito C, Fasoli G, Platin AR, Bellotti N, Conte MM, Cornacchia F, Foschi A, Mazzullo T, Semeraro L, Dal Canton A. Long-term exposure to high glucose up regulates VCAM-induced endothelial cell adhesiveness to PBMC. Kidney Int 2001; 59: 1842-1849.
[12] Keese CR, Wegener J, Walker SR, Giaever I. Electrical Wound-healing assay for cell in vitro. Proc Natl Acad Sci U S A 2004; 101: 1554-1559.
[13] Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. J Biol Chem 2003; 278: 8244-8249.
[14] Lingelbach LB, Mitchell AE, Rucker RB, McDonald RB. Accumulation of advanced glycation endproducts in aging male Fischer 344 rats during long-term feeding of various dietary carbohydrates. J Nutr 2000; 130: 1247-1255.
[15] 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. Graefes Arch Clin Exp Ophthalmol 2002; 240: 996-1002.
[16] Lin WD, Lu SL, Qing C, Yao M, Chen XF, Xiang J, Qiao L, Liu YK, Dong JY, Shi GY, Liao ZJ, Shi JX. The effect of advanced glycation end products modified human serum albumin on the growth of human vascular endothelial cells. Natl Med J China 2003; 83: 572-576.
[17] Luong JH, Habibi-Rezaei M, Meghrous J, Xiao C, Male KB, Kamen A. Monitoring motility, spreading, and mortality of adherent insect cells using an impedance sensor. Anal Chem 2001; 73: 1844-1848.
[18] Cassell OC, Morrison WA, Messina A, Penington AJ, Thompson EW, Stevens GW, Perera JM, Kleinman HK, Hurley JV, Romeo R, Knight KR. The influence of extracellular matrix on the generation of vascularized, engineered, transplantable tissue. Ann N Y Acad Sci 2001; 944: 429-442.
[19] Babaei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ, Stewart DJ. Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide. Am J Pathol 2003; 162: 1927-1936.
[20] Yla-Herttuala S. An update on angiogenic gene therapy: vascular endothelial growth factor and other directions. Curr Opin Mol Ther 2006; 8: 295-300.